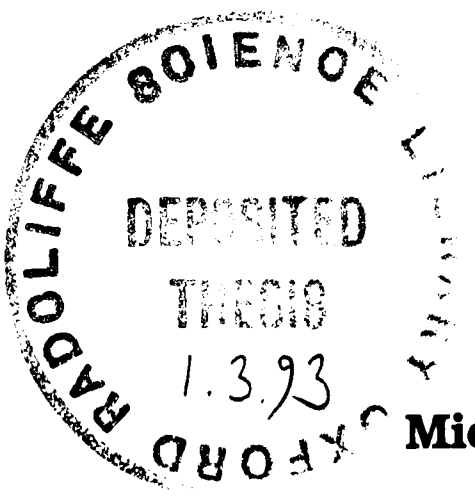
Phosphorylation of the Human Topoisomerase II Protein

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by

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Abstract

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DNA topoisomerase II is an essential enzyme in eukaryotes and is required for many aspects of DNA metabolism including DNA replication, recombination, chromosome segregation and chromosome condensation. It is also a major component of the nuclear scaffold. Topoisomerase II from lower eukaryotes has been shown to be phosphorylated *in vivo* and this phosphorylation leads to a modulation of activity. However, unlike these lower eukaryotes, human topoisomerase II exists as two closely related, but genetically distinct, isozymes which have markedly different expression and localization patterns. Topoisomerase IIα is a 170kDa protein and topoisomerase IIβ is 180kDa. This study set out to analyse the phosphorylation of these specific isozymes and understand how this leads to the regulation of their distinct biological functions.

In order to undertake this study, two polyclonal anti-topoisomerase II antibodies were generated and a series of other polyclonal and monoclonal antibodies characterized. Furthermore, the α isozyme of human topoisomerase II was purified to near homogeneity from cultured HeLa cells. A kinase activity with the biochemical characteristics of casein kinase II co-purified with and could phosphorylate the purified topoisomerase IIα protein.

The α and β isozymes of human topoisomerase II were both shown to be phosphoproteins *in vivo*. The α isozyme is phosphorylated predominantly on serine residues but with a minor proportion of phosphothreonine. Both the α isozyme and a stable 150kDa fragment of the β isozyme are phosphorylated *in vitro* by casein kinase II and the catalytic subunit of PKA (cAMP-dependent protein kinase). The α isozyme can also be phosphorylated *in vitro* by Ca^{2+}-dependent and -independent isozymes of protein kinase C and the cell cycle-regulated $p34^{cdc2}$ kinase.

Two-dimensional tryptic phosphopeptide mapping suggested that the pattern of phosphorylation of human topoisomerase IIα protein *in vivo* is complex with phosphorylation occurring on multiple residues. Comparison with *in vitro* maps suggested that casein kinase II and PKA could account for most of the phosphorylation seen *in vivo*. Using a one-dimensional phosphopeptide mapping approach, a major site of phosphorylation *in vivo* appeared to be within the C-terminal 20kDa, and that casein kinase II, PKA and PKC may all phosphorylate this region.

Phosphorylation of human topoisomerase IIα protein by casein kinase II, PKA and PKC all led to a stimulation of activity as measured by plasmid relaxation and decatenation. In contrast, dephosphorylation led to a marked decrease in activity of the enzyme. The dephosphorylated enzyme could be reactivated by casein kinase II but not PKA phosphorylation. These data suggest that phosphorylation plays a crucial role in the control of DNA tertiary structure in human cells via regulation of the activity of topoisomerase II proteins.

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Abbreviations

ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cdc	cell division cycle
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CHO	Chinese hamster ovary
CK II	casein kinase II
CRB	Cambridge Research Biochemicals
DAB	diaminobenzidine
DMEM	Dulbecco's modified Eagle's minimal essential medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
DTT	dithiothreitol
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol bis(β-aminoethylether) N,N,N',N'-tetraacetic acid
FCS	fetal calf serum
FPLC	fast performance liquid chromatography
GTP	guanosine triphosphate
GST	glutathione-S-transferase
H_2O_2	hydrogen peroxide
H7	1-(5-isoquinolinylsulfonyl)-2-methylpiperazine
HRP	horseradish peroxidase
ICRF	Imperial Cancer Research Fund
kb	kilobase pairs
kDa	kilodaltons
kDNA	kinetoplast DNA
KLH	keyhole limpet haemocyanin
m-AMSA	4'-(9-acridinylamino) methanesulfon-*m*-anisidide
MDR	multidrug resistance
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
NCS	newborn calf serum

PAGE	polyacrylamide gel electrophoresis
PAP	potato acid phosphatase
PBS	phosphate buffered saline
PBS-A	PBS without Ca^{2+} and Mg^{2+}
PCR	polymerase chain reaction
PEG	polyethylene glycol
PKA	protein kinase A (cAMP-dependent protein kinase)
PKC	protein kinase C
PKG	protein kinase G (cGMP-dependent protein kinase)
PKi	protein kinase inhibitor peptide (specific for PKA)
PKI	PKA protein kinase inhibitor protein
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
rDNA	ribosomal DNA
RF	replicative form of DNA
RNA	ribonucleic acid
SCE	sister chromatid exchange
SDS	sodium dodecyl sulphate
ssDNA	single-stranded DNA
TBE	tris / borate / EDTA buffer
TBS	tris buffered saline
TCA	trichloroacetic acid
TLCK	Na-p-tosyl-L-lysine chloromethyl ketone
TPA	12-O-tetradecanoylphorbol 13-acetate
Tris	tris(hydroxymethyl)aminomethane
V8	*Staphylococcus aureus* V8 protease
VM26	teniposide
VP16	etoposide
v/v	volume for volume
w/v	weight for volume

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Chapter 1 INTRODUCTION.

1.1 General Introduction.

The intertwining of the two strands of the DNA double helix imposes a number of topological problems which the cell must overcome to carry out the basic processes of DNA metabolism. Both prokaryotic and eukaryotic cells have solved this problem by utilizing enzymes than can alter the topological state of the DNA. These enzymes are the DNA topoisomerases.

DNA topoisomerases change the degree of supercoiling of DNA by catalysing transient breakage and resealing reactions. The topoisomerases fall into two classes, dependent on the nature of this reaction. Type I DNA topoisomerases create single-strand breaks in the DNA phosphodiester backbone, pass the other strand of the double-helix through this break, and then reseal the break. This leads to a change in the overall linking number of the DNA of one. Type II DNA topoisomerases create double-strand breaks in the double-helix, pass a second helix through this break and then reseal the break. This leads to an overall change in the linking number of two. For general reviews see Wang, 1985; Osheroff, 1989a; Sternglanz, 1989.

Prokaryotic and eukaryotic cells have evolved similar enzymes to fulfill these functions. The first identification of an activity that could relax supercoiled DNA was made from *E. coli* extracts in 1969 (Wang, 1969). This was purified and shown not to be an endonuclease as first thought, but an enzyme capable of relaxing negatively supercoiled DNA. This enzyme did not require ATP (Wang, 1971). It was initially called the ω protein, but later became *E. coli* DNA topoisomerase I. A similar activity was later purified from rat liver cell extracts that was capable of relaxing both positively and negatively supercoiled DNA in the absence of ATP (Champoux and Dulbecco, 1972). This was classified as eukaryotic DNA topoisomerase I.

A new type of topoisomerase was isolated from bacteria in 1976, called DNA gyrase (Gellert *et al.*, 1976). This had the capability to both release and introduce negative supercoils in DNA in an ATP-dependent, double-strand breakage-rejoining reaction. This was the first type II topoisomerase to be identified. A type II topoisomerase was then found in the T4 bacteriophage (Liu *et al.*, 1979), and eukaryotic type II

topoisomerase activity was reported soon after in 1980 (Liu *et al.*, 1980; Baldi *et al.*, 1980; Hsieh and Brutlag, 1980).

1.2 Prokaryotic Topoisomerases.

E.coli topoisomerase I is a monomeric protein of 97kDa. It is encoded by the *topA* gene as identified using mutant analysis by Sternglanz *et al.* (1981). *E. coli topA* mutants are viable and grow only slightly less well than wild-type cells (Sternglanz *et al.*, 1981). Some of the mutants have a more rapid rate of induction and higher level of catabolite-sensitive enzymes such as β-galactosidase, and this is attributed to an increase in negative superhelicity of the DNA. This increase in negative superhelicity is deleterious to the cell though, and several compensatory mutations have been isolated which allow them to grow normally (DiNardo *et al.*, 1982; Pruss *et al.*, 1982). Many of these compensatory mutations mapped to the genes encoding the subunits for the type II topoisomerase DNA gyrase; these mutations led to a lower gyrase activity in the cell. This led to the concept that the relative levels of topoisomerase I and DNA gyrase determine the degree of DNA superhelicity in the bacterial cell (Pruss *et al.*, 1982).

E. coli DNA gyrase is a tetramer (A_2B_2) of two different subunits. The A subunit of 97kDa encoded by the *gyrA* gene, and the B subunit of 90kDa encoded by the *gyrB* gene (Cozzarelli, 1980; Gellert, 1981). The gyrase genes were defined several years prior to the discovery of DNA gyrase, as defined by mutations causing resistance to antibacterial drugs. Mutants in *gyrA* caused resistance to nalidixic acid, and mutants in *gyrB* caused resistance to coumermycin (Gellert, 1981). In *E.coli* the two genes are not linked, but in *Bacillus subtilis* the genes are adjacent and close to the origin of DNA replication (Moriya *et al.*, 1985).

The GyrA subunit contains the site for breakage and rejoining of the DNA; it forms a transient covalent intermediate with DNA through a tyrosine residue. The quinolone family of antibacterial drugs, such as nalidixic acid, oxolinic acid, norfloxacin or ciprofloxacin, prolongs the lifetime of this intermediate, effectively trapping the GyrA subunit on DNA. Drugs such as coumermycin or novobiocin however, bind to the GyrB subunit and compete with ATP for binding to this subunit. Occasionally though, nalidixic acid-resistant mutations have been found in the *gyrB* gene. Two of these mutations have been sequenced and found to cause single amino acid changes in the central region of the GyrB subunit

(Yamagishi *et al.*, 1986). So whilst these drugs appear to preferentially target one subunit or the other, it appears that they can interact with both subunits. Indeed the drug binding pocket may well be formed from parts of both subunits and perhaps also the DNA, in the DNA-$GyrA_2$-$GyrB_2$ complex (Shen and Pernet, 1985).

Recently, a new type I enzyme has been identified in *E. coli*, called DNA topoisomerase III (Dean *et al.*, 1983). Purification showed that its enzymatic properties are fairly similar to *E. coli* topoisomerase I (DiGate and Marians, 1988), but it induces a different cleavage pattern in ssDNA. Topoisomerase III is a monomer of 74kDa encoded by the *topB* gene; it has been cloned and sequenced (DiGate and Marians, 1989). Although smaller than topoisomerase I, it shares 24% identity over a stretch of 308 amino-acids in the middle of both proteins. This region includes the active site tyrosine of topoisomerase I (Lynn and Wang, 1989). Although the function of topoisomerase III has not yet been elucidated it is intriguing that the *topA* and *topB* genes flank, and are just outside, the *E. coli* DNA replication fork terminators, T1 and T2. Topoisomerase III has not yet been identified in other bacterial species.

Two more genes in *E. coli*, *parC* and *parE*, defined by mutants with defects in chromosome segregation, have been found to encode a new type II topoisomerase termed DNA topoisomerase IV (Kato *et al.*, 1988; Kato *et al.*, 1990). The amino acid sequences of the *parC* and *parE* polypeptides are homologous to those of the A and B subunits of DNA gyrase respectively, and indeed, topoisomerase IV also exists as a tetramer of two subunits encoded by each gene. Topo IV is essential though and appears to have a completely independent role from DNA gyrase in the separation of catenated DNA at cell division (Adams *et al.*, 1992)

E. coli and *B. subtilis* are members of the Eubacteria; there is another division, the Archaebacteria, which includes the acidothermophilic bacteria which live in extreme environmental conditions of heat and acidity. A unique type of topoisomerase, called "reverse gyrase", has been identified only in these primitive organisms (Kikuchi and Asai, 1984). This enzyme is the only known topoisomerase able to introduce positive supercoils into DNA, and it is thought that this property may be important in maintaining and controlling chromosome structure under these adverse environmental conditions.

1.3 Eukaryotic Topoisomerases.

Eukaryotic topoisomerase I was first cloned from yeast in 1985 (Thrash *et al.*, 1985) by a series of complementation experiments in a *S. cerevisiae top1* mutant. This mutant strain is viable, suggesting that eukaryotic topoisomerase I is not an essential enzyme. Independently, Goto and Wang (1985) isolated the *S.cerevisiae* TOP1 gene by immunoscreening of *E. coli* infected with a phage λgt11 yeast gene library. The *S. pombe* top1$^+$ gene was cloned by hybridization of a cosmid library of *S. pombe* genomic DNA with the *S. cerevisiae* TOP1 sequence (Uemura *et al.*, 1987b). The gene for human DNA topoisomerase I was then cloned and sequenced (D'Arpa *et al.*, 1988), and shown to be a single copy gene that maps to chromosome 20q12-13.2 (Juan *et al.*, 1988). The cDNA has an open reading frame which would encode a protein of 765 amino acids, similar in size and sequence to the yeast genes. The human enzyme shares 42% amino acid identity with topoisomerase I from *S. cerevisiae*. However eukaryotic topoisomerase I shares no sequence similarity to bacterial topoisomerase I (see yeast TOP3 below).

Unlike topoisomerase I, eukaryotic topoisomerase II is an essential enzyme being required for correct cell division. The *S. cervisiae* TOP2 gene was also isolated by immunochemical screening (Goto and Wang, 1984), and independently cloned by complementing a temperature-sensitive *top2* mutant with a plasmid carrying the TOP2 gene (Voelkel-Meiman *et al.*, 1986). The *S. pombe* top2$^+$ gene was cloned by complementation and hybridization approaches (Uemura and Yanagida, 1986; Uemura *et al.*, 1986). In humans, topoisomerase II was thought to be encoded by a single-copy gene on chromosome 17, which was cloned and sequenced by Tsai-Pflugfelder *et al.* in 1988. However, it has since been recognized that the human genome contains a second functional topoisomerase II gene encoded on chromosome 3. This has also been cloned and sequenced (Chung *et al.*, 1989; Jenkins *et al.*, 1992); see section 1.12 for discussion of topoisomerase IIα and β isozymes.

To confuse matters further, a new yeast type I topoisomerase has been identified in *S. cerevisiae* homologous to bacterial topoisomerase I (Wallis *et al.*, 1989). It has been called DNA topoisomerase III, encoded by the TOP3 gene, not to be confused with the *E. coli topB*-encoded bacterial topoisomerase III. Yeast *top3* mutants are characterized by poor growth and higher frequency of recombination between a class of

repetitive sequences termed δ sequences (Wallis *et al.*, 1989). Sequence data suggest that a yeast gene, HPR1, may encode yet another DNA topoisomerase (Aguilera and Klein, 1990; Wang *et al.*, 1990). It is possible that several more members of the topoisomerase family are waiting to be discovered (Wang, 1991).

1.4 Sequence and Domain Structure of Eukaryotic Topoisomerase Proteins.

Eukaryotic topoisomerase I is a monomeric protein varying in size from 90 to 135kDa, with the yeast topoisomerase I being 90kDa and the human topoisomerase I being 100kDa. Early reports of topoisomerase I being 60-70kDa based on purification results was explained by the rapid breakdown of the full-length protein to a catalytically active fragment (Liu and Miller, 1981; Schmitt *et al.*, 1984).

The human topoisomerase I enzyme is rich in lysyl and glutamyl residues and contains consensus recognition sequences for several protein kinases. Its high content (43%) of charged residues explains why charged molecules, like polyanions *in vitro* or HMG proteins and histone H1 *in vivo*, can modulate its catalytic activity.

Eukaryotic topoisomerase II proteins have a molecular weight ranging from 160 to 180kDa. Hydrodynamic studies indicate that the enzyme exists in solution as a homodimer with a sedimentation coefficient of 9.2 to 11S, and a Stokes radius of 6.4 to 7.8nm. These suggest that in solution the homodimer adopts an elongated, rather than globular, shape.

The full length human topoisomerase IIα protein is 1530 amino-acids long, whilst the *S. cerevisiae* protein is 1429 residues, the *S. pombe* protein 1431 residues and the *Drosophila* protein 1447 residues long. The polypeptides show a high degree of homology, being 49% homologous between the two yeast species and 46% between yeast and *Drosophila* (Wyckoff *et al.*, 1989), with the most divergence in the C-terminal region.

Eukaryotic topoisomerase II consists of three major domains: an N-terminal domain, homologous to the bacterial DNA gyrase GyrB subunit, which contains the ATP-binding site; a central domain, homologous to the DNA gyrase GyrA subunit, which includes the DNA binding and cleavage domain; and a C-terminal domain, which has no equivalent region in the bacterial DNA gyrase protein (Figure 1). It is likely then that

eukaryotic topoisomerase II evolved from bacterial DNA gyrase by fusion of the two gyrase subunits into one polypeptide (Lynn *et al.*, 1986). Hence it is interesting that the genes for the two gyrase subunit genes are adjacent in *B. subtilis*, but not in *E. coli*. The presence of a strong protease sensitive site between these two regions in the yeast topoisomerase II suggests that they still act as independent units (Lindsley and Wang, 1991).

Eukaryotic type II topoisomerases contain sequences, mainly at the C-terminus of the protein, but also at the N-terminus, which have no homologous regions in DNA gyrase, and are poorly conserved between eukaryotes. These regions are characterized by an unusually high concentration of charged residues, and the region is rich in protein kinase consensus sequences. These domains may also include the nuclear localization signals, not necessary in prokaryotes.

Figure 1 shows a comparison of the eukaryotic topoisomerase II protein with the bacterial DNA gyrase subunits and the T4 phage topoisomerase II subunits, aligned for sequence and functional homologies. Limited digestion with papain showed that the yeast, *S. pombe*, topoisomerase II could also be structurally subdivided in a similar manner (Shiozaki and Yanagida, 1991). The "core" region alone, containing only the GyrA and GyrB equivalent domains, but lacking the non-homologous sequences found at the N- and C-termini in the eukaryotic enzyme, still retained enzymatic activity, as would be expected from homology to DNA gyrase. However it was no longer able to rescue a *top2* null mutant, suggesting some essential function *in vivo* for these regulatory domains.

Lindsley and Wang (1991) showed that the protease-sensitive sites in the *S. cerevisiae* topoisomerase II protein changed on binding of an ATP analogue, suggesting that the protein underwent conformational changes, perhaps in respect of one domain to another, upon binding ATP.

Since it is clear that eukaryotic topoisomerase II can only function correctly as a homodimer, it is surprising that so little is known about the region of the eukaryotic topoisomerase II protein involved in dimerization. Based purely on an examination of the human topoisomerase II sequence, Zwelling and Perry (1989) identified a putative leucine zipper motif comprising four leucine residues between residues 994 and 1021 (Figure 1). However, there is no functional evidence supporting this model as yet; but the presence of leucine

zippers in many other DNA binding dimeric proteins makes this an attractive proposal.

As the topoisomerase II protein is such a large molecule, it is beyond the present technology for high resolution structural studies. However, due to the homology with the bacterial DNA gyrase subunits, some very useful parallels can be drawn from high resolution crystallization studies that have been carried out on part of the GyrB molecule. The crystal structure of an N-terminal fragment of the DNA GyrB protein (Figure 1), complexed to a non-hydrolysable ATP analogue, has been solved at 2.5Å (Wigley *et al.*, 1991). This fragment shows some novel protein secondary structures, as well as the residues directly involved in ATP binding. The ability of this fragment to dimerize in the presence of ATP, also suggests a role for the homologous region of the eukaryotic topoisomerase II in protein dimerization. What is also clear is that this fragment, which is only about half of the DNA gyrase protein, itself consists of two distinct domains. Hence it is likely that the full-length eukaryotic topoisomerase II protein also has a highly complex domain structure, where each of the three major 'domains', described earlier in this section, represent only the highest order of domain organization.

1.5 The Enzymatic Reaction Mechanism of DNA Topoisomerases.

The distinction between single-strand and double-strand DNA cleavage defines the mechanistic difference between type I and type II topoisomerases respectively (reviewed in Maxwell and Gellert, 1986; Osheroff, 1989a; Osheroff *et al.*, 1991). Figure 2 shows the enzymatic mechanism for eukaryotic topoisomerase II.

Both catalytic cycles can be broken down into several discrete steps (references refer to topoisomerase II catalysis):

(1) DNA Binding. Topoisomerase I or II binds to the nucleic acid substrate in a non-covalent fashion, that does not need the presence of additional cofactors (Osheroff, 1986). This is known as the non-cleavable complex.

(2) DNA Cleavage. The bound enzyme creates either a single- (topoisomerase I) or double- (topoisomerase II) stranded break in the backbone of the DNA. In the process, the enzyme becomes covalently bound to the DNA via a phospho-tyrosyl linkage. This is known as the

cleavable complex. The double-stranded topoisomerase II-mediated cleavage absolutely requires a divalent cation (Sander and Hsieh, 1983; Osheroff, 1987).

(3) DNA Passage. The intact single (topoisomerase I) or double (topoisomerase II) strand is passed through the transient break made in the nucleic acid backbone. For the topoisomerase II reaction this requires the binding of ATP to the enzyme (Osheroff *et al.*, 1983).

(4) DNA Religation. The topoisomerases religate the cleaved DNA and remain associated with their DNA product in a non-covalent fashion (Osheroff, 1986).

(5) ATP Hydrolysis. Topoisomerase II hydrolyzes the bound ATP molecule to ADP and inorganic phosphate (Miller *et al.*, 1981; Osheroff *et al.*, 1983; Benedetti *et al.*, 1983). This step is only required by the type II topoisomerases. The type I reaction is ATP-independent.

(6) Enzyme Turnover. The topoisomerase-DNA complex turns over and the enzyme regains its ability to reinitiate a new round of catalysis (Osheroff, 1986).

The formation of the covalent "cleavable complex" following DNA breakage is an integral step in the reaction pathway of both type I and type II topoisomerases. It is so called because it can be trapped by certain drug compounds, and in the presence of strong protein denaturants, such as SDS or alkali, the DNA strand breaks are then revealed (Glisson and Ross, 1987). This is of great significance as many of these drugs are known to be effective antitumor agents, and appear to, at least in part, exert their cytotoxic effect through the DNA topoisomerases (see section 1.7).

The nature of the covalent intermediate formed differs slightly between the topoisomerase I and II reactions. Topoisomerase I, after strand nicking, covalently attaches to the 3'-phosphoryl end of the broken DNA strand via a unique tyrosine residue in the protein, Tyr-723 in the human topoisomerase I (Lynn *et al.*, 1989). Topoisomerase II however, which operates as a homodimer, becomes covalently attached at both subunits, one to each free 5'-phosphoryl end from the double-stranded break. Again this covalent linkage is through a specific tyrosine residue in the protein; this has been mapped to Tyr-783 in the *S. cervisiae* topoisomerase II (Worland and Wang, 1989), and through homology identified as Tyr-804 in the human topoisomerase II enzyme (Tsai-Pflugfelder *et al.*, 1988). The double-strand break in topoisomerase

II reactions *in vitro* is staggered by 4 bp, with the 3'-OH end being recessed (Liu *et al.*, 1983; Sander and Hsieh, 1983). It is possible that the length of the stagger is variable *in vivo* (Laemmli *et al.*, 1992).

Prokaryotic type I topoisomerases can only efficiently catalyze the relaxation of negative supercoils. DNA gyrase and eukaryotic type I and II topoisomerases can relax both positive and negative supercoils. Only the bacterial DNA gyrase and reverse gyrase however, can introduce supercoils into DNA. Besides relaxation of supercoiling, type II topoisomerase can carry out catenation/decatenation reactions (i.e. the linking and unlinking of DNA minicircles), and knotting/unknotting reactions on a circular piece of DNA. These form the basis for specific assays for topoisomerase activity *in vitro*: type I topoisomerases are unique in being able to completely relax an excess of supercoiled DNA in the absence of ATP; decatenation or unknotting assays can be used as specific assays for type II topoisomerase activity.

The complete relaxation of a heavily supercoiled plasmid DNA substrate requires multiple topoisomerization reactions. This can be carried out by a single topoisomerase molecule either in a distributive or processive manner depending on the rate of dissociation from the DNA. Under completely distributive conditions, the enzyme relaxes only one supercoil before dissociation, and topoisomers of intermediate superhelicity accumulate. In contrast, under processive conditions the enzyme completely relaxes all the supercoils one after another, before dissociation, so that intermediates do not accumulate. Hence one mechanism by which post-translational modification could alter topoisomerase activity is to shift it towards a more distributive, or alternatively processive, reaction.

Both type I and II topoisomerases appear to have preferred sites of cleavage on DNA, which may be determined *in vivo* by function, chromatin structure, topology and sequence (Sander and Hsieh, 1985; Udvardy and Schedl, 1991; Laemmli *et al.*, 1992). *In vivo* topoisomerase I cleavage sites cluster around the 5' region of actively transcribing genes, whilst topoisomerase II cleavage sites occur predominantly in scaffold-associated regions (SARs) - see section 1.6. However even within these regions, there are preferred primary sequences for cleavage by topoisomerases. The fact that topoisomerase II cleavage sites do not usually show dyad symmetry suggests that the double strand breakage occurs sequentially, with one strand being preferentially cut first.

Perhaps the most interesting question still to be addressed in mechanistic terms, is how the spatial reorganization of the protein-DNA complex is carried out, to allow transfer of the intact single-strand or duplex through the enzyme-bound DNA gate. Crystallization studies on a fragment of the DNA GyrB B protein have suggested that the binding of ATP stabilizes an unfavourable conformation of the Gyr B protein. This in turn might induce a conformational change in the DNA-bound GyrA protein, allowing the DNA to pass into the interior of the complex where stand passage can then occur (Wigley *et al.*, 1991).

1.6 Biological Roles of Topoisomerases in the Cell.

1.6.1 Transcription.

As RNA polymerases move along a stretch of DNA which is being transcribed, either the polymerase must traverse a helical path around the DNA, leading to the nascent RNA molecule getting wrapped around the DNA, or the DNA helix itself must rotate along its long axis during transcription (Cook, 1989). This latter model would lead to the generation of localized domains of supercoiling, positive supercoiling ahead of the RNA polymerase and negative supercoiling behind it. This model of "twin-supercoiled-domains" was proposed by Liu and Wang (1987). Evidence from both bacteria and yeast confirms that this second model is likely to be correct, and that topoisomerases can be used as "swivels" to relieve the torsional stress induced by the localized domains of supercoiling.

The twin-supercoiled domain model illustrates how supercoiling can be introduced into eukaryotic DNA, despite the absence of an enzyme with gyrase activity. No overall change in linking number results in a closed DNA domain though, because equal numbers of positive and negative supercoils are generated, ahead and behind the RNA polymerase, respectively. That is, unless topoisomerases preferentially relax either the positive or negative supercoils generated, in which case a change in linking number would occur. The ability to introduce regions of supercoiling into DNA through transcription may be very important for other DNA processes, such as the initiation of replication.

The appearance of hypernegatively supercoiled DNA in a yeast *top1* mutant or in a *top1.top2*^{TS} double mutant suggests that topoisomerase I, and perhaps also topoisomerase II are required for transcription in

eukaryotes (Brill and Sternglanz, 1988). Other evidence implicates topoisomerase I in transcription. Giaver and Wang (1988) showed that expression of *E. coli* topoisomerase I in a yeast double mutant functionally lacking both topoisomerase genes, led to positively supercoiled plasmids through transcription of the plasmid genes. Micro-injection of anti-topoisomerase I antibodies into *Chironomus tentans* nuclei led to blockage of transcriptional elongation (Egyhazi and Durban, 1987). Camptothecin-induced topoisomerase I cleavage sites are restricted to the transcribed regions of those genes tested, and their presence parallels the degree of transcriptional activation (Zhang *et al.*, 1988). Topoisomerase I protein is also found abundantly in actively transcribing regions.

The evidence for requirement of topoisomerase II in transcription is weaker, and indeed no temperature-sensitive yeast *top2* mutants show any defect in transcription. Furthermore, topoisomerase II is not enriched in actively transcribed regions.

Besides acting as part of the transcriptional machinery itself, topoisomerases could influence the transcription of genes by controlling the level of their supercoiling, particularly in the regions of promoters or enhancers. The positive and negative effects of supercoiling on the transcription of prokaryotic genes has been studied in great detail (reviewed by Pruss and Drlica, 1989). Whether supercoiling affects the induction of eukaryotic genes is not so well documented. Strong topoisomerase II cleavage sites have been found in both the 3' and 5' regions of the *Drosophila* Hsp70 gene (Rowe *et al.*, 1986), and in the SV40 enhancer region (Yang *et al.*, 1985). More data is necessary to understand the relevance of these observations.

1.6.2 Replication.

Topoisomerases play a role in the initiation of replication, the elongation of replication, and the separation of the replicated strands of DNA (Wang and Liu, 1990; Cook, 1991).

In bacteria, initiation of replication appears to be regulated by the degree of supercoiling of the origin of replication, which affects the assembly of initiation proteins, and the unpairing of the DNA strands. Supercoiling may be modulated both by gyrase activity and the relaxation activity of topoisomerase I, and it may be affected by the transcription of genes close to the origin (see section 1.6.1). In eukaryotes neither

topoisomerase I nor topoisomerase II appears to be essential for replication initiation, as replicons still initiate at the restrictive temperature in yeast double mutants (Kim and Wang, 1989a). Whether or not the degree of supercoiling in eukaryotic replication origins is important is not known.

Propagation of the replication fork requires a "swivel", just as in transcription, to prevent the newly-replicated strands from getting tangled on the parental strands. Experiments in cell-free systems suggest that either topoisomerase I or topoisomerase II can function in this role in eukaryotes (Yang *et al.*, 1987). Yeast *top1* mutants can complete the whole replication process, but DNA elongation is temporarily delayed; chain synthesis is not significantly affected in *top2* mutants (Kim and Wang, 1989a). Studies with topoisomerase inhibitors confirm that both topoisomerase I and II assist in elongation of infecting SV40 or adenoviral DNA.

DNA which is organized into fixed loops, where both ends are tethered, yields two intertwined duplexes or catenanes on replication. The segregation of these newly replicated progeny DNA molecules has an absolute requirement for the decatenating activity of a type II topoisomerase (DiNardo *et al.*, 1984; Uemura *et al.*, 1987a). Section 1.6.5 contains a full discussion of topoisomerase II involvement in chromosome segregation. Type I enzymes could only separate replicated DNA strands if one or other of the interlocked pair contained a nick; this may occur *in vivo* in special cases, such as if unpairing of the replicated strands occurred prior to merging of two replication forks (Tse-Dinh and Wang, 1980).

1.6.3 Recombination.

Due to their ability to carry out strand breakage and rejoining reactions, topoisomerases possess the credentials of DNA strand transferases and therefore could play a role in recombination (Ikeda, 1990; Wang *et al.*, 1990). Early work concentrated on the involvement of DNA topoisomerases in illegitimate recombination, the exchange of non-homologous DNA sequences. For instance it was proposed that topoisomerase I might be promoting illegitimate recombination leading to SV40 integration (Bullock *et al.*, 1985). Direct evidence for involvement of topoisomerase I in illegitimate recombination came from

the finding that vaccinia virus topoisomerase I expressed in *E. coli* cells promoted the *int*-independent prophage excision (Shuman, 1989).

There is also evidence that type II topoisomerases from *E. coli*, T4 phage and mammals, can mediate illegitimate recombination. Ikeda (1990) proposed a subunit-exchange model for gyrase-mediated illegitimate recombination, whereby two gyrase/DNA complexes join, and exchange of gyrase subunits occurs, leading to exchange of DNA duplexes. This model could also be used to account for eukaryotic topoisomerase II-mediated illegitimate recombination, where exchange of topoisomerase II monomers between two homodimeric/DNA complexes could take place.

Both illegitimate and homologous recombination have been implicated in the pathway leading to gene amplification (reviewed by Stark, 1986). It is an intriguing possibility that topoisomerase activity may influence the rate of gene amplification, and so be implicated in tumor progression.

Paradoxically, there is now a good deal of evidence suggesting that topoisomerases can also act as suppressors of recombination (Wang *et al.*, 1990). Yeast mutants lacking both the TOP1 and TOP2 gene functions suffer from a massively increased rate of mitotic recombination in the rDNA gene cluster (Kim and Wang, 1989b). Thus the combined action of the topoisomerases must be to suppress recombination in this region. As type I and type II topoisomerases have quite different enzyme mechanisms, the common function here may simply be to reduce the level of DNA supercoiling. In other words topoisomerases might suppress recombination by relaxing regions of high supercoiling.

Mutations in the yeast TOP3 gene also lead to increased recombination (Wallis *et al.*, 1989), and mutants in a new yeast gene, HPR1 (standing for hyperrecombination), which shows homology to eukaryotic topoisomerases, also exhibits the hyperrecombination phenotype (Aguilera and Klein, 1990). However, the ability of single mutants in the TOP3 or HPR1 gene to lead to hyperrecombination argues against the relaxation of supercoils as being the only mechanism of recombination suppression, as these mutants are TOP1$^+$ and show no evidence of elevated supercoiling levels in the genome as a whole. Relaxation of supercoiling may be one mechanism then, but not the only one, by which topoisomerases suppress recombination. The interwound parental strands formed during replication, which topoisomerases are able to decatenate, strongly resemble a recombinational interwound pair

of strands. So perhaps topoisomerases also suppress recombination by disentangling paired strands which form as recombination intermediates.

Interestingly HPR1 also shares homology with the mammalian RAG-1 protein (Schatz *et al.*, 1990), which carries out the sequence-specific recombination in the immunoglobulin genes. It is possible that HPR1 is a sequence-specific topoisomerase. Mutations in RAG-1 impair the recombination of the immunoglobulin genes though, whereas mutations in HPR1 induce recombination. It would be intriguing to find out if RAG-1 has topoisomerase activity. The critical relationship between destabilisation of the genome by recombination and neoplasia is without question, and so this aspect of topoisomerase function is clearly going to be of major interest in the next few years.

Whether or not topoisomerases are involved in the early steps of recombination, Rose *et al.* (1990) recently showed that the segregation of recombined chromosomes in meiosis 1 required topoisomerase II. Temperature sensitive yeast *top2* mutants induced to sporulate at the restrictive temperature underwent premeiotic DNA synthesis and commitment to meiotic levels of recombination but failed to complete the first meiotic division. The introduction of a mutation blocking recombination relieved the requirement for topoisomerase II in meiosis 1. It is highly likely therefore that if recombination does take place in the genome, topoisomerases are going to be needed for the resolution of the recombined molecules.

1.6.4 Topoisomerase II as a Component of the Nuclear Matrix.

The nuclear matrix of eukaryotic cells comprises a dynamic framework on which DNA is organized, and complexes of proteins perform the essential functions of transcription, replication and repair (for reviews see Cook, 1989, 1991 and Jackson, 1991). Topoisomerase II has been identified as a major structural component of mitotic chromosomes, matrices and scaffolds (Berrios *et al.*, 1985; Earnshaw *et al.*, 1985; Earnshaw and Heck, 1985; Gasser *et al.*, 1986). The distinction between a nuclear matrix, scaffold and even skeleton, is purely a technical one dependent on the conditions of preparation. Indeed the non-physiological conditions used in many of these preparation techniques in the past, led to their existence *in vivo* being questioned. However, there is now strong evidence for the existence of such a

structure *in vivo* from studies carried out *in situ* and using molecular genetic methodologies (Jack and Eggert, 1992).

Two major proteins were originally found to comprise the scaffold fraction of higher eukaryotic nuclei; these were called Sc-1, a 170kDa protein, and Sc-2, a 135kDa protein. These proteins held the chromatin in a series of fixed loops or domains. Earnshaw *et al.* (1985) used a polyclonal antibody to identify the Sc-1, 170kDa protein, as topoisomerase II in chicken mitotic chromosome scaffolds. Immunofluorescence and immunoelectron microscopy indicated that the distribution of the Sc-1 topoisomerase II was independent of the bulk chromatin, and was localized to the bases of the radial loop domains of mitotic chromosomes (Earnshaw and Heck, 1985). Also using immunocytochemistry, Gasser *et al.* (1986) confirmed that the Sc-1 topoisomerase II protein is localized to the core of the long axis of the chromatids of metaphase chromosomes in HeLa nuclear scaffolds. Topoisomerase II is also found in matrix or scaffold preparations of interphase nuclei (Earnshaw *et al.*, 1985; Berrios *et al.*, 1985).

The region of DNA which is thought to form the base of radial loops, and to which the scaffold proteins bind, has been called the scaffold-associated region or SAR (reviewed in Laemmli *et al.*, 1992). Two SARs therefore delimit one chromatin loop which can be 5 to 100kb in length. It is thought that one SAR contains several DNA subdomains spaced over several hundred base pairs which act cooperatively to bind the SAR-binding proteins. The AT-rich nature of these regions supports the idea that the proteins recognize structural features of these regions, such as the narrow minor grooves of the dominant A-tracts or DNA bends, rather than specific sequences themselves. Experiments have utilised the drug, distamycin, which binds tightly to narrow minor grooves of A-tracts, to show specific inhibition of SAR-protein binding.

So far the two most important SAR-binding proteins to have been studied are histone H1 and DNA topoisomerase II. SAR sequence motifs include strong topoisomerase II cleavage sites (Gasser and Laemmli, 1986), and topoisomerase II is abundant enough to fulfill a scaffold anchoring function (three copies exist on average per chromatin loop). *In vivo*, topoisomerase II preferentially acts at DNase I-hypersensitive sites, suggesting that its access to DNA is generally restricted to nucleosome-free regions (Laemmli *et al.*, 1992). Histone H1 and topoisomerase II may be interacting competitively for SAR binding, possibly together with other proteins involved in transcriptional regulation. Laemmli *et al.*

(1992) has proposed the "chromatin switch model" whereby AT-binding proteins compete with histone H1 for the SAR regions, and in so doing bring about chromatin opening.

1.6.5 Chromosome Condensation and Segregation at Mitosis.

The arrest at mitosis of yeast temperature-sensitive *top2* mutants incubated at the restrictive temperature, demonstrated that eukaryotic topoisomerase II, unlike topoisomerase I, is an essential enzyme necessary for cell cycle progression. A series of crucial studies revealed that topoisomerase II has two roles at mitosis, namely in chromosome condensation and in segregation of the replicated daughter chromatids (DiNardo *et al.*, 1984; Holm *et al.*, 1985; Uemura and Yanagida, 1986; Uemura *et al.*, 1987a; Adachi *et al.*, 1991).

Adachi *et al.* (1991) studied the role of topoisomerase II in chromosome condensation using chicken erythrocyte nuclei (which have a very low endogenous level of topoisomerase II) added to mitotic *Xenopus* egg extracts immunodepleted for the enzyme. These nuclei entered into mitosis but with partially condensed chromosomes. Addition of purified yeast topoisomerase II to these nuclei caused the chromosomes to condense. Dosage experiments suggested that the condensation process was dose-dependent, as low doses caused only partial and sectored condensation (Wood and Earnshaw, 1990; Adachi *et al.*, 1991). This supports the notion of a structural involvement of topoisomerase II in chromosome condensation. Indeed a recent report suggests that an interaction between topoisomerase II and certain centromeric SARs (see section 1.6.4) might nucleate and establish a polarity for the subsequent events leading to chromosome condensation (Kas and Laemmli, 1992). If topoisomerase II were able to complex other proteins and/or itself while binding SARs, then the bases of radial chromatin loops might be brought together in clusters. This function of topoisomerase II must be carefully regulated through the cell cycle.

However, further work showed that a catalytically active form of topoisomerase II was essential to drive chromosome condensation (Adachi *et al.*, 1991). Perhaps condensation is sterically impossible in the presence of catenated sister chromatids, or a cellular control mechanism exists to prevent condensation until sufficient decatenation has occurred. This does not preclude an additional structural role in condensation though.

Chromosome condensation is more readily observed and better defined in higher eukaryotic cells. Although topoisomerase II mutants are not available, topoisomerase II poisons can be used to block the activity of the enzyme in cultured mammalian cells. Treatment of G_2 phase cells with topoisomerase II poisons prevents chromosome condensation and entry into mitosis (Lock and Ross, 1990b; Roberge *et al.*, 1990; Sumner, 1992). VM26 also halts phosphorylation of histones H1 and H3, which is strictly correlated with chromosome condensation (Roberge *et al.*, 1990), and inhibits $p34^{cdc2}$ kinase, a major regulator of entry into mitosis (Lock and Ross, 1990b). It is not clear whether these changes are the direct result of an inhibition of topoisomerase II activity, or the result of the DNA damage induced by the topoisomerase II poison.

If topoisomerase II is inactivated after chromosome condensation, then yeast cells remain blocked at metaphase unable to achieve chromatid separation (Uemura *et al.*, 1987a). After release from such a block yeast cells show nondisjunction (Holm *et al.*, 1989), just as occurs in meiotic cells (Rose *et al.*, 1990). It appears to be the decatenating activity of topoisomerase II which is essential both for chromosome condensation and segregation, and that is why topoisomerase I cannot substitute in these processes.

In mammalian cells the requirement for topoisomerase II at anaphase for separation of chromatids is less clear. Sumner (1992) was not able to find a clear effect of topoisomerase II inhibitors on the segregation of chromosomes unless colchicine was added to block mitotic spindle formation. Downes *et al.* (1991), using much higher concentrations, showed that topoisomerase II inhibitors do, in fact, interfere with chromosome segregation in mammalian cells. It is possible though that these very high lethal doses have their effect on segregation due to the general unhealthiness of cells, rather than directly via topoisomerase II.

The later events of mitosis still appear to occur in mammalian cells even when chromatid separation is inhibited (Downes *et al.*, 1991). In other words, inhibition of topoisomerase II does not prevent exit from mitosis in mammalian cells, nor does it look as if it affects chromosome decondensation.

Interestingly, the newly discovered topo IV gene products, *ParC* and *ParE*, are essential for chromosome segregation in *E. coli* (Kato *et al.*, 1990; Adams *et al.*, 1992). DNA gyrase is also essential. It is possible that gyrase and topo IV have different substrate specificities, or may be

concerned with different reactions. In fact Topo IV may have a structural role analogous to that of eukaryotic topoisomerase II in the nuclear matrix, in that it can associate with both DNA and membrane (Kato *et al.*, 1988). Bacterial chromosomes are also folded in multilooped structures and organized into nucleoids in which the chromosome is segmented into topologically independent units.

1.7 Topoisomerases as Targets of Anti-Bacterial and Anti-Cancer Agents.

It gradually became clear in the 1980s that topoisomerase I and topoisomerase II are targets for a number of established and investigational antineoplastic agents. This led to the possibility of rapid screening for more potent analogues by *in vitro* testing, and more rational use of existing agents in the light of the biology of topoisomerases. As a consequence, a large slice of the research into topoisomerases was redirected into an understanding of drug-enzyme interaction, an investigation into the mechanisms of sensitivity and resistance to these "topoisomerase poisons", and to rational design of novel drugs. This is reviewed in Drlica and Franco, 1988; D'Arpa and Liu, 1989; Liu, 1989; Smith, 1990.

The discovery of eukaryotic topoisomerases as therapeutic targets followed the earlier identification of the bacterial topoisomerase II, DNA gyrase, as a major target for two classes of anti-bacterial agents: the coumermycin compounds acting through the GyrB subunit, and the quinolones through the GyrA subunit (as described in section 1.2). Indeed the quinolones appeared to trap the gyrase-DNA cleavable complex. Studies suggested that the drug, DNA and enzyme form a ternary complex in which the drug is interacting with both the DNA and enzyme (Shen and Pernet, 1985). The events between the formation of the abortive ternary complex and cell death are not completely understood, but the induction of SOS repair functions may be important (Drlica, 1984).

The action of the quinolones on *E. coli* DNA gyrase serves as a paradigm for the actions of many of the topoisomerase-targeting antitumor agents. Virtually all those so far identified also appear to inhibit the DNA rejoining step thereby causing an accumulation of the cleavable complex (Liu *et al.*, 1989). This reaction is reversible though, with the disappearance of the lesions upon removal of drug (Hsiang and Liu,

1989). The cytotoxic effects of these drugs appears to be manifested, not through a direct inhibition of topoisomerase activity, but through the damage to DNA that occurs because of formation of the cleavable complex. Hence the drugs are better called topoisomerase poisons, than inhibitors. Topoisomerase poisons also serve as powerful investigative tools owing to the unique reversible damage they introduce into chromosomal DNA.

The only clearly identified topoisomerase I poison is camptothecin, an alkaloid from the plant *Camptotheca acuminata*. This compound specifically interferes with the rejoining step catalysed by eukaryotic topoisomerase I, by forming a drug/enzyme/DNA ternary cleavable complex (Hsiang *et al.*, 1985). Impressive antitumor trials in animals led to brief clinical trials in the United States in the early 1970s, but the excessive toxic side-effects prevented its further development. However a number of analogues of camptothecin have now been characterized which do not have such severe side-effects, and look like promising anti-tumour agents.

In designing new drugs much emphasis is being put on finding out whether they are substrates for the well-characterized Multidrug Resistance (MDR) protein. This protein has been so-called because it is the best understood cellular mechanism leading to "multidrug resistance", i.e. the resistance of tumor cells to apparently unrelated drugs. It is a membrane-located pump which binds a wide variety of drugs and expels them from the cell (reviewed in Gottesman and Pastan, 1988). Camptothecin is not an MDR substrate, and one very promising analogue, 9-amino-camptothecin, which is far less toxic has been shown to produce long-term remissions in nude mice carrying human colon cancer xenografts (Giovanella *et al.*, 1989).

Topoisomerase II poisons fall into two classes, those that intercalate into DNA via planar ring structures, and those that do not appear to intercalate. DNA intercalating topoisomerase II poisons include the anthracyclines, Adriamycin and daunomycin, the anthracenediones, mitoxantrone and bisantrene, the amino acridine, m-AMSA (also called amsacrine), and also ellipticines, actinomycins and benzisoquinolinediones. The best known non-intercalator topoisomerase II poisons are the epipodophyllotoxins, etoposide (VP16) and teniposide (VM26).

All these drugs also interfere with the breakage-rejoining reaction by trapping the cleavable complex in a drug/enzyme/DNA ternary complex (Liu *et al.*, 1983; Tewey *et al.*, 1984; Robinson and Osheroff,

1990 and 1991). Except for the epipodophyllotoxins, the importance of intercalation has been shown in the excellent correlation between strength of intercalation and efficiency of cleavable complex formation (Bodley *et al.*, 1989). The intercalative drugs also tend to alter the preferred cleavage sites of topoisomerase II, pointing to an effect on the efficiency of base interaction with the enzyme, and supporting the model of a drug/enzyme/DNA ternary complex (reviewed in Capranico and Zunino, 1992).

A "misalignment model" has been proposed to explain how the intercalation of the drug into DNA promotes cleavable-complex formation by displacing the two transiently broken strands from their aligned position (Liu, 1989). According to this model, specific and high affinity of the drug for the enzyme is not required, which explains the diversity of drug structures of topoisomerase II poisons. This model does not explain the mechanism of action of the non-intercalators though. These must have more specific interactions with the enzyme itself. Although, etoposide has been shown to specifically inhibit the DNA religation step of the eukaryotic topoisomerase II reaction (Osheroff, 1989b), the molecular nature of this interaction has not been studied.

The cellular response to topoisomerase poisons and the mechanism of cell killing are far less well understood. Most groups have concentrated on whether the production of chromosomal breaks by topoisomerase poisons is the cause of cytotoxicity (Liu, 1989). However, in mammalian cells topoisomerase poisons not only lead to site-specific breakage of chromosomal DNA, but also to inhibition of DNA replication and RNA synthesis (Liu, 1989; Zhang *et al.*, 1988), elevated levels of sister chromatid exchanges (SCE) and other chromosomal aberrations (Lim *et al.*, 1986; Pommier *et al.*, 1988), and extensive degradation of chromosomal DNA (Kaufmann, 1989). The most compelling evidence that these poisons kill cells through a topoisomerase-mediated lesion is the correlation between high topoisomerase expression and increased sensitivity to these topoisomerase poisons (Davies *et al.*, 1988; Deffie *et al.*, 1989; Smith and Makinson, 1989; Giaccone *et al.*, 1992).

Topoisomerase poisons, like many other DNA damaging agents, cause G_2 delay or arrest in mammalian cells (Rao, 1980). However it does not appear that G_2 arrest is purely a result of strand breaks or inhibition of RNA synthesis (Rowley and Kort, 1989). It is possible that the G_2 arrest is the result of a cell cycle control checkpoint which blocks the cell from going into mitosis in response to the damage. The pivotal

protein involved in mitotic progression is the $p34^{cdc2}$ protein kinase, first identified in the fission yeast, *Scizosaccharomyces pombe* (reviewed in Nurse, 1990). Its kinase activity, as measured against the *in vitro* substrate histone H1, is cell cycle regulated with maximal activity at mitosis. The activation of $p34^{cdc2}$ appears to be dependent on both its association with cyclin proteins, and its own dephosphorylation on tyrosine and threonine residues. It is thought that phosphorylation of multiple substrates by the activation of $p34^{cdc2}$ initiates the events seen at mitosis, such as chromosome condensation, nuclear lamina disassembly and reorganization of the microtubule networks (Moreno and Nurse, 1990).

Cells arrested in G_2 by VP16, VM26 or as a result of DNA damage by irradiation, show a complete loss of $p34^{cdc2}$ kinase activity, as a result of blocking the tyrosine dephosphorylation of $p34^{cdc2}$, rather than its association with cyclins (Lock and Ross, 1990a; Roberge *et al.*, 1990; Lock, 1992). This inhibition of $p34^{cdc2}$ activity may be involved in the gross chromosomal fragmentation and subsequent cytotoxicity that follows etoposide treatment (Lock and Ross, 1990b).

The G_2 arrest-point corresponds to the time of highest topoisomerase II activity, and the point at which the cell is most sensitive to drug-induced DNA cleavage. However, maximal cytotoxicity occurs during S phase (Chow and Ross, 1987; Estey *et al.*, 1987). This indicates that the topoisomerase-mediated DNA breaks may be necessary but are not sufficient for cytotoxicity, and that other factors are likely to be important as well.

All topoisomerase poisons strongly inhibit DNA synthesis. Hsiang *et al.* (1989) suggested that it is the collision of the replication fork with the drug-induced cleavable complexes which results in replication fork arrest, and possibly breakage. These authors also suggested this could lead to G_2 arrest and cell death. Interestingly, aphidicolin, which blocks replicative fork movement, prevents the killing of cells by camptothecin (Wang and Liu, 1990).

The topoisomerase I poison camptothecin can reversibly inhibit RNA synthesis, most likely through the formation of cleavable complexes which block transcriptional elongation. It is also possible that inhibition of topoisomerase I relaxation activity causes inhibition of RNA synthesis.

The fragmentation of chromosomal DNA induced by topoisomerase poisons is characteristic of the mechanism of programmed cell death or "apoptosis" (for review Williams *et al.*, 1992). However, it is not clear

which topoisomerase poison-induced lesion is responsible for switching on this apoptotic pathway.

Whatever the mechanisms of cytotoxicity, the higher level of topoisomerase II activity in highly proliferating tumor cells (see section 1.9) is likely to be responsible for the efficacy of topoisomerase poisons in cancer chemotherapy. A major reason for studying the regulation of topoisomerases is to be able to improve this therapeutic window still further. For instance, it has been clearly shown that oestrogen treatment potentiates topoisomerase II-mediated cytotoxicity in an activated subpopulation of human breast cancer cells (Epstein and Smith, 1988). It is probable that the recruitment of a subset of the quiescent population into the cell cycle, with a concomitant increase in topoisomerase II levels in these cells, is the explanation for the increased cytotoxicity (Epstein *et al.*, 1989).

1.8 Topoisomerase II and Drug Resistance.

The evidence that topoisomerase II activity correlates with sensitivity of cells to topoisomerase II poisons is strong (see section 1.7). However, what is far more controversial is whether altered topoisomerase II expression in tumour cells actively contributes to the development of drug resistance. At present there is no good evidence that this is in fact the case in tumours; but the isolation of drug-resistant cell lines by chronic exposure to topoisomerase II poisons and the mapping of mutations to the topoisomerase II gene in some of these cell lines, suggests that drug resistance can indeed be achieved through altered topoisomerase II expression, at least in cultured cells (Beck and Danks, 1991). This form of drug resistance has been called at-MDR ("atypical" or "altered topoisomerase" multidrug resistance), to distinguish it from the classical MDR protein-induced multidrug resistance discussed in section 1.7.

Suggestions that altered topoisomerase II expression might lead to drug resistance, initially came from the observation that mutations in the bacterial DNA gyrase genes could lead to cross-resistance to many of the anti-bacterial agents which target the gyrase protein.

A large number of cell lines resistant to topoisomerase II poisons have now been isolated by many different groups (reviewed in Beck and Danks, 1991). These are too numerous to go into in detail, but the various

changes observed in topoisomerase II expression in these cell lines can be summarized into the following areas:

(1) Reduced levels of topoisomerase II mRNA.

(2) Reduced levels of topoisomerase II protein.

(3) Reduced levels of topoisomerase II activity.

(4) Altered association of topoisomerase II with the nuclear matrix.

(5) Altered patterns of topoisomerase II phosphorylation.

(6) Point mutations in the topoisomerase II gene.

(7) Altered requirement for ATP.

(8) Switch in topoisomerase II isozyme expression.

Many of the drug-resistant cell lines exhibit several of the above changes. For instance, a human leukemic CEM cell line selected for resistance to VM26, showed decreased topo II activity, decreased amount of nuclear matrix-associated protein, increased ATP requirement, decreased phosphorylation of topoisomerase II and a single base mutation resulting in a change of Arg_{449} to Gln (Danks *et al.*, 1989; Fernandes *et al.*, 1990; Bugg *et al.*, 1991).

Another point mutation was found in the topoisomerase II gene of two independent m-AMSA-resistant human myeloid leukemia cell lines, HL-60/AMSA and KBM-3/AMSA (Lee *et al.*, 1992). Here the mutation was of Arg_{486} to Lys. This is of great interest as the position of the Arg_{486} mutation in the human topoisomerase II p170 protein, corresponds in sequence to the *nal-31* point mutation in the *E. coli* GyrB subunit, which confers resistance to nalidixic acid. Because m-AMSA and nalidixic acid act on their respective targets by a common mechanism of trapping the cleavable complex, this supports the idea that human topoisomerase II is a major cellular target for m-AMSA and that Arg_{486} is involved in the mAMSA-mediated cleavable-complex trapping.

Not all resistant cell lines are equally resistant to all topoisomerase II poisons. The HL-60/AMSA human leukemia cell line described above for instance, showed 100-fold resistance to mAMSA, but only 2- to 3-fold resistance to etoposide (Zwelling *et al.*, 1989). This suggests that the non-intercalator, etoposide, has a completely different target site on the topoisomerase II protein. However, this means that different resistance mechanisms must explain the phenotype of some mutants such as a CHO cell line strongly cross-resistant to etoposide, mAMSA, mitoxantrone and Adriamycin (Glisson *et al.*, 1986b). In another CHO cell line, also cross-resistant to etoposide, m-AMSA, mitoxantrone and doxorubicin, the level

of resistance correlated to a reduced level of topoisomerase II protein and mRNA suggesting that reduced expression was the sole mechanism of drug resistance in this case (Webb *et al.*, 1991).

Whilst it is clear how point mutations or down-regulation of topoisomerase II mRNA, protein or activity could lead to resistance to topoisomerase II poisons, the mechanism behind other observations, such as altered phosphorylation levels, are less well understood. Whilst the CEM cell line described above had a decreased level of topoisomerase II phosphorylation, two VP16 resistant mutants of human cancer KB cells had increased levels of topoisomerase II phosphorylation (Takano *et al.*, 1991). This latter finding could be explained by studies done on the *Drosophila* topoisomerase II protein *in vitro*, which showed that phosphorylation accelerated the rate of the DNA religation step and reduced the level of drug-induced DNA cleavage products (DeVore *et al.*, 1992).

The potential for a switch in expression from one topoisomerase II isozyme to the other is a novel idea and has yet to be shown (see section 1.12 for discussion of the two isozymes). There has been one report though of a mitoxantrone-resistant HL-60 leukemia cell line associated with a reduced expression of the topoisomerase IIβ p180 isozyme (Harker *et al.*, 1991).

As a generalization, it would seem that point mutations in the coding region of the gene lead to highly resistant forms of topoisomerase II, whereas moderately resistant cell lines are usually explained by more subtle changes in topoisomerase II expression. As a highly resistant form of topoisomerase II has yet to be found in a human tumour cell, it brings into question the relevance of point mutations in drug-resistance in the patient. It also highlights the problem of using chronic exposure regimes to isolate drug-resistant cell lines.

1.9 Differential Expression of Topoisomerase II in Response to Proliferation Status, Transformation and Cell Cycle Progression.

In exponentially growing cells topoisomerase II is a fairly abundant protein with up to 10^6 copies per cell. Many reports have suggested that topoisomerase II is a specific marker for cell proliferation. A link between topoisomerase II activity and cell proliferation was first suggested by studies in which enzyme activity was measured in proliferating and growth-arrested cells. Duguet *et al.* (1983) showed that

the low level of enzyme activity in liver increased significantly after partial hepatectomy. Whilst Tandou *et al.* (1984) found a low level of enzyme activity in G_0 guinea pig lymphocytes that increased dramatically upon mitogenic stimulation. Furthermore, Sullivan *et al.* (1986) observed an increase in drug-induced cleavable complexes in CHO cells following re-entry into the cell cycle after serum-starvation induced arrest.

A clear correlation between topoisomerase II protein level and proliferative status was demonstrated by Heck and Earnshaw (1986) by immunoprecipitating topoisomerase II from a series of chicken cell types. In both normal and transformed cells, the level of topoisomerase II was greatly reduced when cells ceased proliferation. This occurred not only in cells which became quiescent but also in cells which remained transcriptionally active (myotubes). Conversely, mitogenic stimulation of circulating blood lymphocytes, which initially lacked detectable topoisomerase II, expressed topoisomerase II *de novo*. Appearance of the enzyme paralleled the onset of DNA replication. This adds weight to the suggestion that topoisomerase II is not required in large amounts for transcription but is required for DNA replication.

However, whilst the evidence from vertebrate cells is in agreement that topoisomerase II is a good proliferation marker, a report suggested that in *Drosophila* this may not be the case. Using biochemical and immunological techniques, Whalen *et al.* (1991) concluded that apart from in adult males, topoisomerase II content appeared to remain at a relatively constant level throughout *Drosophila* development. It is possible then that topoisomerase II is regulated in a fundamentally different manner in *Drosophila* compared to vertebrates. This has important implications when considering whether lower eukaryotes, such as *Drosophila*, are good models for regulation of human topoisomerase II.

Equally pertinent is the fact that non-vertebrate species appear to have only one topoisomerase II isozyme. This may explain why *Drosophila* topoisomerase II appears to be less proliferation-dependent, as it has to fulfill both the roles carried out by the two vertebrate isozymes. Studies described in section 1.12 show that only the topoisomerase IIα isozyme of vertebrates appears to be strongly proliferation-dependent, whereas the topoisomerase IIβ isozyme level may in fact be slightly higher in non-proliferating cells.

Topoisomerase II levels also appear to be elevated in vertebrate cell lines upon transformation. Transformed chicken fibroblasts had a pool of topoisomerase II molecules roughly 5-fold larger than that of their

normal counterparts (Heck *et al.*, 1988). This was associated with a 4-fold increase in protein half-life, as determined by a pulse-chase labelling experiment. The half-life of the protein in asynchronous normal chicken cells was 3.3 hours, whilst in transformed cells it was 12.0 hours. Kroll and Rowe (1991) measured the half-life of topoisomerase II from the transformed human cell line, HeLa, as 27 hours, suggesting it to be remarkably stable in this cell line. Hence, the elevated topoisomerase II protein levels seen in transformed cells may be primarily a result of increased protein stability. It would be interesting to know whether this is the result of post-translational modification of the protein.

It is not clear whether the increased topoisomerase II protein level seen on transformation in cell lines is reflected in the neoplastic tissue in a tumour. An interesting study was carried out comparing topoisomerase II expression in normal rat prostate with rat prostatic adenocarcinomas (Nelson *et al.*, 1987). Here topoisomerase II levels were found to be elevated in the tumor tissues compared to normal by activity measurements, immunoblot analysis and immunohistochemical studies. However, Holden *et al.* (1990) used a quantitative filter binding assay to demonstrate that whilst the highest level of enzyme activity in neoplasms was found in those that clinically behaved in an aggressive manner and had a high proliferative state, the protein level was still within the same range as that seen in normal tissues. Therefore, proliferative state appears to be a much better marker for topoisomerase II levels in a tumour than neoplastic state.

The response of topoisomerase II protein levels to proliferation and cell cycle position was studied by Hsiang *et al.* (1988). Topoisomerase II levels in primary human skin fibroblasts or mouse NIH-3T3 fibroblasts decreased to an undetectable level following serum starvation or growing to saturation density. The protein could then be returned to the original level by serum refeeding or subculturing respectively, confirming topoisomerase II protein as a proliferation marker. However, these authors found that topoisomerase levels in transformed cells like HeLa, L1210 cells or SV40 T-antigen transformed COS-1 cells, whilst being maintained at high levels, are much less sensitive to growth conditions. They also found that the topoisomerase II level did not vary much across the cell cycle of synchronized HeLa cells. Whilst this suggests that DNA topoisomerase II is regulated at the G_0-G_1 transition, it does not necessarily mean that the level in nontransfomed cells, unlike HeLa, is not also going to be regulated at other points in the cell cycle.

Indeed, Heck *et al.* (1988) used antibodies to look at cell cycle expression of topoisomerase I and II in normal chicken MSB-1 cells, and observed some interesting changes in topoisomerase II, but not topoisomerase I, expression. Following synchronization by centrifugal elutriation, it was seen that the level of topoisomerase II starts to increase just prior to, or at the onset of, DNA replication, continues to increase through S and G_2 phase, and peaks in the late G_2/M population with a level greater than 3-fold more than in G_1. As cells progressed from mitosis back into G_1, there was a rapid degradation of topoisomerase II resulting from a 7-fold decrease in the enzyme's half-life. This is noteworthy in light of the suggestion that topoisomerase II is not necessary for exit from mitosis in mammalian cells (Downes *et al.*, 1991). A similar pattern of topoisomerase II protein expression across the cell cycle was seen in mouse Swiss 3T3 cells (Saijo *et al.*, 1992).

Disassembly of the mitotic chromosome scaffold during chromosome decondensation may lead to the sudden instability in topoisomerase II. It is also possible though that topoisomerase II does play a part in the timing of chromosome decondensation at the end of mitosis, as decondensation may not be able to take place until topoisomerase II has been degraded. Whilst it is likely that the increase in protein from G_1 to G_2/M is the result of transcriptional upregulation (Saijo *et al.*, 1992), the sudden change in topoisomerase II stability at the end of mitosis is likely to be the result of changes in protein modifications. Moreover, there is evidence that phosphorylation of topoisomerase II may be similarly regulated across the cell cycle (Heck *et al.*, 1989; Saijo *et al.*, 1992) as discussed in section 1.11.

1.10 Regulation of Topoisomerases at the Transcriptional Level.

Despite the clear changes in topoisomerase II expression in response to proliferation, transformation and cell cycle progression discussed in the previous section, there have been surprisingly few studies on regulation of topoisomerase II at the transcriptional level. So far, regulation of topoisomerase II mRNA has been shown in response to proliferation (Hsiang *et al.*, 1988; Hochhauser *et al.*, 1992), transformation (Woessner *et al.*, 1990), viral infection (Benson and Huang, 1990), and to certain growth factors, such as oestrogen and G-CSF (Towatari *et al.*, 1990).

Decreased transcription of topoisomerase II mRNA has also been shown in some cell lines with reduced sensitivity to topoisomerase II poisons. An Adriamycin-resistant P388 leukemia cell line had a 7- to 8-fold reduction in the topoisomerase II transcript level as measured by Northern blotting (Deffie *et al.*, 1989), whilst a CHO etoposide-resistant cell line also had reduced levels of topoisomerase II mRNA (Webb *et al.*, 1991). In both these cases the reduction in mRNA correlated with degree of resistance (see section 1.8).

Topoisomerase I expression appears to remain remarkably constant under most conditions. A few reports suggest some transcriptional regulation; for instance, its mRNA was transiently elevated, up to tenfold, during mitogenic or TPA stimulation of human skin fibroblasts, although without any significant change in the protein level (Hwong *et al.*, 1989). In general, there is much more data on regulation through post-translational mechanisms of both topoisomerase I and II.

The recent cloning and characterization of the promoter region of human topoisomerase IIα by Hochhauser *et al.* (1992) opens up the possibility to identify factors or conditions which stimulate transcription from this promoter using fusions of the promoter to a bacterial reporter gene, such as chloramphenicol acetyltransferase.

1.11 Regulation of Topoisomerases by Post-Translational Modifications.

Post-translational modifications can alter the stability of a protein, its activity as an enzyme, or its ability to interact with other proteins or DNA (Krebs and Beavo, 1979). Clearly the transcriptional regulation described in section 1.10 cannot explain all the changes in protein levels seen in response to different cellular conditions. For instance, the depletion of topoisomerase II protein seen during a glucose-regulated stress response was not accompanied by a decline in mRNA levels (Shen *et al.*, 1989). A decreased stability as a result of post-translational modifications might be the answer. However, all the studies carried out so far have concentrated on the effect of modifications on the enzymatic activity or DNA binding efficiency of the topoisomerases (reviewed by Higgins *et al.*, 1990).

Both eukaryotic topoisomerase I and topoisomerase II appear to be regulated by post-translational modifications and in particular by phosphorylation and poly(ADP-ribosylation). Early studies concentrated on topoisomerase I. A phosphoprotein of approximately 110kDa was

purified from ^{32}P-labelled Novikoff hepatoma cells (Durban *et al.*, 1981) and subsequently identified as topoisomerase I (Durban *et al.*, 1983). A protein kinase that phosphorylated topoisomerase I leading to a 3- to 5-fold stimulation in its relaxation activity was purified from an African Burkitt's lymphoma cell line (Mills *et al.*, 1982). This kinase, shown to be casein kinase II, phosphorylated topoisomerase I on serine *in vitro* within the same tryptic peptide as was phosphorylated *in vivo* (Durban *et al.*, 1985). This suggested that topoisomerase I was phosphorylated *in vivo* by casein kinase II on one specific site.

In *Xenopus* ovaries phosphorylation of DNA topoisomerase I may be obligatory for enzyme activity. Relaxation activity was lost upon treatment with phosphatase, but regained after treatment with ATP and a casein kinase II activity (Kaiserman *et al.*, 1988). Phosphorylation of topoisomerase I was reported to be essential for either the DNA binding or cleavage step, as dephosphorylated topoisomerase I failed to nick DNA in the presence of camptothecin (Kaiserman *et al.*, 1988).

However, mammalian topoisomerase I has also been shown to be phosphorylated *in vitro*, and its activity stimulated, by protein kinase C (Pommier *et al.*, 1990). Moreover, a 2 hour stimulation of murine 3T3 cells with 100ng/ml TPA led to a 4-fold increase in phosphate incorporation into topoisomerase I *in vivo*, and the major tryptic peptides from topoisomerase I in TPA-stimulated 3T3 cells comigrated with the same phosphorylated peptides from topoisomerase I phosphorylated by PKC *in vitro* (Samuels and Shimizu, 1992). Hence, it appears that topoisomerase I is the target for multiple protein kinases.

The same has been shown to be true for eukaryotic topoisomerase II. Sander *et al.* (1984) first purified a kinase activity together with *Drosophila* topoisomerase II that phosphorylated the topoisomerase II protein besides other exogenous substrates like the histones and casein. Phosphoserine was the predominant phosphorylation on topoisomerase II. Another subsequent study has reported the copurification of kinase activity with mouse topoisomerase II (Saijo *et al.*, 1990).

Drosophila topoisomerase II was shown to be an *in vitro* target for both casein kinase II (Ackerman *et al.*, 1985) and for protein kinase C (Sahyoun *et al.*, 1986). Both these phosphorylations gave phosphoserine as the only acceptor residue and both led to an increase in enzymatic activity. Two to three phosphates were incorporated per homodimer of topoisomerase II by casein kinase II; whilst approximately 1 mole of phosphate was incorporated per mole of topoisomerase II by protein

kinase C, suggesting just one target site for this kinase in *Drosophila*. The reaction by casein kinase II was moderately specific with an apparent K_m of 0.4μM for topoisomerase II, whilst that for protein kinase C was stronger with a K_m of 0.1μM. Sayhoun *et al.* (1986) also showed phosphorylation *in vitro* of topoisomerase II by calmodulin-dependent protein kinase, but not by cAMP-dependent protein kinase (PKA).

Phosphorylation of eukaryotic topoisomerase II by casein kinase II and protein kinase C was then shown *in vivo* in *Drosophila* (Ackerman *et al.*, 1988) and in the sponge *Geodia cydonium* (Rottmann *et al.*, 1987), respectively. In *Drosophila* again only phosphoserine was seen *in vivo*, and casein kinase II was identified as the major kinase responsible by a series of biochemical assays on cell extracts. The report in *G. cydonium* showed stimulation of the level of phosphorylation of topoisomerase II by TPA treatment of cells.

Phosphorylation of topoisomerase II generally leads to an increase in the specific activity of the enzyme, whilst dephosphorylation has always reduced the specific activity. Phosphorylation by casein kinase II led to an activation of threefold over dephosphorylated enzyme, although dephosphorylated enzyme was still active (Ackerman *et al.*, 1985). The activation of *G. cydonium* topoisomerase II by protein kinase C gave a 2.5 fold increase in enzymatic activity (Rottmann *et al.*, 1987). Likewise, phosphorylation of quail oviduct topoisomerase II *in vitro* by casein kinase II or protein kinase C each led to a 2- to 3-fold increase in specific activity (Schroder *et al.*, 1989). More dramatically, phosphorylation by the co-purifying kinase present in a mouse preparation of topoisomerase II led to an 8.6-fold increase in specific activity over the unmodified enzyme (Saijo *et al.*, 1990). Treatment of the topoisomerase II with phosphatase virtually abolished the activity.

A number of recent reports have looked at phosphorylation of vertebrate topoisomerase II. Heck *et al.* (1989) demonstrated cell cycle-dependent phosphorylation of chicken topoisomerase II, with the level of ^{32}P-incorporation *in vivo* being higher in the G_2+M fraction than earlier in the cell cycle. They suggested that phosphorylation of the 170kDa topoisomerase II protein may be involved in the activation of the enzyme for its role in chromosome condensation or sister chromatid segregation at mitosis. Concomitantly, dephosphorylation of the enzyme, before entry back into G_1, may be important for the dramatic decrease in stability of topoisomerase II seen at this point in the cell cycle; this may be involved in chromosome decondensation and exit from mitosis.

Another study looked at cell cycle-dependent phosphorylation in mouse cells (Saijo *et al.*, 1992) and showed similar changes in phosphorylation levels with an increase beginning in late G_1 and the peak levels being reached again in late G_2/M. The topoisomerase II protein level in these cells followed a similar pattern, as explained earlier (section 1.9). However, inhibition of DNA synthesis by aphidicolin blocked the increase in the level of phosphorylation but not the protein. This suggested that the level of phosphorylation was independent of the level of protein, and that phosphorylation of topoisomerase II may be tightly linked with the enzyme's requirement in DNA replication.

Phosphorylation has also been demonstrated for the human topoisomerase II protein (Kroll and Rowe, 1991). Topoisomerase II was shown to be a phosphoprotein in HeLa cells with phosphoserine again the only acceptor residue. Not only did the protein appear to have a very long half-life (27 hours) in these cells, but the phosphorylation event was relatively stable with a half-life of 17 hours. This suggested that, like the protein, the level of phosphorylation in these transformed cells may not be subject to cell cycle regulation. It should be stressed that the situation may be completely different in untransformed cells.

None of these investigations into vertebrate topoisomerase II phosphorylation has made any attempt to distinguish between the two different isozymes recently identified (see section 1.12). As the isozymes appear to be differentially regulated and as they appear to have quite different biological roles within the cell, it is highly likely that they will be regulated in quite separate ways by phosphorylation.

The most comprehensive recent study on phosphorylation of eukaryotic topoisomerase II was carried out in the yeast, *Saccharomyces cerevisiae* (Cardenas *et al.*, 1992). Many of the earlier results seen in other eukaryotes were shown to be the same in yeast. For instance, levels of phosphorylation are higher in mitotically-arrested yeast cells than in G_1-arrested cells. Interestingly, phosphothreonine as well as phosphoserine is detected in yeast topoisomerase II and the proportion of phosphothreonine goes up at mitosis. In an elegant experiment it was shown that topoisomerase II phosphorylation was nearly undetectable in a yeast casein kinase II temperature-sensitive mutant at the restrictive temperature. Furthermore, two dimensional tryptic phosphopeptide maps showed that the major sites phosphorylated *in vivo* were phosphorylated *in vitro* by casein kinase II. These sites are located in the extreme C-terminus of the protein, the region absent in prokaryotic and

highly divergent in eukaryotic topoisomerase II (see section 1.4). They also showed *in vitro* phosphorylation of yeast topoisomerase II by protein kinase C and the cell cycle-regulated kinase $p34^{cdc2}$.

There have been a few reports over the last ten years of other phosphorylation reactions in topoisomerases, including tyrosine phosphorylations and autophosphorylation of prokaryotic topoisomerases. One group reported tyrosine phosphorylation of *E. coli* topoisomerase I and DNA gyrase, and calf thymus topoisomerase I and II, by the Rous sarcoma virus transforming gene product, $pp60^{src}$ (Tse-Dinh *et al.*, 1984; Goldberg *et al.*, 1985). In the case of topoisomerase I this led to a 90% inhibition of its relaxation activity suggesting that it was the active-site tyrosine involved in covalent binding to DNA that was phosphorylated. There have since been no further reports on this phosphorylation.

Bacteriophage T4 type II topoisomerase and the GyrB subunit of DNA gyrase have protein kinase activity which can lead to autophosphorylation (Higgins *et al.*, 1990). It is not clear whether these activities are a manifestation of the intrinsic catalytic mechanism in which ATP is cleaved as DNA strand passage occurs, or whether it actually serves some regulatory role. Eukaryotic type II topoisomerases do not possess the consensus sequences shown to be present in the kinase domain of all protein kinases identified so far (Hanks and Quinn, 1991).

Whilst phosphorylation has been by far the most heavily studied modification of topoisomerase proteins, both topoisomerase I and II are thought to be poly (ADP-ribosylated) (Ferro *et al.*, 1983; Darby *et al.*, 1985) This modification is carried out by a nuclear enzyme, poly (ADP-ribose) synthetase, which has been extensively characterized in several eukaryotic systems (Ueda, 1987). After the initial transfer of an ADP-ribose moiety from NAD to the substrate by the synthetase, more ADP-ribose residues are added to form a chain of ADP-ribose monomer units. *In vivo* double-strand and single-strand breaks are absolutely required to activate poly (ADP-ribose) synthetase; but once the DNA has been repaired, the poly (ADP-ribose) modification is quickly removed by the enzyme poly (ADP-ribose) glycohydrolase (Ueda, 1987).

The DNA relaxation activity of topoisomerase I, purified from calf thymus, was abolished following poly (ADP-ribosylation) (Jongstra-Bilen *et al.*, 1983). Calf thymus topoisomerase II activity is also inhibited by poly (ADP-ribosylation) (Darby *et al.*, 1985). It is thought that the inhibition of these and other DNA-binding proteins by poly (ADP-ribosylation) is caused by the great increase in negative charge, reducing the proteins

ability to bind DNA as a result of this modification. DNA topoisomerase I is thought to be the major target for poly (ADP-ribose) synthetase in the cell and this may be due to its role in transcription. Stimulation of the poly (ADP-ribose) synthetase by DNA strand breaks could inhibit topoisomerase I and, in so doing, reduce the rate of gene transcription allowing time for DNA repair to occur. In a clever feedback mechanism, the poly (ADP-ribose) synthetase, in the absence of DNA strand breaks, poly (ADP-ribosylates) itself, reducing its own ability to bind DNA.

1.12 Mammalian Topoisomerase IIα and β Isozymes: Differences in Gene, Protein Biochemistry and Regulation.

Studies on yeast *top2* mutants indicated that there is only one topoisomerase II enzyme required in lower eukaryotes to carry out the essential chromosome functions, such as condensation and segregation. In contrast, two distinct isoforms of topoisomerase II have now been identified in mammalian cells (Drake *et al.*, 1987; Drake *et al.*, 1989). These have been designated topoisomerase IIα, which encodes the 170kDa or p170 protein, and topoisomerase IIβ, which encodes a 180kDa or p180 protein. Not only are these two isozymes encoded by different genes on different chromosomes, but they appear to have different biochemical and pharmacological properties, and to be independently regulated.

The human topoisomerase IIα gene was cloned and sequenced by Tsai-Pflugfelder *et al.* (1988) and was mapped to chromosome 17q21-22. Partial human cDNAs of the topoisomerase IIβ gene were then cloned by Chung *et al.* (1989) and by Austin and Fisher (1990), and a full-length clone obtained by Jenkins *et al.* (1992). This gene has been localized to the human chromosome 3p24 (Jenkins *et al.*, 1992). Human cDNAs complementary to unique sequences in the genes of the two isozymes, cross-hybridized to unique sequences of human, rodent and chicken cDNAs in Southern blots, but not in yeast or Drosophila (Tan *et al.*, 1992). This suggests that the existence of these two isozymes may be restricted to vertebrate species.

Whilst the human topoisomerase IIα protein is 1530 amino acids in length, the human topoisomerase IIβ gene would code for a protein of 1621 amino acids (Figure 3). There is 72% amino acid sequence identity between the two human isozymes, with the greatest divergence at the extreme N-terminus and over a C-terminal domain comprising nearly

25% of the total protein. As these are the regions likely to be targets for post-translational modifications, it explains how the two isozymes can be differentially regulated.

Separate purification of the two isozymes allowed their biochemical and pharmacological properties to be measured (Drake *et al.*, 1989). Both enzymes performed type II topoisomerase reactions with equal dependence on ATP. However relaxation of a supercoiled plasmid substrate by topoisomerase IIβ protein was highly processive, while topoisomerase IIα protein performed the same reaction in a distributive manner. This suggests that topoisomerase IIβ has either a more rapid rate of catalysis or a slower dissociation rate from the DNA. However, a key factor in processivity is the salt concentration of the reaction. At lower salt concentrations the enzyme is more processive, while higher concentrations make the enzyme behave more distributively (Osheroff *et al.*, 1983). Yet optimum activity of topoisomerase IIβ occurs at a higher salt concentration than topoisomerase IIα, so the greater processivity of the β isozyme is unlikely to be due to a slower dissociation rate. VM26 and merbarone had a much greater selectivity towards the topoisomerase IIα protein, and the two isozymes appeared to have different preferred sites of cleavage.

Isozyme-specific antibodies have been used to look at the protein levels of the two forms under different conditions and in different cell types. Woessner *et al.* (1990) compared the expression of the two isozymes before and after *ras*-transformation of NIH-3T3 cells. The amount of topoisomerase IIα protein increased dramatically upon transformation and the increase in protein level could be correlated to an increase in mRNA, suggesting that these changes are regulated at the transcriptional level. However, the amount of topoisomerase lIβ mRNA decreased 45-60% upon transformation, yet the amount of topoisomerase IIβ protein remained about the same, suggesting that the topoisomerase IIβ was being regulated at the post-transcriptional level. The topoisomerase IIβ protein from ras-transformed cells was, if anything, less stable than in normal cells though, pointing towards a change in the mRNA stability or rate of translation being the explanation.

ras-transformed cells were also more sensitive to drugs like VM26 and merbarone which target the topoisomerase IIα protein, supporting the increased ratio of topoisomerase IIα to β. However, the increased inhibition by these drugs was greater than would be expected if the change in isozyme ratio was the only cause. One explanation is that

transformation also causes topoisomerase IIα to become more sensitive to the effects of these drugs, possibly through a change in its phosphorylation state.

In agreement with studies done on topoisomerase II prior to the identification of the two isozymes, the level of each isozyme also appears to be dependent upon proliferation status and cell cycle position (Woessner *et al.*, 1991). When serum-starved NIH-3T3 cells were stimulated to enter the cell cycle by addition of fresh medium, the amount of topoisomerase IIα was undetectable until the cells reached late S-phase, peaked in G_2-M phase cells, and decreased as the cells completed mitosis. The level of topoisomerase IIβ did not vary very much across the cell cycle, perhaps just peaking in late G_1. Forcing cells into quiesence by serum starvation, led to a drop in topoisomerase IIα protein levels until it became undetectable. The level of the topoisomerase IIβ protein however dropped only marginally and could still be detected even after all cells had reached G_0. It is likely that most of the earlier studies into proliferation and cell cycle dependence, which did not distinguish between the two isozymes, were looking only at topoisomerase IIα, as much of this work was done using antibodies specifically raised to purified topoisomerase IIα protein.

The different patterns of expression of the two isozymes suggest that they perform different functions within the cell. It is suggested that the topoisomerase IIα protein is involved in events leading up to mitosis, such as chromosome condensation and chromosome segregation. The topoisomerase IIβ isozyme is more likely to be involved in some aspect of transcription. Indeed a report from Zini *et al.* (1992) showed that the β isozyme of topoisomerase II is localized in the nucleolus, where it may perform a structural role in the spatial organization and regulation of expression of the ribosomal RNA genes. Topoisomerase IIα is more generally localised in the nucleoplasm. Previous studies in *S. pombe* had revealed a structural role for the yeast topoisomerase II in the nucleolus (Hirano *et al.*, 1989). It is possible that topoisomerase IIβ fulfils this role in human cells, in a manner analogous to that in which the human topoisomerase IIα isozyme is thought to be a structural component of the nuclear matrix (see section 1.6.4).

A number of groups are now looking at the different patterns of expression of the two isozymes in tissues and in tumours. One recent report looking in mouse, showed that expression of the α isozyme was high in proliferating tissues, such as bone marrow and spleen, but low or

undetectable in other tissues (Capranico *et al.*, 1992). In contrast, high or intermediate levels of expression of the β isozyme were seen in a variety of both proliferating and non-proliferating tissues, including lung and brain. The same paper reported a general decrease in tissue expression of both isozymes during murine development, with levels highest in early embryogenesis. There were slight post-natal inductions of topoisomerase IIα expression in liver, and topoisomerase IIβ expression in brain, suggesting tissue-specific roles for the two different isozymes in the development of differentiated tissues such as the brain or liver.

Jenkins *et al.* (1992) used RNase protection to measure topoisomerase IIβ mRNA levels in a panel of human tumor cell lines. Topoisomerase IIβ mRNA was detectable in haemopoietic, epithelial and fibroblast cell lines, although to different extents, with U937 cells (a promonocytic leukaemia cell line) showing a particularly high level. In general though they did not see any obvious relationship between the level of expression of the two genes, and concluded that it was unlikely that strong coordinate regulation of the two topoisomerase II genes occurs in human tumor cell lines under normal growth conditions.

1.13 Strategy for the Study of Phosphorylation of the Human Topoisomerase II Proteins.

So far, all the phosphorylation studies on topoisomerase II have been carried out either in lower eukaryotes, such as yeast or *Drosophila*, which appear to only have one topoisomerase II isozyme, or in vertebrates, but without addressing the existence of the two differentially-regulated isozymes. Hence, the aim of this work was to identify which kinases are involved in phosphorylation of the individual isozymes of human topoisomerase II protein *in vivo* and how this phosphorylation regulates their biological activity and function.

In order to identify a topoisomerase II isozyme as a true substrate for a kinase *in vivo*, three basic criteria need to be met. Firstly, the purified kinase should be able to efficiently phosphorylate the purified topoisomerase II isozyme *in vitro*. Secondly, the phosphorylation sites generated *in vitro* by a purified kinase should match the phosphorylation sites *in vivo*, and phosphorylation of these sites *in vivo* should change in response to conditions which stimulate or inhibit this kinase. Thirdly, phosphorylation of the topoisomerase II isozyme should cause changes in

its biochemical activity that have appropriate consequences for its topoisomerase II function *in vivo*.

Initially, this required the preparation and characterization of antibodies which specifically recognized each isozyme of human topoisomerase II in order that they could be studied independently. At the same time, for the purpose of carrying out *in vitro* phosphorylation studies, we developed a procedure for purifying to near-homogeneity the topoisomerase IIα isozyme. Using the antibodies and purified protein as tools, we were then able to carry out a series of experiments to identify the protein kinases which lead to phosphorylation of the topoisomerase IIα isozyme *in vivo* as well as *in vitro*. We were also able to do some preliminary studies on the phosphorylation of the topoisomerase IIβ protein.

Having identified the important kinases *in vivo*, we proceeded to look at how the phosphorylation state regulates the activity of the topoisomerase II enzyme and which domains of the topoisomerase II protein are involved in this regulatory role. Ultimately, we aimed to build up a model of how regulation through phosphorylation might enable the individual isozymes to fulfill their apparently independent biological roles within the cell.

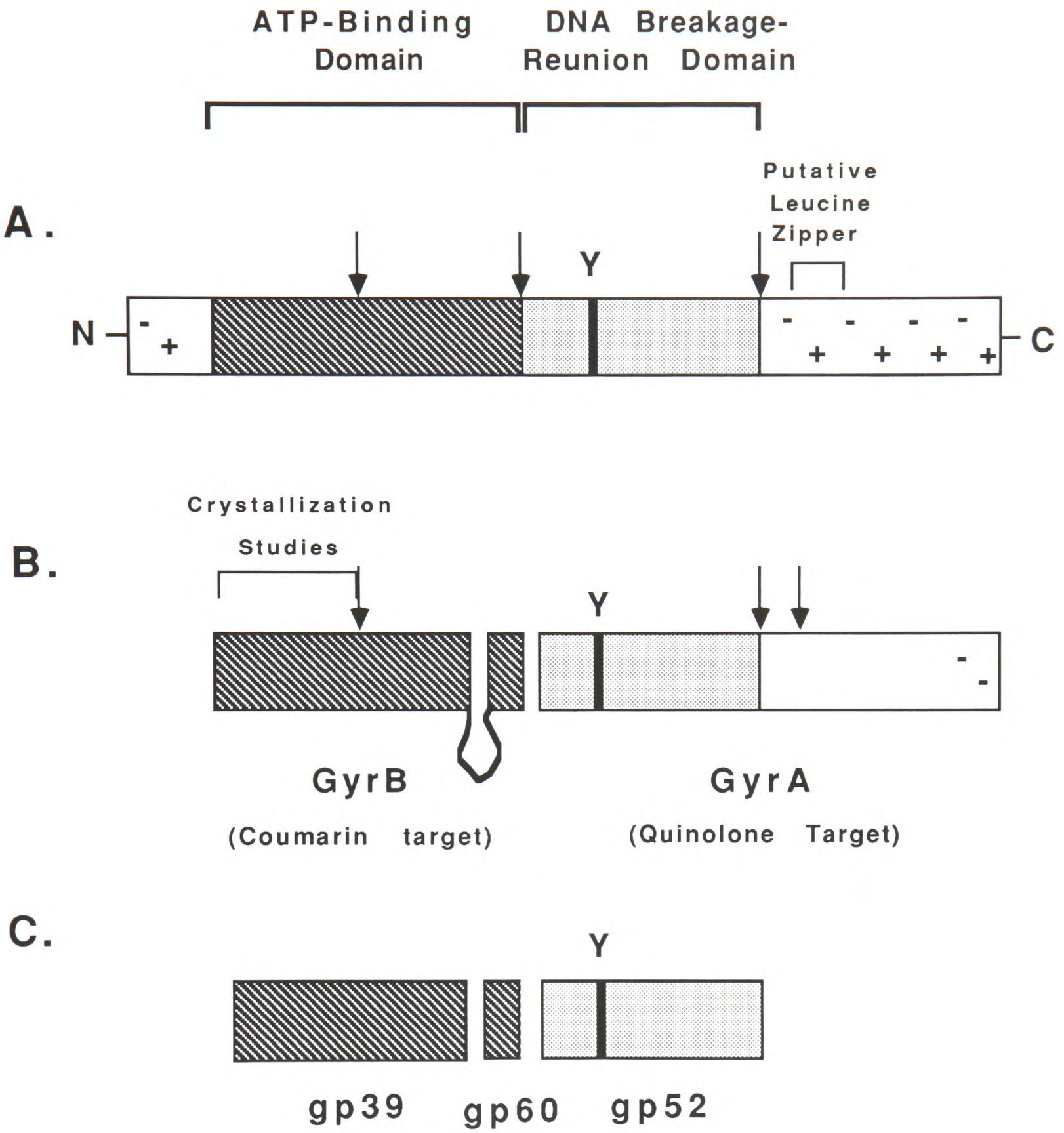


Figure 1 The domain structure of prokaryotic and eukaryotic type II topoisomerases. The Gyr A and B subunits of *E. coli* DNA gyrase (B), and the 3 subunits of the T4 phage topoisomerase II (C) are aligned with the yeast topoisomerase II (A) to highlight the conservation of the ATP-binding and DNA breakage-reunion domains. The positions of the active-site tyrosine (Y), protease sensitive sites (arrows), and highly charged sequences (-/+), are shown. The region of the Gyr B subunit that has been crystallized and the position of the proposed leucine zipper dimerization region are also indicated.

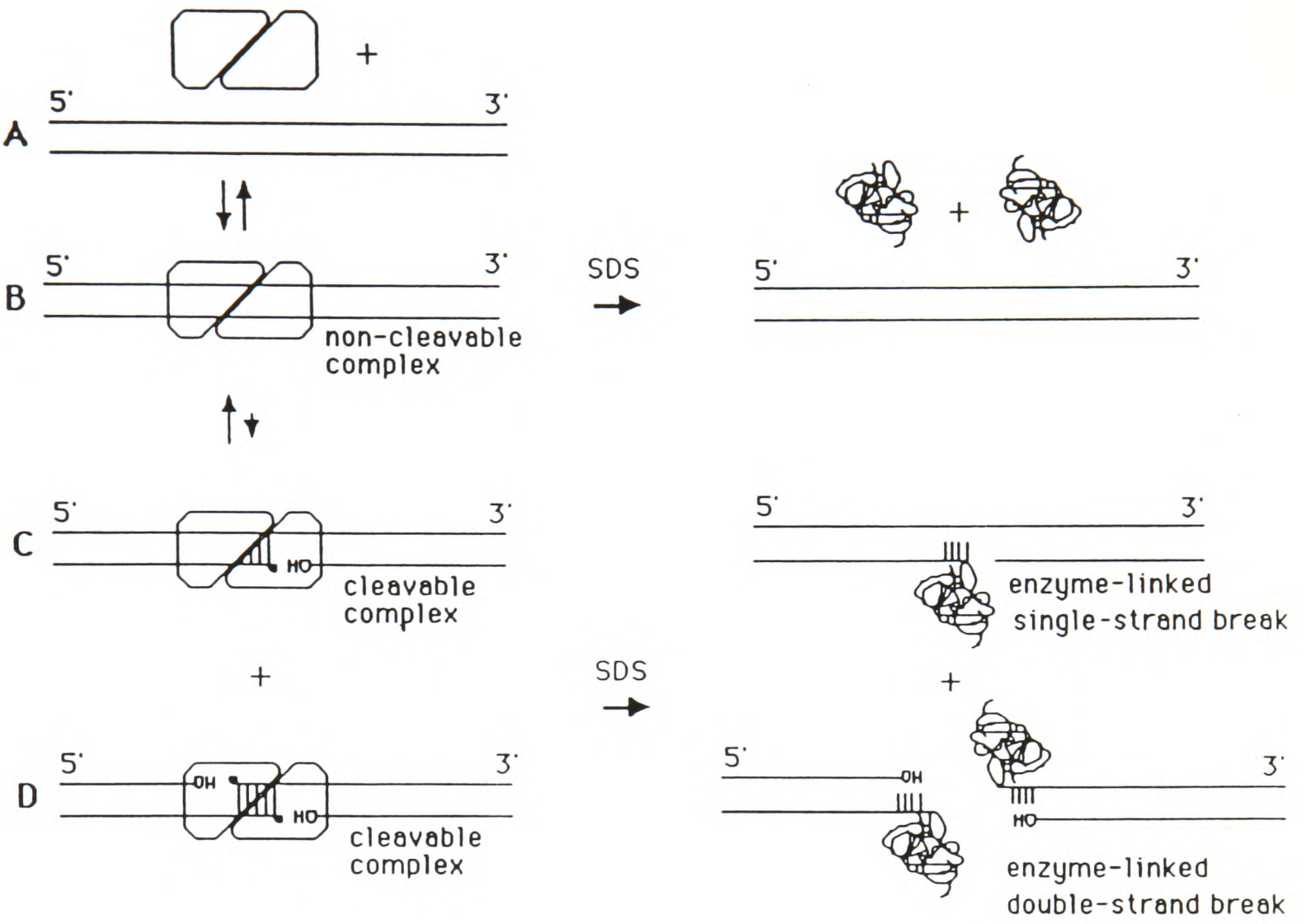


Figure 2 The enzymatic mechanism of eukaryotic type II topoisomerase. Reproduced from D'Arpa and Liu (1989). (A) Topoisomerase II homodimer and substrate DNA. (B) Enzyme bound to DNA in noncleavable complex. (C and D) Enzyme bound to DNA in the cleavable complex. Treatment of the cleavable complex with SDS or alkali can result in a single- (C) or double-strand (D) break in which each 5' broken DNA strand is linked to denatured enzyme. The reaction is completed by passage of a duplex through the double-strand break, religation of the break and enzyme turnover.

```
TopIIα    1          mevsplqpvnenmqVN kiKK        neDakKrLSVERiYQKKTQLEHILL
                                   ||   ||          |  | |||||  ||||||||||||
TopIIβ    1 maksggcgagagvgggngaltwVNnaaKKeesetanknDssKkLSVERvYQKKTQLEHILL

TopIIα   46 RPDTYIGSVElvTQqMWVYDEDVGiNyREVTFVPGLYKIFDEILVNAADNKQRDpkMsCIr
            ||||||||||  || |||||||| | ||||||||||||||||||||||||||  | ||
TopIIβ   62 RPDTYIGSVEplTQfMWVYDEDVGmNcREVTFVPGLYKIFDEILVNAADNKQRDknMtCIk

TopIIα  107 VtIDPEnNlISIWNNGKGIPVVEHKVEKmYVPALIFGQLLTSSNYDDDEKKVTGGRNGYGA
            | |||| | |||||||||||||||||| ||||||||||||||||||||||||||||||||
TopIIβ  123 VsIDPEsNiISIWNNGKGIPVVEHKVEKvYVPALIFGQLLTSSNYDDDEKKVTGGRNGYGA

TopIIα  168 KLCNIFSTKFTVETAsrEYKkmFKQTWMdNMgragEmelKpFnGEDYTCITFQPDLSKFKM
            ||||||||||||||  |||  |||||| ||    |   | | ||||||||||||||||||
TopIIβ  184 KLCNIFSTKFTVETAckEYKhsFKQTWMnNMmktsEakiKhFdGEDYTCITFQPDLSKFKM

TopIIα  229 qsLDKDIVALMvRRAYDiAGStkdVKVflNGnKLPVkGFRSYVDmYlKDKLDETGnsLKVI
              |||||||| |||| |||   |||  || |||| ||||||| | ||||||||  ||||
TopIIβ  245 ekLDKDIVALMtRRAYDlAGScrgVKVmfNGkKLPVnGFRSYVDlYvKDKLDETGvaLKVI

TopIIα  290 HEqvNhRWeVCLTmSEKGFQQISFVNSIATsKGGRHVDYVaDQiVtKLvdVVKKKNKgGVa
            ||  | || |||| |||||||||||||| |||||||| || | ||  ||||||| ||
TopIIβ  306 HElaNeRWdVCLTlSEKGFQQISFVNSIATtKGGRHVDYVvDQvVgKLieVVKKKNKaGVs

TopIIα  351 VKahQVKNHmWiFvNaLIENPTFDSQTKENMTLQPKSFGStCQLSEKFiKAAigCGIVESI
            ||  ||||| | | | |||||||||||||||||||| ||||||| |||  |||||||
TopIIβ  367 VKpfQVKNHiWvFiNcLIENPTFDSQTKENMTLQPKSFGSkCQLSEKFfKAAsnCGIVESI

TopIIα  412 LNWVKFKAQvQLNKKCSaVKhnrIKGIPKLDDANDAGGrnStECTLILTEGDSAKtLAVSG
            ||||||||| ||||||| ||   |||||||||||||  | |||||||||||| |||||
TopIIβ  428 LNWVKFKAQtQLNKKCSsVKyskIKGIPKLDDANDAGGkhSlECTLILTEGDSAKsLAVSG

TopIIα  473 LGVvGRDkYGVFPLRGKILNVREASHKQIMENAEINNIIKIVGLQYKKnYeDedSLKTLRY
            ||| ||| |||||||||||||||||||||||||||||||||||||||| | |  |||||||
TopIIβ  489 LGViGRDrYGVFPLRGKILNVREASHKQIMENAEINNIIKIVGLQYKKsYdDaeSLKTLRY

TopIIα  534 GKIMIMTDQDQDGSHIKGLLINFIHHNWPSLLrHrFLEEFITPIVKvSKNKQEmaFYSlPE
            |||||||||||||||||||||||||||||| | |||||||||| |||||  ||| ||
TopIIβ  550 GKIMIMTDQDQDGSHIKGLLINFIHHNWPSLLkHgFLEEFITPIVKaSKNKQElsFYSiPE

TopIIα  595 FeEWKsstpNhKkWKvKYYKGLGTSTsKEAKEYFADMkRHRIqFkYsGPEDDAAIsLAFSK
            | |||    | | || |||||||||| |||||||||| |||| | | |||||||| |||||
TopIIβ  611 FdEWKkhieNqKaWKiKYYKGLGTSTaKEAKEYFADMeRHRIlFrYaGPEDDAAItLAFSK

TopIIα  656 KqIDDRKEWLTNFMEDRRQRkLlGLPEdyLYGqtTtyLTYNDFINKELILFSNSDNERSIP
            | |||||||||||||||| | ||||  |||  |  ||||||||||||||||||||||||
TopIIβ  672 KkIDDRKEWLTNFMEDRRQRrLhGLPEqfLYGtaTkhLTYNDFINKELILFSNSDNERSIP

TopIIα  717 SmVDGlKPGQRKVLFTCFKRNDKREVKVAQLAGSVAEMSsYHHGEmsLMMTIiNLAQNFVG
            | ||| ||||||||||||||||||||||||||||||||| |||||  |||||  ||||||||
TopIIβ  733 SlVDGfKPGQRKVLFTCFKRNDKREVKVAQLAGSVAEMSaYHHGEqaLMMTIvNLAQNFVG

TopIIα  778 SNNlNLLQPIGQFGTRLHGGKDsASPRYIFTMLSsLARLLFPpkDDhtLKFLYDDNQRVEP
            ||| |||||||||||||||| |||||||||| |||||||  ||  ||||||||||||
TopIIβ  794 SNNiNLLQPIGQFGTRLHGGKDaASPRYIFTMLStLARLLFPavDDnlLKFLYDDNQRVEP

TopIIα  839 EWYIPIIPMVLINGAEGIGTGWsCKiPNfDvREIVNNiRRlmDGeePlPMLPsYKNFKGTI
            |||||||||||||||||||| || || | |||||| ||  ||  | |||| ||||||||
TopIIβ  855 EWYIPIIPMVLINGAEGIGTGWaCKlPNyDaREIVNNvRRmlDGldPhPMLPnYKNFKGTI
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TopIIα  900 eELapNQYviSGEvailnstTiEIsELPVRTWTQtYKEQVLEPMLNGTeKTPpLItDYrEY
TopIIβ  916 qELgqNQYavSGEifvvdrnTvEItELPVRTWTQvYKEQVLEPMLNGTdKTPaLIsDYkEY

TopIIα  961 HTDTTVKFVVKMTEEKLAeAErvGLHKVFKLQTsLTCNSMVLFDHvGCLKKYdTVlDILrd
TopIIβ  977 HTDTTVKFVVKMTEEKLAqAEaaGLHKVFKLQTtLTCNSMVLFDHmGCLKKYeTVqDILke

TopIIα 1022 lFeLRLkYYGLRKEWLlGMLGAESaKLNNQARFILEKIdGKIiIENkpKKeLIkvLiQRGY
TopIIβ 1038 fFdLRLsYYGLRKEWLvGMLGAEStKLNNQARFILEKIqGKItIENrsKKdLIqmLvQRGY

TopIIα 1083 dSDPVKAWKEAQqkvpdeEEneEsdNeketekSDSvTdSGPtFNYlLdMpLWyLTKEKkdE
TopIIβ 1099 eSDPVKAWKEAQ  ekaaEE dEtqNqhddssSDSgTpSGPdFNYiLnMsLWsLTKEKveE

TopIIα 1144 LcrlRneKeqEldtLKRKSPSDLWKEDLAtFiEELeaVEakEkqDeqvGlpGK  gGK
TopIIβ 1157 LikqRdaKgrEvndLKRKSPSDLWKEDLAaFvEELdkVEsqEreDvlaGmsGKaikGKvgk

TopIIα 1200 aKgKKtQmaEvlPSPrGqRvIPrITiemkaeaeKknKKK       iknentegspqedgV
TopIIβ 1218 pKvKKlQleEtmPSPyGrRiIPeITamkadaskKllKKKkgdldtaavkvefdeefsgapV

TopIIα 1254 E       leglkqrlekKqKR   EPGTktkKqtTlafKP iKKgKKRNPW    pdSES
TopIIβ 1279 EgageealtpsvpinkgpkpKRekkEPGTrvrKtpTssgKPsaKKvKKRNPWsddeskSES

TopIIα 1300 D               RssdEsnfdvppretEprraAtktkftmdldsdedfsdfdektD
TopIIβ 1340 DleetepvviprdsllrraaaerpkytfdfseEedddAddddddnndleelkvkaspitnD

TopIIα 1345 dED FVPSD     asppktktSpklsnKeLkpqKS     vvSdleaddvkgsvplsssp
TopIIβ 1401 gEDeFVPSDgldkdeytfspgkSkatpeKsLhdkKSqdfgnlfSfpsysqkseddsakfds

TopIIα 1395 pathfpdeteitnpvpkknvtvkKT AAKsqsstStTgaKkraAPKgtKrdpAlNS gvSq
TopIIβ 1462 needsasvfspsfglkqtdkvpsKTvAAKkgkpsSdTvpKpkrAPKqkKvveAvNSdsdSe

TopIIα 1454 kpdPaKT       knrrKRKpStSddsdsnfekivskavTSKK     skgeSD  dFhm
TopIIβ 1523 fgiPkKTttpkgkgrgakKRKaSgSenegdynpgrktsktTSKKpkktsfdqdSDvdiFps

TopIIα 1501 DFdsavapraksvRAkKpiKYleESDE  DDlf.
TopIIβ 1584 DFpteppslprtgRArKevKYftESDEeeDDvdfamfn.
```

Figure 3 Alignment of the human topoisomerase IIα and β isozyme protein sequences, taken from Jenkins *et al.* (1992). The single letter amino acid code is used. Identical residues are in upper case letters and separated by a vertical line. Non-identical residues are in lower case letters. Residue numbers are indicated on the left.

Chapter 2 MATERIALS AND METHODS

2.1 Materials.

2.1.1 Enzymes.

Casein kinase II, purified from rabbit skeletal muscle, and heparin, were kindly provided by Dr. J. R. Woodgett (Ludwig Institute for Cancer Research, London, U.K.). Protein kinase C isotypes α, ß1, β2, γ and ε, purified from bovine brain, were kindly provided by Dr. P. J. Parker (I.C.R.F., London, U.K.). The catalytic subunit of PKA (cAMP-dependent protein kinase) and the α isozyme of PKG (cGMP-dependent protein kinase) were obtained from Promega. Baculovirus-expressed chicken $p34^{cdc2}$ was kindly provided by Dr. Erich Nigg (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland). Potato acid phosphatase, PAP (Grade 1), was obtained from Boehringer Mannheim as an ammonium sulphate suspension. This was spun at 10,000g for 2 minutes and the pellet resuspended in PAP buffer (20mM MOPS, pH 6.5, 100mM NaCl, 1mM $MgCl_2$) at 100 units/ml. The solution was then dialysed overnight at 4°C against PAP buffer. Endoproteinase GLU-C (*S. aureus* V8 protease) was from Promega. TPCK-treated trypsin was from Worthington.

2.1.2 Antibodies.

The rabbit polyclonal antiserum raised to a C-terminal peptide of topoisomerase IIα was from Cambridge Research Biochemicals (CRB). The six monoclonal antibodies to human topoisomerase II (three specific for the α isozyme, three for the β isozyme) were kindly provided by Dr. G. Astaldi-Ricotti (Institute of Genetics, Biochemistry and Evolution, Pavia, Italy). Rabbit polyclonal antisera to a C-terminal peptide (LDNQIKKM) of human $p34^{cdc2}$ and pre-immune serum from the same rabbit were kindly provided by Dr. C. Norbury (I.C.R.F., Oxford, U.K.). "x-fos", a rabbit polyclonal antiserum raised to the N-terminal 17 residues of *c-fos*, was kindly provided by Dr. G. Evan (I.C.R.F., London, U.K.). Affinity purified rabbit polyclonal antiserum raised to a 23 residue synthetic peptide corresponding to residues 70-91 of the human casein kinase II α(catalytic)-subunit was obtained from Upstate Biotechnology, Inc.

(U.S.A.). Peroxidase-conjugated secondary antibodies were from DAKO immunoglobulins (Denmark).

2.1.3 Chemicals.

Protein A-sepharose CL-4B, casein (dephosphorylated), histone (type III-S, lysine-rich fraction), PKi (the PKA inhibitor peptide), H-7 and all protease inhibitors were obtained from Sigma. Histone H1 for $p34^{cdc2}$ kinase assays and nuclease-free bovine serum albumin (BSA) were from Boehringer Mannheim. PKG substrate heptapeptide, RKRSRAE, was from Peninsula Laboratories. Nocodazole was from Janssen Biochimica. Catenated kinetoplast DNA (kDNA) was purified from the kinetoplast of the insect trypanosome *Crithidia fasciculata* by TopoGEN, Inc. (U.S.A.) and PhiX174 RF I DNA was from New England Biolabs. Hydroxylapatite Bio-Gel HT was from Bio-Rad, phosphocellulose P11 was from Whatman, and the FPLC MonoQ column was from Flowgen. All other FPLC columns and equipment were from Pharmacia. FCS was from Advanced Protein Products; all other cell culture reagents were provided by GIBCO.

2.2 Mammalian Cell Culture.

2.2.1 Human Cell Lines.

HeLa-S3 cells were obtained from I.C.R.F Cell Production stocks. Cells grown as adherent monolayers had a doubling time of around 12 hours in the growth medium used, and cells were routinely subcultured at 2 to 3 day intervals. Cells grown in suspension were grown by the I.C.R.F. Cell Production Services at I.C.R.F. Clare Hall (South Mimms, U.K.) and supplied as fresh cell pellets.

The testis and bladder tumor cell lines described in section 3.2.3 were cultured by Dr. J. R. W. Masters, Institute of Urology, St. Paul's Hospital (London, U.K.), and provided for analysis as frozen cell pellets.

2.2.2 Chinese Hamster Ovary Cell Lines.

The wild-type Chinese hamster ovary (CHO) cell line, CHO-K1, was originally obtained from Flow Laboratories Ltd. The mutant cell lines, ADR-1 (Davies *et al.*, 1988), ADR-2 and Adr^R (Hoban *et al.*, 1992), were isolated in this laboratory from CHO-K1 by chemical mutagenesis as

described by Davies (1989). These cells are epithelial in origin. The cells displayed a doubling time of around 13 hours in the growth medium used, and cells were routinely subcultured at 3 to 4 day intervals.

2.2.3 Mammalian Cell Culture Conditions and Media.

Human cells were maintained either as a subconfluent monolayer, or in suspension, in DMEM medium (I.C.R.F Cell Culture supplies) with 10% (v/v) fetal calf serum (FCS), 3mM glutamine and antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml and nystatin 50 units/ml). CHO cells were maintained as a subconfluent monolayer in Ham's F10 medium (I.C.R.F Cell Culture supplies) supplemented with 5% (v/v) fetal calf serum (FCS), 5% (v/v) newborn calf serum (NCS), 3mM glutamine and antibiotics (as described for human cell lines). All cells were grown in petri-dishes or flasks at 37°C in a humidified atmosphere containing 5% CO_2.

For passaging, cell monolayers were detached using 0.02% (w/v) EDTA in PBS-A. Frozen stocks were maintained in liquid nitrogen in growth medium containing 20% (v/v) FCS and 10% (v/v) DMSO. All cell lines were routinely tested for mycoplasma contamination and found to be negative.

2.3 Protein Gel Electrophoresis.

Many of the techniques described elsewhere in this chapter require the separation of proteins by polyacrylamide gel electrophoresis. This method was always carried out under denaturing conditions (in the presence of SDS and 2-mercaptoethanol) using the discontinuous buffer system of Laemmli (1970). Prior to electrophoresis, protein samples were boiled for 3 minutes in 1 x SDS sample buffer (0.125M Tris-HCl pH 6.8, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, 0.005% (w/v) bromophenol blue).

For analysis of topoisomerase II proteins, samples were resolved by SDS-PAGE on 7.5% minigels; phosphorylated casein or histones were separated on 12% minigels; phosphorylated synthetic peptides were resolved on 22% minigels. All SDS-polyacrylamide gels were prepared on the BIORAD MiniProtean vertical gel electrophoresis apparatus, except for the 17.5% SDS-polyacrylamide gels used for one-dimensional separation of phosphopeptides (section 2.12). These were run on Hoefer

Scientific Instruments 16cm vertical gel electrophoresis apparatus. Resolving gels were prepared with 0.75M Tris-HCl pH 8.8, 0.2% (w/v) SDS, and the 4% stacking gel made up of 0.25M Tris-HCl pH 6.8, 0.2% (w/v) SDS. The running buffer contained 0.129M glycine, 0.025M Tris and 0.1% (w/v) SDS. For the one-dimensional separation of phosphopeptides, 1mM EDTA was added to both resolving and stacking gels and the running buffer.

For detection of unlabelled proteins following electrophoresis, gels were stained with 0.1% (w/v) Coomassie blue in 50% methanol, 10% acetic acid for 30 minutes at room temperature, and then destained in 30% methanol, 10% acetic acid. For detection of radiolabelled proteins following electrophoresis, gels were dried on a Hoefer slab gel dryer, model SE11560 and then exposed to X-ray film (FUJI or Kodak XAR) either at room temperature or at -70°C in a cassette with intensifying screens for autoradiography. Autoradiographs were quantified by densitometric scanning on a Bio-Image Analyzer (MillGen/Biosearch).

2.4 Immunoblotting.

Following protein gel electrophoresis, immunoblotting was performed according to the method of Towbin *et al.* (1979) by electroblotting onto nitrocellulose filters (Hybond-C Super, Amersham) at 30v overnight in transfer buffer (50mM Tris-HCl, pH 7.5, 380mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol) in a BIORAD Trans-Blot Cell. The filters were incubated for 60 minutes in blocking buffer (20mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20, 1% (w/v) MARVEL low fat milk powder). For 'x-fos' western blots, 0.5% (w/v) BSA was also included in the blocking buffer. Primary antibody reactions were carried out for 2 hours in blocking buffer containing the antibody at the stated dilution. The filters were washed in blocking buffer for 60 minutes, with at least three changes of buffer, before addition of the detection reagent.

2.4.1 Radiolabelled Detection using ^{125}I-Protein A.

For radiolabelled detection, the filters were incubated with affinity purified Protein A, ^{125}I-labelled with Bolton and Hunter reagent, at 1μCi/ml in blocking buffer for 3-4 hours, washed extensively in blocking

buffer without MARVEL, air-dried, covered in Saran-Wrap, and exposed to X-ray film for autoradiography.

2.4.2 Chemical Detection using Diaminobenzidine (DAB).

For enzyme-linked detection, the filters were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (either rabbit anti-mouse immunoglobulins or swine anti-rabbit immunoglobulins), at a 1 in 50 dilution, for 60 minutes at room temperature, washed several times with Tris-buffered saline, TBS, (20mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl) and then added to the detection mix (0.6mg/ml 3,3'-Diaminobenzidine, 0.03% (w/v) $NiCl_2$, 0.03% (v/v) H_2O_2 in TBS). After 15 minutes, or when protein bands were clearly seen, the reaction was stopped by rinsing the filters in distilled water. Blots were allowed to air-dry.

2.4.3 Enhanced Chemiluminescence (ECL) Detection.

Enhanced chemiluminescence works on the principle that HRP can catalyse the oxidation of the substrate, luminol, which, in the presence of a chemical enhancer, produces a sustained light emission which is readily detected on standard X-ray film.

For ECL detection, the filters were also incubated with HRP-conjugated secondary antibodies, as described in section 2.4.2, for 60 minutes at room temperature and washed several times with TBS. Filters were then treated with ECL Western Blotting Reagents (Amersham, U.K.) according to the Amersham ECL Western blotting protocol. Filters were immediately wrapped in Saranwrap and exposed to X-ray film for 60 seconds. Longer or shorter exposure times were carried out as required based on the intensity of the 60 second exposure.

When comparisons of the topoisomerase II protein levels were being made, crude nuclear extracts were first equalised for protein content before electrophoresis. Equalised protein samples were then checked by electrophoresing and staining the gel with Coomassie blue.

2.5 Preparation of Crude Nuclear Extracts.

2.5.1 Nuclear Extracts for Immunoblotting.

Crude nuclear extracts were made from approximately 10^8 cells in exponential growth phase using the method of Glisson *et al.* (1986a). All procedures were performed at 4°C, with the addition of protease inhibitors, as descibed under purification of human topoisomerase II (section 2.10). After harvesting and washing cells twice in PBS-A, cell pellets were resuspended in 5ml TMN buffer (10mM Tris-HCl pH 7.5, 1.5mM $MgCl_2$, 10mM NaCl) with 1% (v/v) Nonidet P40, and left on ice for 45 minutes. This hypotonic buffer caused cell swelling and lysis. The extent of lysis was checked by phase microscopy. If lysis was not complete, the cells were homogenized by 20 strokes of a Dounce homogenizer. Nuclei were isolated by centrifugation at 600g for 10 minutes and resuspended in 2ml TKMC buffer (50mM Tris-HCl pH 7.5, 25mM KCl, 3mM $MgCl_2$, 2mM $CaCl_2$). They were layered over a 0.6ml TKMC buffer containing 0.6M sucrose in Corex tubes, and centrifuged at 2000g for 10 minutes. The nuclear pellet was washed with 2ml TKM buffer (50mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 25mM KCl) containing 0.25M sucrose, and centrifuged at 2000g for 10 minutes. The pellet was then resuspended in 0.15ml TKM buffer, 15µl of 0.2M EDTA added, and then two volumes of TEN buffer (80mM Tris-HCl pH 7.5, 2mM EDTA, 0.53M NaCl, 20% (v/v) glycerol, 1mM DTT) added (final NaCl concentration 0.35M). The extraction was allowed to continue on ice for 30 minutes, before pelleting the precipitate by microcentrifugation at 10,000g for 25 minutes. Protein concentration in the supernatants was determined immediately using the method of Bradford (1976). Extracts were stored in 50% (v/v) glycerol with 1mg/ml BSA at -70°C.

One experiment was carried out comparing the differences in efficiency of topoisomerase II protein extraction using 0.35M and 1M NaCl (Figure 7, section 3.2.7). As there did not appear to be any additional topoisomerase II extracted from the nuclei with 1M NaCl then 0.35M NaCl was used routinely.

2.5.2 Nuclear Extracts for Immunoprecipitation.

Crude nuclear extracts were prepared for immunoprecipitation from approximately 10^7 cells in exponential phase, essentially by the

method of Kroll and Rowe (1991). All procedures were performed at 4°C. The monolayer of cells was washed once on the plate with Tris buffered saline (TBS, see section 2.4.2), and then harvested by scraping with a Costar disposable cell scraper into 2ml TBS containing 1mM EDTA, 1mM PMSF and 10mM β-glycerophosphate. Inclusion of protease and phosphatase inhibitors minimized any degradation or dephosphorylation of the proteins during harvesting. TBS was chosen, rather than PBS, in *in vivo* labelling experiments to maintain a phosphate-free environment, thus preventing incorporation of cold phosphate into the topoisomerase II protein during extraction.

The cells were centrifuged at 1000g, and the cell pellets washed again in 2ml of the harvesting buffer. The cell pellets were then resuspended in 0.36ml nuclei isolation buffer, NIB (30mM Tris-HCl pH 7.5, 1.5mM $MgCl_2$, 10mM KCl, 20% (v/v) glycerol and all protease and phosphatase inhibitors as described under purification of human topoisomerase II, section 2.10). Cells were lysed by addition of 40µl of 10% (v/v) Triton-X-100 and briefly vortexing. After incubating the lysate for 5 minutes on ice, nuclei were microcentrifuged at 1000g in an Eppendorf Microfuge for 90 seconds. The pelleted nuclei were then resuspended in 0.36ml of ice-cold NIB buffer containing 0.35M NaCl. After 30 minutes on ice, the samples were microcentrifuged for 5 minutes at 10,000g and the resulting supernatants used in the immunoprecipitation reactions (section 2.8).

2.6 Production of Rabbit Polyclonal Antisera.

The peptides designed for the generation of anti-topoisomerase II antibodies were synthesized and checked for purity by HPLC analysis by the ICRF Peptide Synthesis Laboratory (ICRF, Lincoln's Inn Fields, London). These peptides were then coupled by the same laboratory to either bovine thyroglobulin for the first round of immunizations, or to keyhole limpet haemocyanin (KLH) for the second round of immunizations. The conjugated peptides were then sent to the ICRF Animal Unit (ICRF, Clare Hall, South Mimms) and incorporated by them into a standard rabbit immunization schedule.

The coupled peptides were prepared in complete Freund's adjuvant for the first injection, or in incomplete Freund's adjuvant for all subsequent injections. The peptides were administered by subcutaneous injection into rabbits. The immunization protocol involved five injections

of 200μg of the peptide (at 1mg/ml) coupled to thyroglobulin, with a test bleed after the third and fifth injections, followed if positive, by four more injections with 200μg of the peptide (at 1mg/ml) coupled to KLH, with a test bleed after the seventh and ninth injections. A prebleed of 20ml was taken on the day of the first injection; injections were then given at 14 day intervals, with the test bleed (25ml) being taken 10 days after the injection. After this, the animals were sacrificed and bled out. Blood samples were left to clot at room temperature for a minimum of 4 hours, decanted, spun at 2500 RPM for 10 minutes, and the serum removed and stored at 4°C for analysis.

2.7 Metabolic Labelling of Cultured Cells.

For *in vivo* phosphorylation studies, cells were washed twice in phosphate-free medium 1 hour before addition of label. At the time of labelling this medium was removed and the cells transferred to phosphate-free medium, with 5% (v/v) dialysed serum, containing 100μCi/ml ^{32}P-orthophosphate (carrier-free; Amersham, U.K.) for 16 hours. For ^{35}S-methionine labelling, cells were washed twice in PBS-A and then incubated with 25μCi/ml ^{35}S-methionine (>1000Ci/mmol, Amersham, U.K.) in methionine-free medium for 16 hours.

2.8 Immunoprecipitation.

2.8.1 Immunoprecipitation from Labelled Cells.

Immunoprecipitations from crude nuclear extracts (prepared as in 2.5.2) were carried out essentially according to the method of Kroll and Rowe (1991), except that, after preparation, the nuclear extracts were precleared by incubating with preswelled Protein A-sepharose beads (see below), with shaking, for 60 minutes at 4°C. All procedures were carried out at 4°C, and protease and phosphatase inhibitors were as for purification of human topoisomerase II protein (section 2.10). Precleared nuclear extracts were added to an equal volume of immunoprecipitation buffer, IPB (100mM Tris-HCl pH 8.0, 500mM NaCl, 0.75% (v/v) Triton-X-100, 10mM EDTA, 0.02% (w/v) NaN_3), and the antiserum at the required dilution. Immunoprecipitation of topoisomerase IIα for *in vivo* phosphorylation studies was carried out with the CRB anti-topoisomerase II antibody at a 1 in 50 dilution. This antibody specifically

immunoprecipitates the α (170kDa) isoform of topoisomerase II (see Figure 5). Immunoprecipitation of topoisomerase IIβ protein for phosphorylation analysis was done with a 1 in 10 dilution of the 8D7 monoclonal antibody raised against purified human topoisomerase IIβ (see section 3.2.5).

After incubation of the extracts with the antiserum for 60 minutes, 50µl of preswelled 6% (w/v) Protein A-sepharose beads in IPB buffer was added and the samples incubated with shaking for 60 minutes. The beads were then pelleted by microcentrifugation at 10,000g for 10 seconds and washed three times with 1ml of IPB buffer containing 0.1% (w/v) SDS, with a final wash of 10mM Tris-HCl pH 7.5. The resulting immunoprecipitates were resuspended in 30µl of 2 x SDS sample buffer (250mM Tris-HCl pH 6.8, 10% glycerol, 2% 2-mercaptoethanol, 2% SDS, 0.01% (w/v) bromophenol blue), boiled for 3 minutes and electrophoresed as described in section 2.3.

2.8.2 Immunoprecipitation of $p34^{cdc2}$ Kinase.

Immunoprecipitation of either human or hamster $p34^{cdc2}$ kinase for topoisomerase II phosphorylation assays or histone H1 kinase assays was carried out with a polyclonal anti-$p34^{cdc2}$ kinase antibody kindly provided by Dr. C. Norbury (ICRF, Oxford) and based on the method used by Norbury *et al.* (1991). Again all procedures were carried out at 4°C, and protease and phosphatase inhibitors were as for purification of human topoisomerase II protein. Cells were harvested as described in section 2.2.3 and resuspended at $2x10^7$ cells/ml in Histone Kinase Buffer (HKB) (25mM Tris-HCl pH 7.4, 15mM $MgCl_2$, 15mM EGTA, 1mM DTT) containing 1% (v/v) Triton-X-100. Cells were left on ice for 30 minutes to lyze and then the lysate pelleted by microcentrifugation at 10,000g for 10 minutes. Supernatant (0.5ml) was transferred to a 1ml polycarbonate tube and spun at 100,000 RPM (approx. 250,000g) in a TL100.2 rotor, in a Beckman TL100 benchtop ultracentrifuge for 30 minutes. This supernatant (designated S100) was then taken for the immunoprecipitation reaction.

Either 2µl of anti-$p34^{cdc2}$ antiserum or 2µl of pre-immune serum from the same rabbit was added to 200µl of the S100 supernatant and incubated for 60 minutes at 4°C. Then 20µl of preswelled 6% (w/v) ProteinA-sepharose beads in RIPA buffer (20mM Tris-HCl pH 7.4, 5mM EDTA, 100mM NaCl, 1mM DTT, 1% (v/v) Triton-X-100) was added, and

the tubes rotated for 30 minutes. The beads were then pelleted by microcentrifugation at 10,000g for 10 seconds and washed firstly with 0.5ml RIPA buffer, then 0.5ml RIPA buffer+1M NaCl, then 0.5ml RIPA buffer, and lastly 0.5ml HKB buffer. The immunoprecipitated $p34^{cdc2}$ was then used in a kinase assay (see section 2.11).

2.9 Phosphoamino Acid Analysis.

This method determines the relative content of phospho-serine, -threonine or -tyrosine in a phosphoprotein, and is essentially as described by Woodgett (1992), based on the original method of Cooper *et al.* (1983). Experiments were carried out with the help of Dr. Bernd Pulverer and Dr. Jim Woodgett at the Ludwig Institue for Cancer Research, London.

2.9.1 Elution of Proteins from Polyacrylamide Gels.

Phosphorylated proteins were electrophoresed on SDS-polyacrylamide gels and the gels dried down; the position of the radiolabelled topoisomerase II protein was detected by autoradiography. The appropriate region of the dried gel was then excised and hydrated for 5 minutes in 0.5ml freshly prepared 50mM NH_4HCO_3. The swollen gel piece was then homogenized with a small teflon pestle (Kontes), which was rinsed with a further 0.5ml NH_4HCO_3. To this was added 50µl 2-mercaptoethanol and 10µl 10% (w/v) SDS, followed by boiling for 3-5 minutes and vortexing the slurry. Protein was eluted by rotation for 3-16 hours and the supernatant removed after centrifugation in a microfuge for 2 minutes. A further wash with the 2-mercaptoethanol/SDS/NH_4HCO_3 mixture (boil, vortex, rotate 3 hours, and microfuge) extracted residual protein to give a total volume of 1.2ml supernatant. This was reclarified by microcentrifugation for 10 minutes. The supernatant was carefully removed and cooled on ice. To this 20µg of RNaseA was added as a carrier, followed by 250µl cold 100% (w/v) trichloroacetic acid. The protein was precipitated for at least 1 hour at 0°C, after which it was pelleted by microcentrifugation for 10 minutes at 4°C. The pellet was carefully washed with cold absolute ethanol and air-dried. Cerenkov counting was used to determine the yield of extraction.

2.9.2 Hydrolysis of Phosphoprotein.

The TCA-precipitated protein was hydrolysed by adding 100µl of constant-boiling HCl (5.7 N). The tube was sealed and incubated at 110°C for exactly 60 minutes. The reaction was stopped by adding 400µl of cold water and freezing on dry-ice to prevent further hydrolysis leading to removal of phosphate from phosphoamino acids. After rotary evaporation with a Speedvac equipped with an acid-trap, the sample was carefully resuspended in 10µl pH 1.9 electrophoresis running buffer (see below) containing 70µg/ml each of cold pSer, pThr and pTyr standards.

2.9.3 Two-Dimensional Separation of Phosphoamino Acids.

The samples were microcentrifuged and no more than 8µl removed for loading, being careful not to take up any particulate matter. Loading onto precoated cellulose plates (Merck) was achieved by repeated spotting of 0.5µl volumes followed by drying with a stream of air. Electrophoresis was carried out with the pH 1.9 buffer (88% formic acid/acetic acid/dH_2O at a ratio of 0.025 : 0.078 : 0.897) in the first dimension for 20 minutes at 1500v, after which the plate was left to air-dry. Electrophoresis in the second dimension was at 1300v for 20 minutes in pH 3.5 buffer (acetic acid/pyridine/dH_20 in a ratio of 0.05 : 0.005 : 0.945). The sample was focused into a spot for the first dimension and along the first dimension for the pH 3.5 electrophoresis. Focusing was achieved for the first dimension by overlaying the plate with a sheet of Whatman 3MM paper with a hole cut at the origin which had been soaked in pH 1.9 running buffer, or for the second dimension with strips of 3MM paper soaked in pH 3.5 running buffer. After drying, the plates were sprayed with 0.5% (w/v) ninhydrin in acetone and heated to 65°C for 20 minutes to visualize the phosphoamino acid standards. Plates were exposed directly to X-ray film (Kodak) with intensifying screens at -70°C for up to 14 days.

2.10 Purification of Human Topoisomerase II Protein.

The method was based on those of Miller *et al.* (1981), Drake *et al.* (1987), and Strausfeld and Richter (1989). All steps were carried out at 4^oC. Buffers contained the following protease and phosphatase inhibitors unless stated otherwise: 1mM PMSF, 2µg/ml leupeptin, 1µg/ml

aprotinin, 1μg/ml pepstatin A, 1μg/ml chymostatin, 1μg/ml soybean trypsin inhibitor, 1mM benzamidine, 1μg/ml antipain, 50μg/ml TLCK, 0.1mM β-glycerophosphate, 0.1mM *p*-nitrophenylphosphate, 0.5mM glucose-1-phosphate and 10mM 2-mercaptoethanol. Topoisomerase II activity was identified in the fractions from each column using a decatenation assay (see section 2.14).

2.10.1 Nuclear Extraction.

40 litres of exponentially growing HeLa cells in suspension (approx. 5 x 10^{10} cells) were provided by ICRF Cell Production Services (Clare Hall, South Mimms, U.K.). Cells were harvested by centrifugation and washed with PBS-A. The cell pellet was resuspended in 300ml swelling buffer (5mM potassium phosphate, pH 7.0, 2mM $MgCl_2$, 0.1mM EDTA, 0.5mM DTT, 1mM 2-mercaptoethanol) and left on ice for 1 hour. Nonidet P40 was added to 0.5% (v/v) and the cells lysed by 20 strokes of a Dounce homogenizer. Lysis was monitored by phase microscopy. The lysed cells were centrifuged at 200g for 10 mins to pellet nuclei. Nuclei were then washed once with 100ml wash buffer (5mM potassium phosphate, pH 7.0, 0.5mM DTT, 1mM 2-mercaptoethanol), repelleted, resuspended in 100ml wash buffer with 4mM EDTA, and held at 4°C for 15 mins. 100ml nuclear lysis buffer A (50mM Tris-HCl, pH 7.5, 2M NaCl, 10mM 2-mercaptoethanol) was then added slowly with constant stirring and the solution held at 4°C for 15 mins. 100ml nuclear lysis buffer B (50mM Tris-HCl, pH 7.5, 1M NaCl, 18% (w/v) PEG 6000, 10mM 2-mercaptoethanol) was then added slowly with constant stirring and the solution held for a further 40 mins. The nuclear extract was clarified by centrifugation at 12,000g for 30 mins, the supernatant filtered through gauze, and re-centrifuged.

2.10.2 Column Chromatography.

The supernatant (Fraction I) was immediately loaded onto a 200ml hydroxylapatite column equilibrated with HA buffer (50mM potassium phosphate, pH 7.5, 10% (v/v) glycerol). The column was washed with 2 bed volumes of HA buffer and a 1000ml gradient run from 100% HA buffer to 100% HB buffer (700mM potassium phosphate, pH 7.5, 10% (v/v) glycerol). Active fractions eluting at 400mM potassium phosphate were pooled (Fraction II), diluted three-fold with 10% (v/v)

glycerol/0.1mM EDTA and loaded onto an 80ml phosphocellulose P11 column equilibrated with P11A buffer (50mM potassium phosphate, pH 7.5, 10% (v/v) glycerol, 0.1mM EDTA). The column was washed with 2 bed volumes of P11A buffer and a 400ml gradient run from 100% P11A buffer to 100% P11B buffer (700mM potassium phosphate pH 7.5, 10% (v/v) glycerol). Active fractions eluting at 300mM potassium phosphate were pooled (Fraction III) and diluted four-fold with 20mM Tris-HCl, pH 7.5/0.1mM EDTA/10% (v/v) glycerol ready for the first FPLC purification step.

The diluted Fraction III was loaded via a 40ml superloop onto a 4ml FPLC MonoQ column equilibrated with MA buffer (20mM Tris-HCl, pH7.5, 50mM NaCl, 0.1mM EDTA, 10% (v/v) glycerol). The column was washed with 6 bed volumes of MA buffer and a 40ml gradient run from 100% MA buffer to 100% MB buffer (20mM Tris-HCl, pH 7.5, 750mM NaCl, 10% (v/v) glycerol, 0.1mM EDTA). Active fractions eluting at 300mM NaCl were pooled (Fraction IV), diluted two-fold with 20mM Tris-HCl, pH 7.5/2M $(NH_4)_2SO_4$/0.1mM EDTA and loaded onto a 1ml FPLC phenylsuperose column equilibrated with PSA buffer (20mM Tris-HCl, pH 7.5, 1M $(NH_4)_2SO_4$, 10% (v/v) glycerol, 0.1mM EDTA). The column was washed with 6 bed volumes of PSA buffer and a 10ml gradient run from 100% PSA buffer to 100% PSB buffer (20mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 0.1mM EDTA). Active fractions eluting at 400mM $(NH_4)_2SO_4$ were pooled (Fraction V), diluted four-fold with 20mM Tris-HCl, pH 7.5/0.1mM EDTA/10% (v/v) glycerol and loaded onto a 1ml FPLC MonoS column equilibrated with MA buffer. The column was washed with 6 bed volumes of MA buffer and a 10ml gradient run from 100% MA buffer to 100% MB buffer. Active fractions (Fraction VI) eluting at 350mM NaCl were aliquoted and stored separately in 50% (v/v) glycerol with 0.5mg/ml BSA at -80°C. Enzymatic activity was stable for approximately 6 months.

2.11 Protein Kinase and Phosphatase Assays.

All kinase and phosphatase assays were performed for 10 mins at 30°C in a total reaction volume of 30µl. Each kinase required a specific reaction buffer, which in each case also contained 1mM DTT, 200µM ATP and the protease and phosphatase inhibitors described under purification of topoisomerase II (see section 2.10). Phosphatase assays were performed similarly except buffers lacked phosphatase inhibitors.

For radiolabelled phosphorylation reactions, 0.1μCi [γ^{32}P]-ATP (3000Ci/mmol, Amersham, U.K.) or 0.1μCi [γ^{32}P]-GTP (>5000Ci/mmol) was added to each reaction as described. Where required, purified human topoisomerase IIα (10ng) was added to each kinase or phosphatase assay. Reactions were stopped by addition of 3μl 10 x SDS-PAGE sample buffer and electrophoresed as described in section 2.3.

Due to the presence of a co-purifying heparin-dependent kinase in some of the preparations of human topoisomerase IIα protein (chapter 4), heparin was included at 10μg/ml in those phosphorylation reactions not involving CK II.

Casein Kinase II. 0.01 units of partially purified rabbit CK II was added to each phosphorylation reaction. The reaction buffer was 50mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 50mM NaCl, 25mM KCl. Casein was used as the control substrate at 0.2mg/ml. Heparin was used as a strong inhibitor at 10μg/ml, or the concentration given.

PKA and PKG. 10 units of PKA catalytic subunit or PKG α isozyme were added to each reaction. The reaction buffer was 40mM Tris-HCl pH 7.4, 20mM magnesium acetate. Casein was used as a control substrate for PKA at 0.2mg/ml, whilst the heptapeptide, RKRSRAE, at 0.1mg/ml, was the control substrate for PKG. The peptide PKi, based on the physiological protein kinase inhibitor protein, PKI, was used at 5μM to inhibit PKA activity.

Protein Kinase C. 0.1 unit of purified bovine PKC isotype α, β1, β2, γ or ε was added to each reaction. The reaction buffer was 50mM Tris-HCl pH 7.4, 0.25mM EDTA, 0.75mM $CaCl_2$, 12.5mM $MgCl_2$, 0.005% (v/v) Triton-X-100. In a final reaction volume of 30μl, PKC reactions also contained 7.5μl of a lipid preparation. The lipid preparation was made fresh for each assay by adding 100μl of 10mg/ml Grade 1 phosphatidylserine (Lipid Products, Surrey, U.K.) to 1μl of 1mg/ml TPA. This was dried down by blowing with nitrogen, and redissolved in 200μl of 20mM Hepes pH 7.5, 1% (v/v) Triton-X-100, with vortexing and heating to 30°C for 4 hours. Histone type III-S at 0.5mg/ml was used as the control substrate for PKC-α, β1, β2 and γ, whilst a peptide [Ser]^{25}PKC(19-31) from GIBCO was used at 0.1mg/ml as a control substrate for PKC-ε. PKC-ε activity could be blocked by the inhibitory peptide PKC(19-31) also from GIBCO.

p34^{cdc2} kinase. Human p34^{cdc2} was immunoprecipitated from a cytoplasmic extract of HeLa cells using either immune or pre-immune anti-p34^{cdc2} antisera as described in section 2.8.2. Recombinant chicken p34^{cdc2} (100pmol/min/µl) was purified from insect cells following overexpression in a Baculovirus expression system. 0.2µl recombinant p34^{cdc2} kinase was used per assay. The reaction buffer was 25mM MOPS pH 7.2, 15mM $MgCl_2$, 15mM EGTA. Histone H1 at 0.1mg/ml was used as a control substrate. Histone H1 kinase assays are based on the method of Moreno *et al.* (1989).

Potato Acid Phosphatase. PAP reaction buffer is described in section 2.1.1. To demonstate PAP activity, casein at 0.1mg/ml was phosphorylated by CK II and then dephosphorylated by addition of 30 units/ml PAP in the presence of 10µg/ml heparin (to inhibit the CK II activity). Dephosphorylation of topoisomerase IIα protein was carried out with 30 units/ml PAP at 30°C for 10 minutes. For rephosphorylation experiments following PAP treatment of topoisomerase IIα, PAP was inhibited with 10mM sodium phosphate before addition of the kinase.

2.12 One-Dimensional Phosphopeptide Mapping.

This method allows a comparison of the phosphopeptides generated from different samples following the partial digesion with a protease such as V8 protease. It is designed specifically for the digestion of proteins in gel slices and is essentially according to the method of Cleveland *et al.* (1977).

Phosphorylated proteins were electrophoresed on a 7.5% SDS-PAGE minigel, the gel was then wrapped in Saranwrap and exposed to X-ray film. By lining up the exposed film with the gel the ^{32}P-labelled topoisomerase IIα gel slice could be cut out. The excised gel slice was immersed in Soaking Buffer (0.125M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 1mM EDTA) for 30 minutes and then inserted into the well of a 16cm SDS-PAGE gel also containing Soaking Buffer. The gel slice was then overlayed with 20µl Soaking Buffer containing 20% (v/v) glycerol and finally 20µl of 20µg/ml Endoproteinase GLU-C (*S. aureus* V8 protease) in Soaking buffer + 10% (v/v) glycerol was added.

Electrophoresis was performed normally on 17.5% SDS-polyacrylamide gels (containing 1mM EDTA) as described in section 2.3. As digestion proceeds directly in the stacking gel during electrophoresis,

best results were obtained on 1.5mm-thick gels which were cast with longer than usual stacking gels (up to 5cm). During electrophoresis, the current was turned off for 30 minutes when the dye front neared the bottom of the stacking gel. This increases the amount of digestion of the protein. Following electrophoresis, the gel was fixed in 30% methanol/10% acetic acid for 30 minutes, dried and exposed to X-ray film at -70°C.

2.13 Two-dimensional Tryptic Phosphopeptide Mapping.

This method allows the two-dimensional separation of phosphopeptides according to charge and hydrophobicity, and is essentially as described by Woodgett (1992), based on the original method of Cooper *et al.* (1983). These experiments were also carried out with the help of Dr. Bernd Pulverer and Dr. Jim Woodgett at the Ludwig Institue for Cancer Research, London. Elution of phosphorylated proteins from polyacrylamide gels is described in section 2.9.1.

2.13.1 Performic Acid Oxidation and Trypsinisation.

Proteins that contain methionine or cysteine residues in the phosphopeptides must be oxidised to prevent separation of the various oxidation states upon chromatography. This was achieved by the addition of 100µl freshly prepared performic acid (900µl 100% formic acid + 100µl 30% (v/v) H_2O_2, incubated at room temperature for 45 minutes) to the TCA-precipitated protein sample on ice and incubation for 60 minutes. The acid was removed by diluting with 400ml cold water, freezing on dry ice and lyophilisation in a Speedvac. Trypsinisation was then carried out by resuspending the pellet in 50µl NH_4HCO_3 (pH 8-8.5) and addition of 10µl 1mg/ml TPCK-treated trypsin. The reaction was allowed to proceed to completion by incubation at 37°C for 16 hours followed by addition of fresh trypsin and incubation for a further 3 hours after extensive vortexing. To remove all NH_4HCO_3 salt which would interfere with separation, 0.5ml of dH_2O was added to the sample which was then vortexed, microcentrifuged, transfered to a fresh tube and lyophilised in a Speedvac. This was then repeated except using filtered pH 4.72 electrophoresis running buffer (see below) instead of dH_2O.

2.13.2 Two-dimensional Separation of Phosphopeptides.

The final pellet was Cerenkov-counted and then resuspended in 10µl of pH 4.72 electrophoresis running buffer (n-butanol/pyridine/acetic acid/dH_2O at a ratio of 0.05 : 0.025 : 0.025 : 0.9). Samples usually contained about 1000 cpm which was near the lower limit for detection of complex maps following a 14 day exposure. Loading and focusing of samples on cellulose plates was as described in section 2.9.3. The wet plate was then electrophoresed in a HTLE-7000 apparatus (CBS Scientific, U.S.A) in the presence of marker dye (5mg/ml DNP-lysine + 1mg/ml xylene cyanol FF) at 1000v constant voltage for 30 minutes. After electrophoresis, the plate was allowed to air-dry and then put into an air-tight chromatography tank pre-equilibrated with 300ml (2cm deep) phosphochromatography buffer (n-butanol/pyridine/acetic acid/dH_2O in a ratio of 0.375 : 0.25 : 0.075 : 0.3). Chromatography was carried out for 16 hours at room temperature and then the plates allowed to air-dry completely before exposing to X-ray film (Kodak XAR) at -70°C with intensifying screens for 14 days.

2.14 Decatenation and Plasmid Relaxation Assays.

The decatenation of catenated (interlinked) DNA minicircles or the ATP-dependent relaxation of negatively supercoiled plasmid DNA are both assays for type II topoisomerase activity. In a decatenation assay, catenated DNA remains exclusively at the origin of a 1% agarose gel, while the minicircles released by decatenation migrate into the gel. In a plasmid relaxation assay, the relaxation of supercoiled DNA (Form I DNA) to completely relaxed DNA (Form II DNA) can also be detected on a 1% agarose gel. As topoisomerase I is also capable of carrying out plasmid relaxation in an ATP-independent reaction, decatenation activity was used as a specific assay for topoisomerase II activity during the purification of human topoisomerase IIα protein (described in section 2.10).

Topoisomerase II decatenation assays were carried out by the method of Davies *et al.* (1988). Total reaction volume was 50µl, containing 50mM Tris-HCl pH 7.5, 120mM KCl, 10mM $MgCl_2$, 0.5mM DTT, 0.5mM EDTA, 1.0mM ATP, 30µg/ml nuclease-free BSA, and 1µg of kinetoplast DNA. After incubation at 30°C for 30 minutes or the time stated, the reaction was stopped by the addition of 12.5µl of 4 x Stop

solution (5% (w/v) SDS, 25% (w/v) Ficoll, 0.25mg/ml bromophenol blue) and the samples run on a 1% TBE-agarose gel.

Relaxation assays, based on the method of Osheroff *et al.* (1983), were also carried out in a total reaction volume of 50µl, containing the same reaction mix as for decatenation assays, except that 500ng PhiX174 RF I DNA was used as the substrate instead of the kinetoplast DNA. The reactions were carried out at 30°C for the stated time, and then stopped by the addition of 5µl 10 x SDS/proteinase K mix (5% (w/v) SDS, 50mM EDTA, 1.5mg/ml proteinase K). Samples were incubated at 30°C for 30mins, 5µl of 10 x DNA loading buffer (50% (w/v) sucrose, 50mM EDTA, 0.01% (w/v) SDS, 0.1% (w/v) bromophenol blue) added and samples electrophoresed on a 1% TBE-agarose gel.

For decatenation assays of column fractions during the purification of human topoisomerase IIα protein, 5µl of each fraction was added to the 50µl reaction mix and the reaction carried out as described above. For decatenation and plasmid relaxation assays of purified topoisomerase IIα following phosphorylation or dephosphorylation, 2µl of topoisomerase IIα protein was used per 30µl kinase or phosphatase assay and, after incubation at 30°C for 10 minutes, 500µl of either decatenation or plasmid relaxation mix (10x the 50µl reaction mix described above) was added. 50µl aliquots were removed and stopped at times indicated in specific experiments. These were then run on 1% agarose gels.

For both assays, following electrophoresis the gels were stained with ethidium bromide, destained and photographed with a polaroid camera. For quantitation, the negatives of the polaroid photographs were scanned on a Bio-Image Analyzer (MillGen/Biosearch).

Heparin was never used in activity assays as it inhibited the reaction. Instead, a preparation free of co-purifying kinase activity was used for all topoisomerase II activity experiments (see section 5.2.3).

2.15 Clonogenic Assays.

500 HeLa cells, in exponential growth phase, were seeded into 6-well tissue culture dishes, allowed to adhere for 6 hours, and then exposed to the cytotoxic agent. Cells were exposed to drugs in normal medium for 60 minutes before being washed twice in PBS-A and then returned to normal growth medium. As VM-26 was prepared in DMSO, all wells were equalised for the amount of DMSO added. Cells were incubated at 37°C for 10 days to allow colony formation.

Washed colonies were then fixed in methanol: acetic acid (3:1 v/v) and stained with crystal violet (400μg/ml). Colonies of greater than 2mm diameter were considered survivors. Each point on a survival curve represents the mean of at least three independent experiments.

2.16 Cell Cycle Studies.

2.16.1 Mitotic Arrest and Cell Synchronization.

To arrest cells in mitosis the mitotic spindle inhibitor nocodazole was used. Stocks of 100μg/ml nocodazole were made up in ethanol (at pH>7) and stored at -20°C. Nocodazole was added to normal growth medium to give a final concentration of 1μg/ml, the medium was then pH balanced and filter-sterilized, before adding to cells for the required length of time. Mitotic figures were counted to confirm the build-up of cells in mitosis.

CHO cells were synchronized by serum starvation. The exponentially growing cells were rinsed twice in serum-free growth medium and then grown for 36 hours in the serum-free medium. This is more than two doubling times for both CHO cell types being analysed. Returning the cells to normal serum-supplemented growth medium induced them to reenter the cell cycle. It took approximately 16 hours for quiescent cells to reach mitosis following serum-refeeding.

2.16.2 Flow Cytometry.

The DNA content of cells was determined by flow cytometry. Cells in exponential growth phase were harvested and stained with propidium iodide (50μg/ml in 1% (v/v) Triton-X-100, 1mg/ml RNase in PBS-A). The DNA content was then determined as 620nm fluorescence using a Fluorescence Activated Cell Sorter (Becton Dickenson).

2.16.3 Mitotic Figure Counts.

The proportion of cells in an asynchronous population undergoing mitosis was measured by staining the chromosomes and counting the number of cells with mitotic figures. Mitotic figures can be visualized by fluorescence microscopy after staining of the chromosomes with a DNA fluorochrome stain, in this case Hoechst 33258. A 10,000x stock of this

stain was prepared by dissolving 50mg of stain plus 10mg thimerosal in 100ml of 1 x Hanks balanced salt solution (HBSS).

Cells were grown on glass coverslips in petri-dishes under normal growth medium. Cells in exponential growth phase were then fixed in methanol: acetic acid (3:1 v/v), and the coverslips allowed to air-dry. 1ml of 1 x Hoechst 33258 in HBSS was then applied to the cells on the coverslips, and left for 20 minutes in the dark. The stain was then pipetted off, and the cells washed three times with distilled water. The coverslips were blotted gently with tissue-paper and mounted on a slide. Mitotic figures were then counted using a fluorescent microscope. All cells with condensed chromosomes were counted.

Chapter 3 GENERATION AND CHARACTERIZATION OF TOPOISOMERASE II ANTIBODIES.

3.1 Introduction.

Immunological techniques form the basis of many structural and functional studies on cellular proteins. Indeed, the availability of a specific antibody to a protein is often essential to allow a detailed investigation into the expression of that protein within the cell. However, at the onset of this work there were only a very few antibodies to topoisomerase II protein available from other laboratories, most of which were not specific for one particular isozyme of topoisomerase II. All of these antisera were only available to us in small quantities.

Previous studies on topoisomerase II protein levels carried out in this laboratory, relied on a rabbit polyclonal antiserum raised to recombinant human topoisomerase II polypeptide by Dr. L. F. Liu (Baltimore, U.S.A.), or a rabbit polyclonal antiserum raised to calf thymus topoisomerase II by Dr. C. A. Austin (St. George's Hospital, London). Neither of these antisera had been specifically raised against one or other isozyme of topoisomerase II.

Following the original purification of the two different isozymes of topoisomerase II by Drake *et al.* (1987), this group raised isozyme-specific rabbit polyclonal antisera using the two different isozymes, purified from gels, as the immunogens. The same group, after isolating cDNA clones for the separate isozymes, also went on to use synthetic dodecapeptides, representing corresponding but dissimilar protein stretches in the alignment, to raise rabbit polyclonal antibodies, which again appeared to be specific for one or other isozyme (Chung *et al.*, 1989). However, it was not clear whether any of these antibodies recognised the individual isozymes in their native form, essential for immunoprecipitation experiments, as only immunoblot data was presented (Drake *et al.*, 1987; Chung *et al.*, 1989); furthermore these antibodies were also only available in very small amounts.

Therefore, due to the general lack of availability of large quantities of anti-topoisomerase II antibodies, we decided both to raise our own antibodies and to characterize any other anti-topoisomerase II antibodies which became available to us in sufficient quantity.

The major choice in generating antibodies is whether to opt for polyclonal antibodies, usually raised in rabbits, or monoclonal antibodies, raised in mice. The advantages and disadvantages of each system are complex and tend not to rate one approach as being definitively better than the other (reviewed in Harlow and Lane, 1988). Ultimately, it is best to have a panel of both polyclonal and monoclonal antibodies for different applications. As monoclonal antibodies are generally more time-consuming and costly to prepare than polyclonal antibodies, and do not necessarily offer a better choice for many immunochemical applications, we initially set out to generate some rabbit polyclonal anti-topoisomerase II antibodies.

At this stage, we did not have any purified human topoisomerase II protein to use as an immunogen, so instead, had to decide between generating some bacterially-expressed protein fragments, or using synthetic peptides based on the human topoisomerase II sequence, as the potential immunogens. We opted to use a synthetic peptide approach, as this has proved highly successful in the generation of antibodies to many prokaryotic and eukaryotic proteins (reviewed in Walter, 1986, and in Ciba Foundation, 1986). Because of the way in which peptides are displayed to the immune system, many peptides ellicit a good response. Furthermore, we wanted to raise the antibodies against potential protein kinase target sites within the human topoisomerase IIα protein (see below), for which only an anti-peptide approach would be suitable. Unfortunately, the percentage of antibodies raised against peptides that will bind to native protein varies from antigen to antigen, and so the success rate could not be predicted.

In choosing peptides as immunogens, it is important to remember that the production of antibodies by an animal relies on the *in vivo* humoral response to the immunogen (see Harlow and Lane, 1988). For a compound to ellicit a primary antibody response and a strong secondary response, it must contain an epitope that can bind to the cell-surface antibody of a virgin B cell, and it must promote cell-to-cell communication between B cells and helper T cells. This requirement for both an epitope and an MHC class II-T-cell receptor binding site imposes a minimum size limit on an immunogen, and molecules of less than about 3000-5000 daltons are generally not good immunogens. Compounds smaller than this, termed haptens, can often bind the B cell surface antibody, but may not have suitable sites for the simultaneous binding of a class II MHC protein and a T-cell receptor. Hence small molecules, such

as peptides, are usually coupled to larger immunogenic compounds, termed carriers, to provide the missing class II-T-cell receptor binding sites. A modern development is the ability to synthesize the peptide epitope directly in sequence with a peptide known to be a synthetic class II-T-cell receptor site. The synthetic peptide, now containing both sites, can then be injected without coupling to a carrier.

Peptide sequences are more likely to generate antibodies which recognise the native protein if they are located on the surface of the protein. Early work suggested that peptides containing hydrophilic amino acids and proline residues were more likely to be exposed on the surface of the native protein than other sequences (Hopp and Woods, 1981). Hydrophilic residues are required on the surface to solubilise non-membrane proteins like topoisomerases, however, many strongly hydrophilic residues are buried in water pockets, and so this does not necessarily guarantee surface location. Prolines are known to cause the characteristic β-turn in protein secondary structure, and these often form portions of epitopes. Highly mobile regions of a protein, often including the N- or C-termini, are also likely to be on the surface. X-ray crystallography or NMR studies can highlight these mobile regions of a protein. Lastly, sites known to interact with other proteins, DNA or membranes must be exposed on the surface in at least one conformation of the protein; this includes sites of post-translational modification.

Whilst a crystallographic study of a fragment of the *E. coli* DNA gyrase B subunit has recently been published (Wigley *et al.*, 1991), there is no structural datum on any of the eukaryotic topoisomerases to suggest highly mobile regions of the protein. Therefore, in choosing peptides, we placed emphasis on the other criteria. Also, as we were particularly interested in the possible phosphorylation of topoisomerase II by the cell-cycle regulated kinase $p34^{cdc2}$, we decided to use potential $p34^{cdc2}$ kinase sites from within the human topoisomerase IIα sequence to make peptides for raising antibodies. Not only were potential kinase sites likely to be exposed on the protein surface, but the consensus target site for $p34^{cdc2}$ contains an absolutely conserved proline residue in a reasonably hydrophilic environment. The consensus being Ser/Thr-Pro with basic residues after, and sometimes before, this core sequence (see section 7.2.7). Thus, even if they were not *in vivo* phosphorylation sites, these sequences were likely to be on the surface of the protein. A further incentive to using this approach was that if $p34^{cdc2}$ was shown to phosphorylate topoisomerase IIα, then any antibodies raised to these

peptides could be used to try and block the phosphorylation, and hence identify a site of phosphorylation within the protein. Four peptides were chosen based on these criteria, designated T2K1 to T2K4.

Once partial protein sequences became available for both the α and β isozyme of human topoisomerase II, then two more peptides were chosen from highly divergent regions of the two isozymes, one being α-specific, designated T2α, the other being β-specific, designated T2β.

The six peptide sequences chosen and their location within the human topoisomerase IIα and β protein sequences are shown in Table 1. A cysteine residue not present in the topoisomerase IIα sequence was added at the N-terminal end of each peptide for coupling to a carrier molecule. For all peptides, the carrier molecule used first was bovine thyroglobulin, coupled via the sulfhydryl group of the N-terminal cysteine residue. Those peptides which raised positive anti-topoisomerase II antisera after immunization were then coupled separately to keyhole limpet haemocyanin (KLH) for further immunization. Immunizing with the peptide coupled to a different carrier boosts the anti-peptide antibodies with respect to antibodies the animal has raised to the carrier molecule.

The degree of divergence of an epitope from one species to another determines the degree of cross-reactivity of the antibody with the same antigen from different species. In general, whilst there is often reasonable cross-reactivity of an antibody between species which are closely related from an evolutionary standpoint, there is less certainty over cross-reactivity between widely divergent species e.g. human to yeast. Hence, the degree of cross-reactivity of an antibody can give a good indication of how well the protein, or at least the epitope-containing domain of the protein, has been conserved across evolution. If a conserved sequence is already known, then raising an antibody to a synthetic peptide containing this sequence gives the best chance of an antibody with wide cross-reactivity. For example, the extremely highly conserved PSTAIRE motif from the $p34^{cdc2}$ sequence has been used to generate antibodies which recognise the *S. pombe cdc2* homologue from virtually every species studied (Norbury and Nurse, 1989).

Finally, as well as raising antibodies by immunization in animals, human antibodies may be available to a required antigen in patients with a particular auto-immune disorder. For instance, patients suffering from the autoimmune connective tissue disease, systemic sclerosis, have high levels of anti-topoisomerase I antibodies (Shero *et al.*, 1986). Meliconi *et*

al. (1989) detected anti-topoisomerase II antibodies, by ELISA-screening, in 37% of sera from patients with cryptogenic fibrosing alveolitis (CFA), in sera from one case (8%) of sarcoidosis and in 31% of sera from systemic lupus erythematosus (SLE) patients. Patients of other connective tissue diseases, such as rheumatoid arthritis or systemic sclerosis, were negative for anti-topoisomerase II antibodies. As a limited number of CFA patients were available to us, their sera were tested for anti-topoisomerase II antibodies.

3.2 Results.

3.2.1 Screening Patients with Cryptogenic Fibrosing Alveolitis for Anti-topoisomerase II Antibodies.

Sera from three CFA patients were tested for the presence of anti-topoisomerase II antibodies, by immunoblotting crude nuclear extracts made from either a human cell line, HeLa, or from Chinese hamster ovary cell lines Whilst these extracts were clearly shown to contain topoisomerase II protein by other antibodies, none of the three sera gave any band of 170kDa on a Western blot, suggesting the absence of anti-topoisomerase II antibodies in these sera (data not shown). Whilst the ELISA-positive CFA sera all reacted with a 170kDa protein by immunoblotting in the studies of Meliconi *et al.* (1989), the ELISA-positive SLE sera reacted only very poorly in immunoblots. It is more likely that autoantibodies will react positively in assays using native protein such as ELISA or immunoprecipitation, than in assays using denatured protein such as immunoblotting. These three sera were not tested though against native protein as we wanted to obtain antibodies that could be used for immunoblotting in a variety of studies.

3.2.2 Characterization of Rabbit Anti-topoisomerase II Polyclonal Antibodies.

The test bleeds from the six rabbits immunized with the topoisomerase II peptides were initially assayed by immunoblotting, for recognition of denatured protein. The bleeds were tested against crude nuclear extracts from both human, HeLa, and hamster, CHO, cell lines. HeLa cells are known to contain a high level of topoisomerase II protein, possibly as a result of their high rate of proliferation. Two mutant Chinese

hamster ovary (CHO) cell lines that had been raised in this laboratory, designated ADR-2 and Adr^r, had been shown to have altered levels of topoisomerase II protein, by immunoblotting with the polyclonal antibody from Dr. L. F. Liu. The ADR-2 cell line had about twice the level of topoisomerase II protein as the wild-type (Davies, 1989), whist the Adr^R cell line had a reduced level of topoisomerase II protein level compared to wild-type (Hoban *et al.*, 1992). Hence, if the antibodies generated cross-react with hamster topoisomerase II, then they should demonstrate the difference in topoisomerase II protein levels between these two cell lines.

After the initial injections with the peptide coupled to the thyroglobulin carrier, three of the six peptides, designated T2K1, T2K2 and T2β, gave reasonably good signals on immunoblots. These were then coupled to KLH and the same rabbits given further immunizations. After the full course of injections these three rabbits were bled out.

Figure 4 shows Western blots of crude nuclear extracts made from HeLa cells, ADR-2 cells and Adr^r cells, run out on a 7.5% SDS-polyacrylamide gel and blotted onto nitrocellulose. ^{125}I-Protein A was used to detect the bound antibody. T2K1 appears to recognize both human and hamster topoisomerase II, in that (at a 1 in 1000 dilution) it not only reveals a 170kDa band in all three tracks, but it also shows up the dramatic difference in topoisomerase II protein levels between the two CHO cell lines. T2K2 (at a 1 in 1000 dilution) reveals a much stronger band at 170kDa in the HeLa extract, but only recognizes a 170kDa band very weakly in the hamster extracts. T2K2 also does not appear to recognize a difference in topoisomerase II protein levels between the two CHO cell lines.

T2K1 and T2K2 were then tested for their ability to recognise native human protein in an immunoprecipitation assay. HeLa cells were labelled for 16 hours with ^{35}S-methionine in methionine-free medium. Nuclear extracts were then prepared and the antisera used to immunoprecipitate the labelled topoisomerase II protein from the extract using the method of Kroll and Rowe (1991). The immunoprecipitates were washed and electrophoresed on a 7.5% SDS-polyacrylamide gel and the gel fixed, stained in AMPLIFY (Amersham), dried and exposed to X-ray film. A composite figure of some of the antibodies under investigation, including T2K1, is shown in Figure 5. Neither T2K1, nor T2K2 (data not shown), immunoprecipitated a protein of 170kDa. The labelling, extraction and immunoprecipitation methods

used were clearly working, as other antibodies tested did immunoprecipitate a 170kDa band. Likewise, both T2K1 and T2K2, were tested for their ability to stain fixed human cells but with negative results.

The antiserum raised to T2β, the topoisomerase IIβ specific peptide, appeared to be recognizing a protein of 170kDa in the human crude nuclear extract by immunoblotting, but with weaker affinity than either T2K1 or T2K2. As it was not as good an antibody in terms of affinity as T2K1 and T2K2 and neither was it clear whether it was in fact recognizing topoisomerase IIα or β, this antibody was not pursued further.

3.2.3 Use of Peptide-derived Antisera to Compare Topoisomerase II Protein Levels in Bladder and Testis Tumour Cell Lines.

Patients with metastatic testis tumors are generally curable using chemotherapy, whereas those with disseminated bladder carcinomas are not. Bladder and testis tumor cell lines reflect the clinically observed drug sensitivities of the two tumor types. For example, testis tumor cell lines are more sensitive to killing by *cis*-platinum and Adriamycin than are bladder tumor cell lines (Walker *et al.*, 1987). Adriamycin is thought to exert its cytotoxic effects through topoisomerase II, causing protein-associated strand breaks (see section 1.7 for a review of drug interactions). We wished to determine whether the differential sensitivity of these tumor types to topoisomerase II inhibitors, observed both clinically and *in vitro*, was due to differences in the level of topoisomerase II protein. This would support the current practise of using VP-16 as a drug in the treatment of nonseminomatous, testicular germ cell tumors (Fitzharris *et al.*, 1980).

The topoisomerase II protein levels were compared in three testis (SuSa, 833K and GH) and three bladder (RT4, RT112 and HT1376) tumor cell lines which differed in their sensitivity to topoisomerase II inhibitors (Fry *et al.*, 1991). Table 2 shows the drug sensitivities of these cell lines as measured using the dimethylthiazol-diphenyltetrazolium bromide (MTT) assay. Figure 6 shows a representative Western blot of nuclear proteins from the bladder and testis cell lines using the T2K2 antiserum. A high level of topoisomerase II protein was found in both SuSa and GH (testis) cells, a level 2- to 3-fold above that seen in the third testis line, 833K. RT112 and HT1376 cells, both bladder tumor cell lines, expressed very much lower levels of protein than SuSa or GH cells

(6- to 7-fold). However, 833K (testis) and RT4 (bladder) showed similar topoisomerase II protein levels. A similar relative level of topoisomerase II protein was seen by Western blotting with T2K1 antiserum and with the polyclonal antiserum from Dr. C. A. Austin (data not shown). Similar differences in protein levels were also seen on Western blots of whole cell extracts (data not shown).

Figure 6 shows that the predominant form of topoisomerase II in the bladder cell extracts is of a slightly higher molecular weight than that in the testis extracts. Whether this represents a difference in the expression of the topoisomerase IIα and β isozymes is not known. It is possible that a change in expression from the β to the α isozyme in the testis cells could sensitize them to topoisomerase II inhibitors, as the α isozyme has been shown to be preferentially sensitive to at least some of these drugs. (Drake *et al.*, 1989). However, as discussed in section 3.2.5, the topoisomerase IIβ protein appears to degrade very rapidly to a 150kDa form in usual extraction procedures (Negri *et al.*, 1992) and neither the T2K1 nor T2K2 antiserum appear to recognize this 150kDa band. So it is perhaps more likely that this blot is only detecting the α isozyme, but either in full-length or partially degraded form, and differences between the cell lines reflect a difference in susceptibility to degradation of the topoisomerase IIα protein.

Additional experiments were carried out to measure the frequency of DNA single-strand breaks produced by a range of m-AMSA concentrations in the testis and bladder cell lines and to compare the level of *in vitro* DNA:protein cross-linking produced by m-AMSA (Fry *et al.*, 1991). These data are also summarized in Table 2. There was a good correlation betwen m-AMSA-induced strand breakage in cellular DNA and m-AMSA-specific DNA:protein cross-linking in nuclear extracts. Furthermore, the variation in topoisomerase II strand breakage activity in the cell lines reflected the relative level of expression of topoisomerase II protein seen by Western blotting.

Although 833K cells expressed a relatively low level of topoisomerase II protein compared with that of the other two testis cell lines, and only slightly higher than that of the highest expressing bladder cancer cell line, RT4, they were markedly more sensitive to VP16 and Adriamycin than any of the bladder cell lines. IC_{50} values for these two drugs in the testis and bladder tumor cells fell into two narrow ranges and did not directly correlate with topoisomerase II protein expression. This indicates that a component of the drug sensitivity in these cell lines

must be an intrinsic property not explained by variation in topoisomerase II activity. Although VP16 and Adriamycin in part exert their cytotoxic effects via topoisomerase II, it is likely that other mechanisms, such as free radical generation, are also important (Katki *et al.*, 1987; Sinha and Mimnaugh, 1990).

In contrast to the findings with VP16 and Adriamycin, it was found that with m-AMSA, an apparently more specific topoisomerase II inhibitor, that there was a continuum of cell sensitivity that broadly reflected drug-induced DNA damage and topoisomerase II protein levels (Fry *et al.*, 1991). These results taken together suggest that topoisomerase II levels are an important component, but not the sole mechanism through which clinical topoisomerase II inhibitors exert their cytotoxic effects.

Testis tumor cell lines are sensitive to a wide range of drugs, including *cis*-platinum and vincristine, which appear to act via mechanisms independent of topoisomerase II. It has been demonstrated that there is reduced repair of *cis*-platinum-induced DNA-DNA intrastrand cross-links in SuSa cells, relative to 833K and RT112 cells (Bedford *et al.*, 1988), and this probably contributes to the extreme sensitivity to *cis*-platinum seen in this cell line.

It seems likely therefore, that the high level of topoisomerase II protein expressed in these testis tumor cell lines contributes to their sensitivity to m-AMSA, Adriamycin and VP-16. Conversely, the low level of topoisomerase II protein seen in bladder cancer cell lines probably contributes to their relative resistance to chemotherapeutic agents. This may be of clinical relevance in that measurement of topoisomerase II levels in individual tumors might predict response to chemotherapy. However the extreme and uniform hypersensitivity of the testis cell lines to certain topoisomerase II inhibitors is unlikely to result solely from the degree of expression of topoisomerase II protein.

3.2.4 Use of Cambridge Research Biochemical Polyclonal Topoisomerase II Antibody for Immunoprecipitation.

A polyclonal antiserum to topoisomerase II was raised in rabbits by Cambridge Research Biochemicals (Cheshire, U.K.), using a synthetic 18-mer peptide, based on the very C-terminus of the human topoisomerase IIα protein sequence. This antiserum, designated CRB, had been affinity-purified, and, according to the manufacturers, recognised a single band of

175-180kDa on Western blots. It had also been used successfully in FACS studies.

The peptide used to raise the antibodies, is not in fact a perfect match for the α-isozyme C-terminus, as it misses out a lysine residue, present in the protein, near the N-terminal end of the peptide. However, this region of the α isozyme differs markedly from the β isozyme C-terminus, with only about half the residues being conserved in both isozymes. It was likely then that, despite the size of the protein band recognized, this antibody was specifically recognizing the topoisomerase IIα isozyme.

We tested the ability of the CRB antiserum, not only to western blot human topoisomerase II, but also to immunoprecipitate the protein from crude nuclear extracts of ^{35}S-methionine-labelled HeLa cells. The CRB antiserum recognised a single band of approximately 170kDa on Western blots of crude nuclear extracts of HeLa cells (data not shown). This lined up with a band Western blotted by an α-specific, but not β-specific, monoclonal antibody (see section 3.2.5), confirming it to specifically recognize the α isozyme of human topoisomerase II.

Likewise, on immunoprecipitation analysis, the CRB antiserum specifically precipitated a band of 170kDa, which comigrated with a protein band immunoprecipitated by the α-specific monoclonal antibodies (Figure 5). The β-specific monoclonal antibodies immunoprecipitated a protein band of 150kDa, not present in immunoprecipitates with the CRB antiserum. Indeed, the CRB antiserum appeared to be the most efficient antibody at specifically immunoprecipitating the human topoisomerase IIα isozyme.

3.2.5 Characterization of Anti-topoisomerase IIα and β Monoclonal Antibodies.

Six monoclonal antibodies specific to one or other of the two human DNA topoisomerase II isozymes were obtained from Dr. G. C. B. Astaldi Ricotti (Pavia, Italy). Their production and preliminary characterization is described in Negri *et al.* (1992). They were raised in female Balb/c mice, following injection with a partially purified HeLa preparation containing major bands corresponding to both topoisomerase II isozymes.

Immunoblot analysis.

The antibodies were tested by immunoblot analysis against crude nuclear extracts of HeLa cells prepared either with 0.35M NaCl, or 1M NaCl (Figure 7). Three of the monoclonal antibodies, designated 7E6, 6F3 and 1F6, recognised the 170kDa topoisomerase IIα isozyme, though with different affinities: 6F3 and 1F6 gave similar signals, but 7E6 was considerably weaker.

The other three antibodies, 8D7, 8F8 and 9H10, recognised a 150kDa protein, which has been identified as a degradation product of the very labile 180kDa topoisomerase IIβ isozyme (Negri *et al.*, 1992). Topoisomerase IIβ protein was shown by Drake *et al.* (1989) to be twice as heat-labile as the topoisomerase IIα protein, and this greater instability probably at least partially accounts for why the two isozymes were not distinguished earlier. All three β-specific antibodies had comparable affinities, with 8F8 perhaps being the weakest. The 150kDa band in each case appeared to be associated with a smear from about 100-150kDa of immunologically associated protein. The strong band at 150kDa suggests that the full length 180kDa protein is cleaved quite specifically to give a product of 150kDa. This is then susceptible to cleavage by exopeptidases leading to a continuum of degraded fragments.

For both topoisomerase IIα and β isozymes, there did not appear to be a difference in the amount of protein extracted using either 0.35M or 1M NaCl. This confirmed that 0.35M NaCl is a sufficient concentration to remove all the salt-extractable topoisomerase II protein from isolated nuclei.

Immunoprecipitation analysis.

The six monoclonal antibodies described above were also tested for their ability to immunoprecipitate human topoisomerase II isozymes from crude nuclear extracts of ^{35}S-methionine labelled HeLa cells (Figure 5). The antibodies 6F3 and 1F6 immunoprecipitated a 170kDa protein from human cells, but with poor efficiency compared to the CRB polyclonal antibody. Both 6F3 and 1F6 also immunoprecipitated a band of approximately 140kDa, which was not present on the immunoblots with these antibodies, nor was present with the β-specific antibodies. This could represent a protein that specifically associates with the topoisomerase IIα protein.

The three antibodies which recognized the 150kDa fragment of topoisomerase IIβ on immunoblot also immunoprecipitated a 150kDa

protein from metabolically labelled cells. However, whilst the affinities on immunoblotting were the same for the three antibodies, 8D7 was clearly the best antibody on immunoprecipitation, followed by 8F8, with 9H10 being a very poor antibody. Again there was a strong co-precipitating band present with 8D7 and 8F8, not present on the immunoblots; the molecular weight of this co-precipitating protein was approximately 75kDa.

Immunostaining analysis.

The monoclonal antibodies were also used to stain both fixed HeLa cells and a number of different tissue sections. In tissue sections the staining patterns were identical when using any one of the three anti-topoisomerase IIα antibodies, and likewise the three anti-topoisomerase IIβ antibodies gave similar staining patterns to each other. Figure 8 shows the staining of a human tonsil section with an α-specific and a β-specific topoisomerase II antibody. The result is somewhat surprising as both antibodies seem to preferentially stain proliferating cells compared to the non-proliferating cells. This would suggest that not only topoisomerase IIα, but also topoisomerase IIβ, may be a proliferation marker in certain cells.

Staining of fixed HeLa cells confirmed the data of Zini *et al.* (1992) which showed that the anti-topoisomerase IIβ antibodies gave strong staining of the nucleolus (data not shown). The anti-topoisomerase IIα antibodies gave diffuse staining of the nucleoplasm, but there was also a surprisingly high level of cytoplasmic staining as well (data not shown).

Immunostaining of fixed cells and tissue sections was carried out by Dr. Carol MacGregor at the Institute of Molecular Medicine, Oxford.

3.3 Discussion.

The generation of two polyclonal anti-topoisomerase II antisera using a peptide approach, and the characterization of one other rabbit polyclonal antisera and six mouse monoclonal antibodies to human topoisomerase II proteins is presented. These are summarized in Table 3, where the usefulness of each antibody in different immunological applications is shown. We were unable to detect any anti-topoisomerase II antibodies in the three sera taken from patients suffering from the auto-immune disorder, cryptogenic fibrosing alveolitis.

The success in generating our own anti-topoisomerase II antibodies was limited; we obtained two polyclonal antisera which recognized a 170kDa protein on western blots from a total of six peptide sequences tried. Some of the possible reasons why the other four peptides were not strongly immunogenic have been discussed in the introductory section, 3.1. The two antisera which were positive on western blots appeared to recognize only the denatured topoisomerase II protein, as neither worked at all on immunoprecipitation or immunostaining. This means that the sequences represented by these peptides can only act as epitopes following denaturation of the protein. In other words, the peptides used to raise the antibodies do not appear to be presented on the surface of the native protein or else the conformation adopted in the short peptide molecule is different from that which the sequence adopts in the native protein.

The strong cross-reactivity of T2K1 with human and hamster topoisomerase II suggests a high level of conservation at the region to which this peptide was synthesized, residues 944-957 in the human topoisomerase IIα sequence. However T2K2, which has a stronger affinity for human topoisomerase II than T2K1, cross-reacts only poorly to hamster topoisomerase II suggesting a much weaker degree of conservation at the region to which this peptide was synthesized, residues 1155-1168 in the human topoisomerase IIα sequence. Comparing the human topoisomerase IIα sequence with other known eukaryotic topoisomerase II sequences, including the *Drosophila*, *S. cerevisiae* and *S. pombe* sequences, then in the region covered by the T2K1 peptide there are three residues absolutely conserved, whilst in the region covered by the T2K2 peptide there are only two residues absolutely conserved. This in itself only reflects the primary sequence conservation and it suggests that perhaps the tertiary structure of the T2K1 region is much better conserved than that of the T2K2 region.

The T2K1 and T2K2 peptides were designed before the sequence for the human topoisomerase IIβ protein became available. Later comparison showed that, of the 14 amino-acids in T2K1, 10 are absolutely conserved between the α and β sequences, with 3 of the other 4 being conservative changes interspersed thoughout the sequence. Of the 14 amino-acids in T2K2, 12 were absolutely conserved in a contiguous run with only the first two residues being different. Hence it was possible that both T2K1 and T2K2 would also recognize the human topoisomerase IIβ isozyme. However, there was no evidence for this as

neither revealed a band at 150kDa in Western blots, which was shown to be present by the β-specific monoclonal antibodies.

The two polyclonal antibodies generated, T2K1 and T2K2, have been used to look at the amount of topoisomerase II protein present in a variety of cells. Results are presented here for a comparison of topoisomerase II protein levels in a series of testis and bladder tumour cell lines, relating the protein level to the sensitivity to topoisomerase II poisons. This study demonstrated the correlation between sensitivity to these drugs and a high topoisomerase II protein content. These antibodies have also been used to study the topoisomerase II protein levels in a series of lung cancer cell lines with differing sensitivities to topoisomerase II poisons. However, in these cells there did not appear to be a good correlation between topoisomerase II protein content and sensitivity to these drugs suggesting that some other factor, such as MDR expression, is more important in these cells (S. Houlbrook and J. Carmichael, personal communication).

Characterization of the six monoclonal antibodies by western blot, immunoprecipitation and immunostaining showed that the α-specific antibodies, 6F3 and 1F6, recognize the denatured topoisomerase IIα protein relatively well, but have only a weak affinity for the native protein. 7E6 has a poor affinity both for denatured and native protein. The β-specific monoclonal antibodies all have a good affinity for the denatured topoisomerase IIβ protein, and 8D7 and to a lesser extent 8F8, have good affinities for the native protein.

It will be extremely useful for many reasons to identify the domains of the isozymes in which the epitopes for these monoclonal antibodies lie. The β-specific antibodies, 8D7 and 8F8, did not appear to cross-react with hamster topoisomerase II, suggesting that the epitopes these antibodies recognise are in regions of the protein that are not well conserved across species (data not shown). The identification of the co-precipitating proteins, p140 with topoisomerase IIα and p75 with topoisomerase IIβ, may also prove to be of great interest if they are shown to interact biologically with the different topoisomerase II isozymes.

In order to carry out a detailed analysis of the regulation of the human topoisomerase IIβ protein by phosphorylation it is important to obtain full-length protein. Otherwise major *in vivo* sites of phosphorylation may be lost, non-physiological sites may be revealed, and allosteric conformational changes induced by phosphorylation may be interrupted. The detection of human topoisomerase IIβ protein, both on

western blots and immunoprecipitates, as a 150kDa band, suggests that a 30kDa fragment has been cleaved off from either the N- or C-terminus. As these are the regions rich in potential phosphorylation sites, it makes isolation of the full-length protein still more critical. The identification of which end of the protein is lost, say through the use of antibodies generated to one or other end of the protein, will be a useful piece of information.

It is conceivable that cleavage of the topoisomerase IIβ protein is a physiological event in some cell types, explaining why even in buffers containing multiple protease inhibitors, only a 150kDa band is extracted. During studies on the cell cycle expression of topoisomerase II in chicken cells, Heck *et al.* (1988) identified a highly reproducible ladder of fragments of 80-150kDa, which were immunologically related to topoisomerase II. The authors suggested that these fragments were likely to be the result of *in vivo* proteolysis as they were present even after lysing cells straight into hot SDS/PAGE sample buffer. Whether these are all fragments of topoisomerase IIα, topoisomerase IIβ, or a mixture of both is not known, but the sizing of the largest fragment as 150kDa is suggestive that this at least is topoisomerase IIβ.

The immunostaining results with the monoclonal antibodies were slightly surprising in that firstly, both α-specific and β-specific topoisomerase II antibodies appeared to preferentially stain proliferating cells in tonsil tissue sections. Previous data in NIH-3T3 cells had suggested that topoisomerase IIβ is not proliferation-dependent (Woessner *et al.*, 1991). This difference might be species-specific or dependent upon the cell-type, but it suggests that the expression pattern of topoisomerase IIβ protein is more complex than first thought. Secondly, all three topoisomerase IIα-specific antibodies showed diffuse cytoplasmic, as well as nuclear, staining. Whether this cytoplasmic staining represents a biological important form of topoisomerase IIα protein is not known, but differential localization within the cell could be an important mode of regulation of nuclear topoisomerase II activity. This would need further investigation and is beyond the scope of this work.

The combination of the polyclonal and monoclonal antibodies against topoisomerase II means that we now have a good source of antibodies for many different applications. The polyclonal antibodies, T2K2 and CRB, have extremely high affinities for topoisomerase II protein in Western blots. T2K1, unlike all the other antibodies tested, appears to be strongly cross-reactive with hamster topoisomerase II and

presumably with other more closely related mammmalian species. The monoclonal antibodies will be very useful for immunoblotting and immunostaining the individual isozymes of human topoisomerase II. For immunoprecipitation purposes, CRB has a high affinity for the topoisomerase IIα isozyme and 8D7 reacts strongly with the topoisomerase IIβ isozyme.

Unfortunately, preliminary data suggest that CRB and 8D7 do not immunoprecipitate active protein as assayed by a decatenation assay (data not shown). This may be a steric problem if the antibodies interact with and block the active site tyrosine or ATP-binding site; or it could be due to not enough enzyme being immunoprecipitated in these assays to measure activity. Hence, for a detailed investigation of the effect of phosphorylation on topoisomerase II isozyme activity it was still necessary to purify the human enzyme chromatographically.

Name	Topo IIα Amino Acid Position (NB *)	Peptide Sequence
T2K1	944-957	(C)NGTEK**T**PPLITDYR
T2K2	1155-1168	(C)DTLKRK**S**PSDLWKE
T2K3	1356-1369	(C)KTKT**S**PKLSNKELK
T2K4	598-610	(C)KSS**T**PNHKKWKVK
T2K5	797-811	KDSA**S**PRYIFTMLSS
T2K6	1345-1359	EDFVPSDA**S**PPKTKT
T2K7	1208-1221	EVLP**S**PRGQRVIPR
T2K8	1350-1366	SDA**S**PPKTKT**S**PKLSNK
T2K9	566-579	HRFLEEFI**T**PIVKV
T2α	874-885	(C)NIRRLMDGEEPL
T2β	* 785-797 (Topo IIβ)	(C)EIFVVDRNTVEIT
H1K	* (from Histone H1 sequence)	(C)AKAKK**T**PKAKK

Table 1 Peptides used for antibody production and $p34^{cdc2}$ kinase assays. 10 peptides (T2K1-T2K9 and T2α) from the topoisomerase IIα sequence, 1 peptide (T2β) from the topoisomerase IIβ sequence, and 1 peptide (H1K) from the histone H1 sequence were synthesized. Peptides T2K1-T2K4, T2α and T2β, were conjugated via the additional N-terminal cysteine residue (C) and used to raise anti-topoisomerase II antibodies in rabbits. The 'Y' in T2K5 is the position of the active site tyrosine of topoisomerase IIα. Peptides T2K1-T2K9 and H1K were tested as substrates for $p34^{cdc2}$ kinase (see section 7.2.7); the potential phosphoacceptor residue is in bold type.

Cell Line	Doubling Time (hours)	Relative Topo II Protein Level[a]	m-AMSA-specific DNA:protein Cross-links[b]	ssDNA breaks (rad equiv.) at 50ng/ml m-AMSA	Relative m-AMSA sensitivity (IC^{50})
Bladder					
HT1376	31	1.0[c]	0.50	37.5 (1.0[c])[d]	1.0[c]
RT112	24	1.2	0.75	60.0 (1.6)	4.1
RT4	37	2.2	2.5	210 (5.6)	8.4
Testis					
833K	22	3.0	3.5	293 (7.8)	16
Susa	20	6.6	12	480 (13)	38
GH	25	7.2	14	480 (13)	16

Table 2 Summary of properties of bladder and testis cell lines (taken from Fry *et al.*, 1991).

[a] Based upon densitometric scanning of Western blots (Figure 6). Values represent mean of 2 independent determinations.

[b] Percentage of DNA bound at 6mg/ml of m-AMSA minus control in the absence of m-AMSA.

[c] Arbitrarily given a value of 1.0.

[d] Numbers in parentheses, relative values.

Antibody	Substrate Specificity	Type	Source*	Application		
				Blotting	I.P.	Staining
T2K1	Topo IIα	Polyclonal	Fry	+++	-	-
T2K2	Topo IIα	Polyclonal	"	++++	-	-
CRB	Topo IIα	Polyclonal	CRB	++++	++++	N.D.
7E6	Topo IIα	Monoclonal	Negri	+	-	++
6F3	Topo IIα	Monoclonal	"	+++	+	++
1F6	Topo IIα	Monoclonal	"	+++	+	++
8D7	Topo IIβ	Monoclonal	"	+++	++++	+++
8F8	Topo IIβ	Monoclonal	"	++	+++	+++
9H10	Topo IIβ	Monoclonal	"	+++	+	+++

Table 3 Summary of antibody characterization. The table lists those antibodies which we, or others, have generated which show degree of positivity towards topoisomerase II proteins. The isozyme-specificity and usefulness of each antibody in different immunological techniques is given (scale - to ++++ represents bad to extremely good). "Source*" refers to the group which generated each antibody; i.e. "CRB", Cambridge Research Biochemicals; "Negri", published in Negri *et al.* (1992); or "Fry", this work. N.D. were not determined.

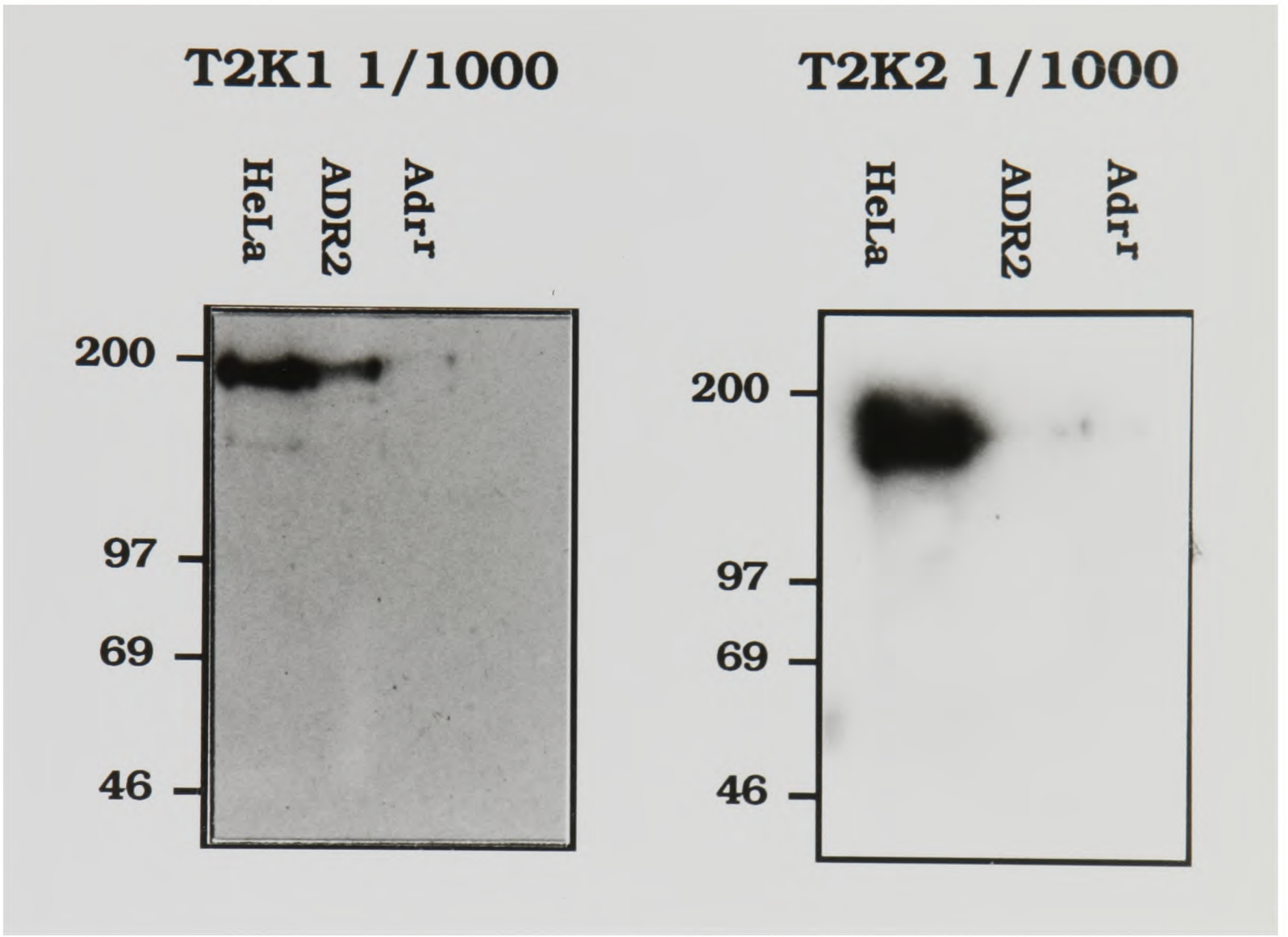


Figure 4 Western blots of human and hamster topoisomerase II using polyclonal antisera raised to peptides based on the human topoisomerase IIα sequence. Crude nuclear extracts of HeLa cells and the two CHO mutant cell lines, ADR-2 and Adr^r, were electrophoresed on 7.5% SDS-polyacrylamide gels, alongside molecular weight markers (kDa, shown on the left of each panel), and electroblotted onto nitrocellulose membrane. These were then blotted with either the T2K1 antiserum (left-hand panel) or the T2K2 antiserum (right-hand panel) at 1 in 1000 dilution. Bands were detected using ^{125}I-Protein A.

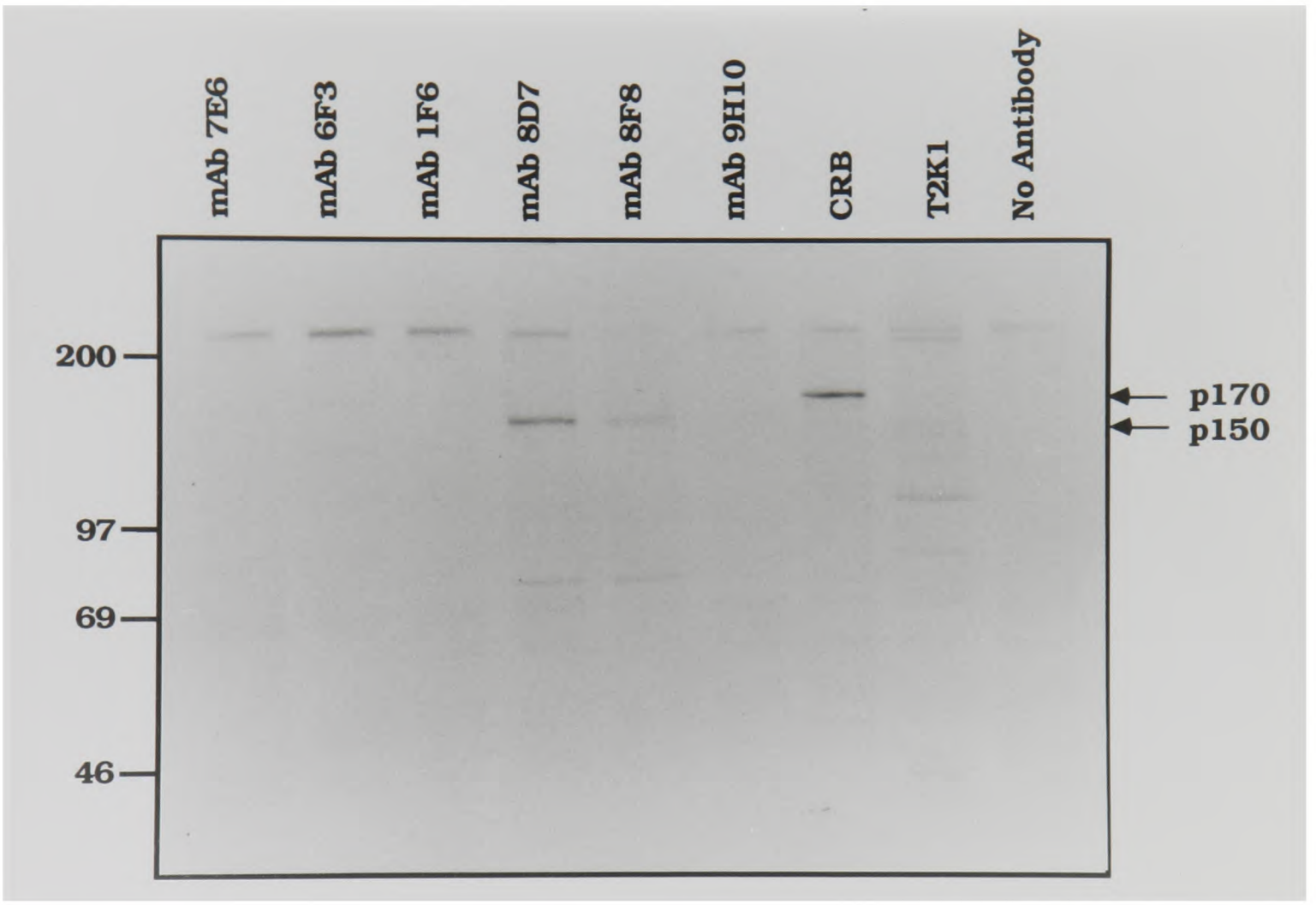


Figure 5 Immunoprecipitation of human topoisomerase II isozymes with a range of antibodies. Topoisomerase II proteins were immunoprecipitated from nuclear extracts of ^{35}S-methionine labelled cells with the antibodies shown above each track. The positions of the topoisomerase IIα protein (p170) and the stable fragment of the topoisomerase IIβ protein (p180) are shown on the right, and the positions of the molecular weight standards (kDa) shown on the left.

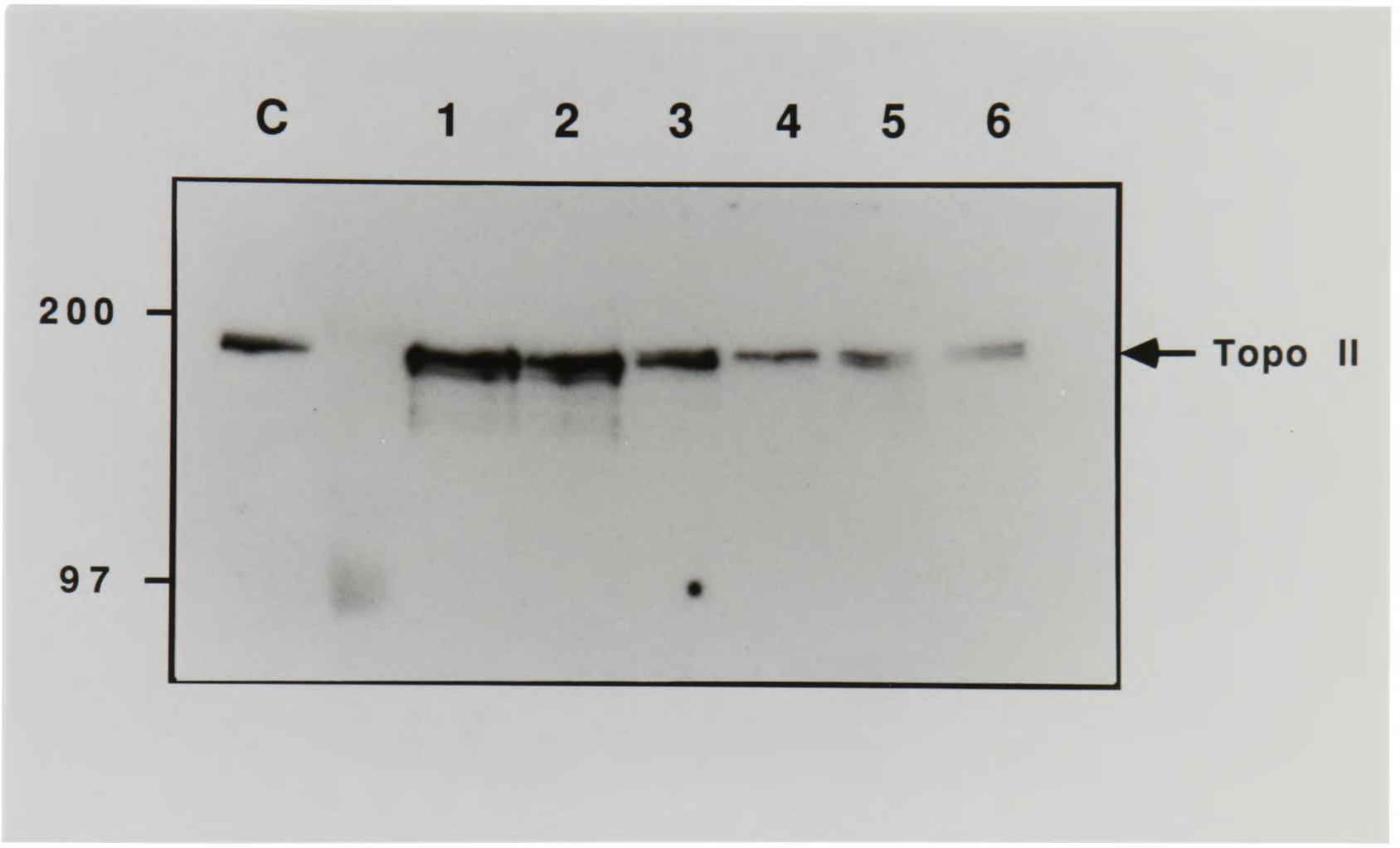


Figure 6 Western blot of nuclear extracts of testis and bladder cell lines using the T2K2 anti-topoisomerase II polyclonal antiserum. Lanes 1-3 show testis cell lines, SuSa (lane 1), GH (lane2), 833K (lane 4); lanes 4-6 show bladder cell lines, RT4 (lane 4), RT112 (lane 5), HT1376 (lane 6). Molecular weights (kDa) are shown on the right. Lane 'C' shows an extract from a control human cell line, HeLa. Bands were detected using ^{125}I-Protein A.

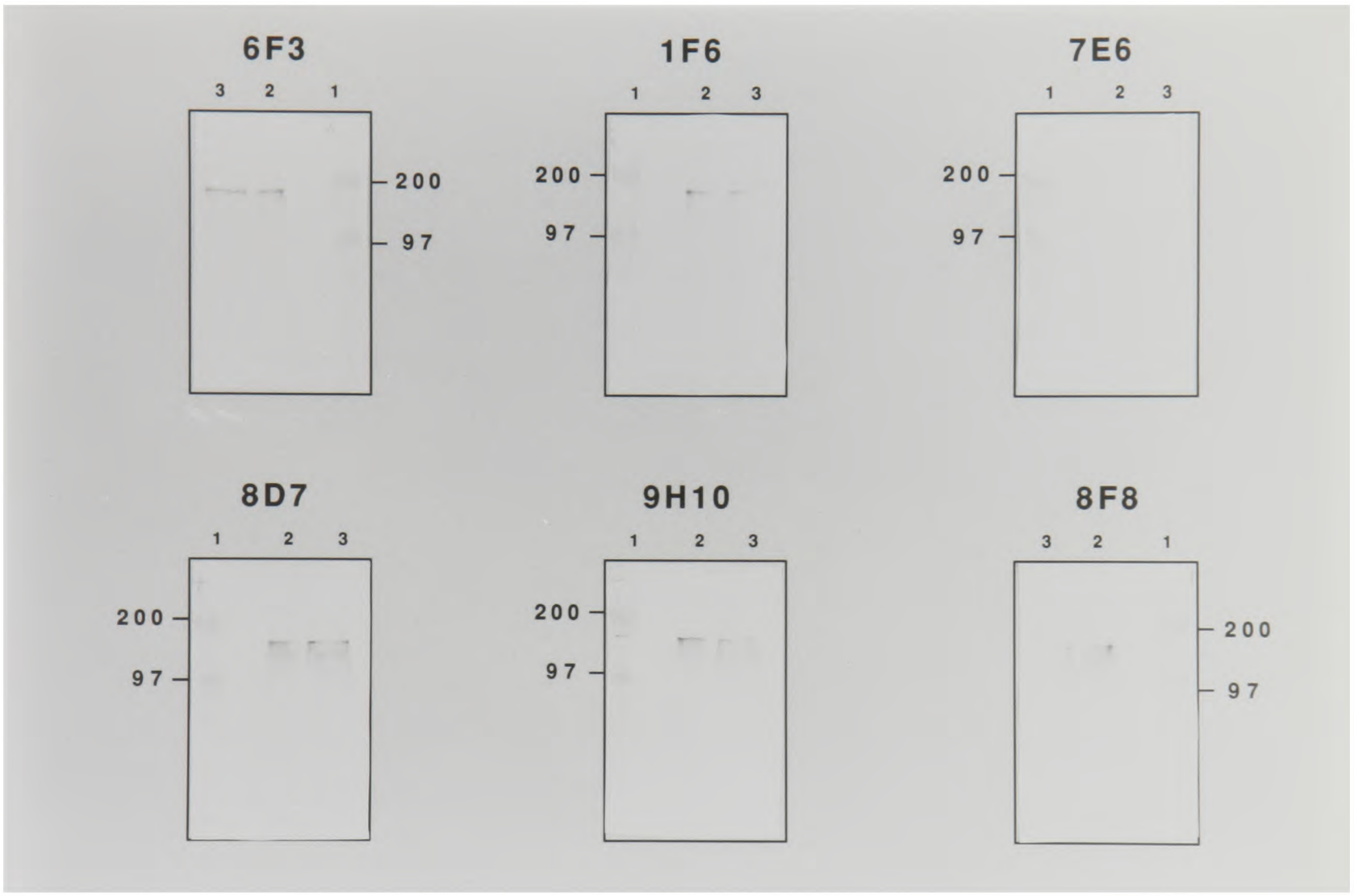
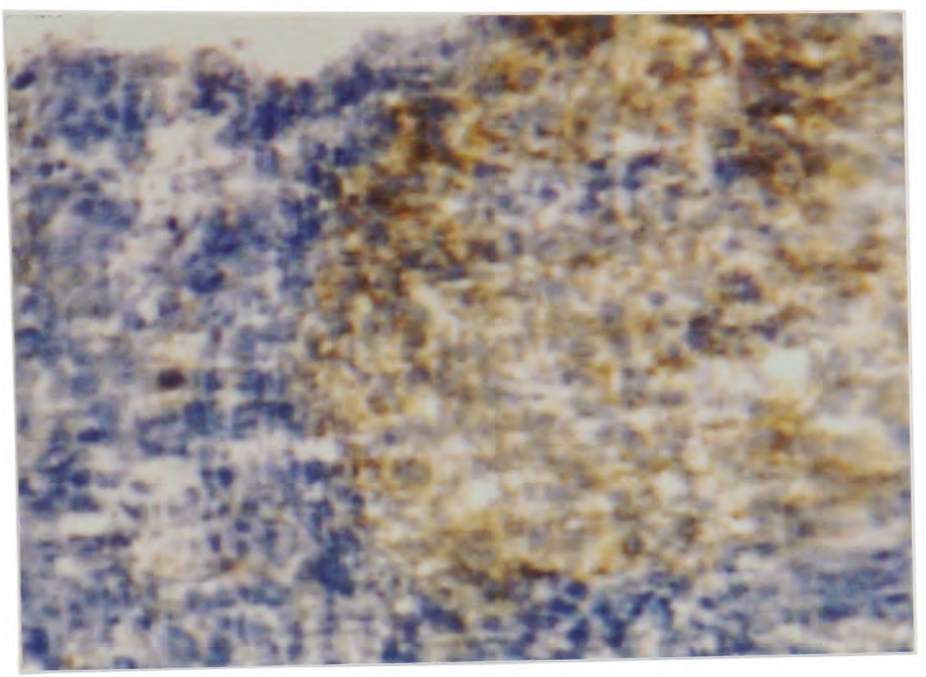


Figure 7 Western blot of human topoisomerase II isozymes using isozyme-specific monoclonal antibodies. Topoisomerase II proteins were extracted from HeLa cell nuclei, either with 0.35M (lane 2) or 1.0M (lane 3) NaCl, and electrophoresed against molecular weight standards, kDa (lane 1), on 7.5% SDS-polyacrylamide gels. After electroblotting onto nitrocellulose, the membranes were blotted with one of the six isozyme-specific monoclonal antibodies as shown above each panel. The bands were detected using an HRP-conjugated secondary antibody and diaminobenzidine.

A



B

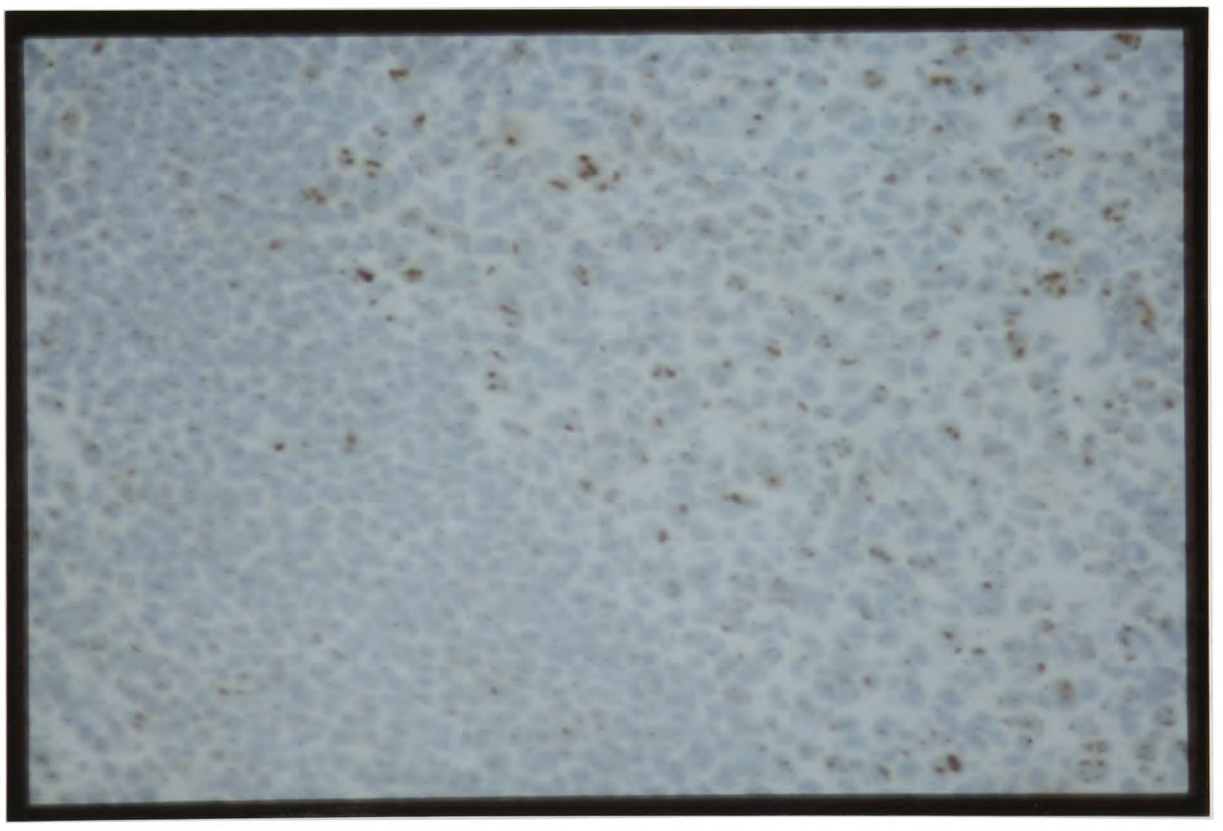


Figure 8 Immunoperoxidase staining of human tonsil sections with either a topoisomerase IIα (A) or topoisomerase IIβ (B) specific monoclonal antibody. The region of low cell density (top right) is the germinal centre containing highly proliferating cells, whilst the area of packed cells (bottom left) is the surrounding stromal tissue in which cells are not proliferating. Immunostaining was carried out by Dr. Carol MacGregor at the Institute of Molecular Medicine, Oxford.

Chapter 4 PURIFICATION OF HUMAN TOPOISOMERASE IIα PROTEIN: CO-PURIFICATION OF A PROTEIN KINASE ACTIVITY.

4.1 Introduction.

It has been clearly shown that regulation of topoisomerase II activity in response to many conditions is controlled through post-translational modifications (section 1.11). However, the two isozymes of mammalian topoisomerase II appear to be regulated in an independent manner as manifested by their different localization and expression patterns. The primary aim of this project was to investigate the specific regulation of the individual isozymes of the human topoisomerase II protein by phosphorylation. This should not only reveal how the biological functions of each isozyme are regulated by phosphorylation but also to what extent these differences in isozyme function are controlled by differential phosphorylation patterns.

To study the regulation of human topoisomerase II isozymes by phosphorylation, it was considered necessary to purify the proteins to near homogeneity. Purification of a protein enables very specific biochemical experiments to be carried out, which would not be possible in crude extracts or intact cells. In particular, the study of many enzymes has depended on their complete purification to allow analysis in defined systems where enzyme, substrate and product concentration can all be controlled and monitored, and in turn manipulated. Not only does it then become possible to measure the catalytic activity of the enzyme but, importantly in the context of this work, to also study the responsiveness of the enzyme to different forms of post-translational modification. Purification of a protein may also be necessary to allow a study of its interactions with other proteins or DNA, to crystallize the protein for subsequent structural analyses, or to obtain sequence of the protein with the aim of cloning the gene.

The realistic aim of purification is to increase the ratio of enzymatic activity to total protein to the maximum possible. Further purification may yield less contaminants but also less total activity. In order to study changes in the activity of purified topoisomerase II protein following changes in phosphorylation status, purity is perhaps a greater priority than yield. It is particularly important to obtain a preparation lacking proteins which migrate at the same position on SDS-PAGE which could

lead to confusion over which protein is being phosphorylated. It should be stressed that no protein is purified to absolute homogeneity, as even when the best possible detection methods show up no contaminating proteins, there are probably millions of foreign molecules in the purified fractions. Hopefully, such contaminants will not matter if they have come through the purification procedure by coincidence, unless they are highly reactive toward the protein or its substrate. Repeated copurification over many different types of column matrix of two proteins is suggestive of an interaction between the proteins which may merit further investigation.

After the first report of eukaryotic type II topoisomerase activity (Liu *et al.*, 1980; Baldi *et al.*, 1980; Hsieh and Brutlag, 1980), the first homogenous preparation of a eukaryotic type II topoisomerase soon followed (Miller *et al.*, 1981). Since then, nuclear topoisomerase II has been purified and characterized from a wide variety of eukaryotic sources, including protozoans (Shlomai and Zadok, 1983; Riou *et al.*, 1986), fungi (Goto and Wang, 1982; Goto *et al.*, 1984), insects (Hsieh, 1983; Shelton *et al.* 1983), amphibians (Benedetti *et al.*, 1983), and mammals, including calf thymus (Halligan *et al.*, 1985; Schomberg and Grosse, 1986; Strausfeld and Richter, 1989), rodent (Duguet *et al.*, 1983; Minford *et al.* 1986; Saijo *et al.*, 1990) and a variety of human cell lines (Miller *et al.*, 1981; Holden and Low, 1985; Drake *et al.*, 1987). Mitochondrial topoisomerase II has also been purified from the trypanosomatid *Crithidia fasciculata* (Melendy and Ray, 1989).

Comparable polypeptide molecular weights for the major species have been obtained: 172kDa (human), 168kDa (mouse), 175kDa (calf), 166kDa (*Drosophila*) and 150kDa (yeast). In addition, the *Drosophila*, calf and human enzymes have very similar sedimentation coefficients (9.2 - 9.5S) and Stokes radii (6.4 - 7.8nm) indicating that they may have a common tertiary structure. These hydrodynamic studies also first suggested the dimeric nature of the native eukaryotic enzyme. However, whilst there is agreement over the size of the major species, many workers have obtained multiple lower molecular weight immunoreactive bands when purifying topoisomerase II. Purified topoisomerase II from 6- to 18-hour old *Drosophila* embryos gave a major polypeptide of 166kDa (Shelton *et al.*, 1983), but also a group of peptides between 132kDa and 145kDa, and several small peptides of 30-40kDa (Heller *et al.*, 1986). Calf thymus preparations yielded either two bands of 175 and 150kDa (Schomberg and Grosse, 1986) or three bands of 165, 150 and 140kDa (Strausfeld and Richter, 1989). One mouse preparation gave two bands of

167 and 151kDa (Saijo *et al.*, 1990), whilst another yielded a cluster of lower molecular weight proteins of 100-150kDa (Minford *et al.*, 1986).

It has generally been thought that these lower molecular weight forms are *in vitro* breakdown products, and in some cases the concentration of these forms has indeed increased with storage of the purified protein (Minford *et al.*, 1986). However, there have also been suggestions that these lower molecular weight forms may exist in cells (Heller *et al.*, 1986), possibly playing a role in mitosis (Heck *et al.*, 1988).

It is possible that all of these observations can be explained in one of three ways. Firstly, the eukaryotic topoisomerase II protein appears to be highly susceptible to proteolysis and is rapidly degraded during isolation if great care is not taken to minimize proteolysis by the use of protease inhibitors and low temperatures. The ability to isolate a virtually homogeneous preparation of the 170kDa form, as has been achieved by several laboratories, argues against breakdown products being present naturally in the cell, but it is not proof. Degradation, be it *in vivo* or *in vitro*, probably accounts for the majority of the lower molecular weight products. The second explanation is the presence of two isozymes of topoisomerase II in vertebrate species. Drake *et al.* (1987), using amsacrine-resistant P388 leukaemic cells, specifically purified both the α and β isozymes and assigned them molecular weights of 170kDa (p170) and 180kDa (p180) respectively. Due to estimates of protein molecular weights being fairly inaccurate, it is possible that these two forms have been seen before but not distinguished as such. Equally the breakdown products identified by anti-topoisomerase II polyclonal antibodies could have come from either isozyme. In fact, the p180 protein appears to be even more unstable than the p170, and some preparations could have been a mix of full-length p170 together with degraded p180.

The third explanation is modification of the protein leading to a change in molecular weight. It has been well-documented that phosphorylation of a protein can alter its mobility on SDS-PAGE, and it is quite possible that two immunoreactive bands migrating closely near 170kDa represent different phosphorylation states of topoisomerase II.

Thus with this information in mind, our aim was to purify human topoisomerase II proteins for phosphorylation studies. In this respect the purified protein must fulfil at least two criteria:

(i) it must be clearly identifiable on an SDS-PAGE gel, well-separated from other contaminants, and preferably by far the major protein in the preparation.

(ii) it must still retain enzymatic activity, so that changes in activity in response to phosphorylation can be measured.

The major topoisomerase II-reactive band should also preferably be full-length for several reasons. One is that the protein can adopt its native structure in solution maintaining the structural integrity of target kinase sites, and not revealing sites which would be masked in the holoenzyme. Alternatively real target sites might be lost if domains of the protein are clipped off.

The presence of the two topoisomerase II isozymes must also be considered. So far this has not been done when studying the regulation by phosphorylation. Results presented in chapters 5 and 6 demonstrate that both topoisomerase IIα and β are phosphoproteins *in vivo*, and must therefore both be subject to regulation by phosphorylation. As standard enzymatic assays do not distinguish between the two isozymes, then once a type II topoisomerase activity has been purified it is essential to identify whether this represents either α or β, or a mix of both. This can be done by immunoblotting the purified material with monoclonal antibodies specific for each isozyme.

Furthermore, when purifying proteins for phosphorylation analyses, it is likely that the purified protein may already be partially or fully phosphorylated. If the protein is a phosphoprotein *in vivo*, as are both human topoisomerase II isozymes (see chapters 5 and 6), then at the point when cells are harvested for the preparation, a certain proportion of the molecules will be in a phosphorylated state dependent upon the various factors which stimulate phosphorylation *in vivo*. As phosphorylation is a covalent modification, a phosphorylated residue will generally remain intact throughout the purification procedure, unless it comes into contact with a active form of a phosphatase capable of removing the phosphate. This aspect will be dealt with more fully in chapters 5 and 6.

4.1.1 Strategy for purification of human topoisomerase II protein.

Choice of cell type.

Initially the decision had to be made whether to use a cultured cell line, or human tissue as a source for human topoisomerase II. Studies into the proliferation-dependence of topoisomerase II protein levels point to the α isozyme being virtually undetectable in quiescent cells. As the bulk

of cells in childhood and adult tissues are quiescent, they would be unlikely to be good sources of topoisomerase II.

One tissue that does have high levels of proliferating cells is the placenta and a preliminary purification on human term-placenta, obtained from the maternity department of the John Radcliffe Hospital, Oxford, was carried out. Success was limited as their was a large excess of non-proliferating and fibrous tissue cells compared to readily accessible proliferating placental tissue. Also term-placenta is perhaps not the best source as the cells stop proliferating towards the end of pregnancy, and a mid-term placenta would be a better source. Tissues also yield a mixed population of cell types, and this would lead to a mixed population of topoisomerase II proteins with respect to cell specificity.

With cell lines, it is possible to grow up very large numbers of cells of a homogenous cell type, with the result that, in general, a reasonable yield of cell-specific protein is possible. Cell lines do have some disadvantages, in that those that grow well are nearly all immortalized, if not also virally transformed. This could lead to a protein which is abnormally regulated *in vivo*.

Taking these things into account then it was decided to use a cultured cell line despite the drawbacks mentioned. The human cervical carcinoma cell line, HeLa, was chosen as the source for a number of reasons. Firstly, it is a human cell line, and the aim was to study the phosphorylation of the human topoisomerase II isozymes. Secondly, it is a cell line that grows rapidly in suspension allowing more than 10^{10} cells to be grown up for the start of the preparation. Thirdly, as the cell line grows equally well as a monolayer and in suspension, it will be easy to standardise on all experiments. In other words follow-up experiments carried out *in vivo*, based on results with the purified HeLa enzyme, can also be done on HeLa cells. Fourthly, it is known that HeLa cells, like other transformed cells, maintain intracellular topoisomerase II at high levels (Hsiang *et al.*, 1988), although the isozyme-specific abundance has not been studied in this cell type. Fifthly, topoisomerase II has been previously purified from HeLa cells (Miller *et al.*, 1981), and thus some of the chromatographic properties of HeLa topoisomerase II are already known. However, information on the chromatographic properties of the individual isozymes of topoisomerase II from HeLa cells was not known.

Choice of columns.

Figure 9 shows the choice of columns used to purify human topoisomerase II protein. All of these columns had been shown previously to bind mammalian topoisomerase II protein. The first steps of the purification protocol were based on the method of Miller *et al.* (1981), who prepared a 1M NaCl extract from isolated HeLa nuclei, and then separated this on hydroxylapatite and phosphocellulose chromatographic columns, and then an FPLC MonoQ column. The use of two further FPLC columns, phenylsuperose and Mono S, was deemed necessary after preliminary experiments showed the preparation to be relatively impure at this stage. The importance of maximizing purity at the expense of yield for these experiments was discussed in section 4.1.

FPLC phenylsuperose had been used in the purification of calf thymus topoisomerase II (Strausfeld and Richter, 1989). FPLC Mono S had been used in the purification of murine topoisomerase II by Drake *et al.* (1987). Furthermore this latter chromatographic step appeared to separate the two isozymes of topoisomerase II (Drake *et al.*, 1987). This separation is a prerequisite for a study of the phosphorylation of each isozyme independently.

The five chromatographic steps chosen rely on a variety of different physicochemical properties for retention and elution, so maximizing the chance of complete purification away from all proteins which do not physically interact with the topoisomerase II protein. Hydroxylapatite is a calcium phosphate gel, and provides separation based on a form of adsorption chromatography, probably involving dipole-dipole or purely electrostatic interactions with the Ca^{2+} and PO_4^{3-} ions. Phosphocellulose is a highly negatively charged matrix, having some of the properties of DNA; it therefore has a particularly high affinity for DNA binding proteins like topoisomerases. Mono Q and Mono S are ion exchange columns of opposite charge, Mono Q being anionic and Mono S cationic. Hence very few proteins bind to both columns at pH 7.5, and together these two columns make a good chromatographic combination. Those proteins that do bind both columns, like topoisomerase II, must have both strongly basic and strongly acidic regions of the protein. In contrast to the ion exchange columns, phenylsuperose relies on hydrophobic interactions for retention on this column; in other words exploiting yet another feature of the protein.

4.2 Results.

4.2.1 Purification of Human Topoisomerase II Protein.

Topoisomerase II enzyme activity was purified by conventional chromatography and FPLC on the columns shown in Figure 9 and the activity followed using a decatenation assay which is specific for type II topoisomerases. The approximate salt concentration that topoisomerase II activity eluted off each column is also shown in Figure 9 and documented in section 2.10.2 of the Materials and Methods. Following elution from one column, the active fractions were diluted down to a salt concentration which allowed the topoisomerase II activity to be retained on the subsequent column. In this way, the active fractions could be immediately loaded onto the next column. This was done in preference to prolonged dialysis as the protein appeared to be highly unstable upon extended dialysis at 4°C.

A representative trace of protein eluting off the column together with the appropriate decatenation assay photograph is shown for the two conventional chromatographic steps, hydroxylapatite (Figure 10) and phosphocellulose P11 (Figure 11). Figure 10 shows that using hydroxylapatite chromatography, the bulk of protein either did not bind to the column or came off in the early gradient fractions prior to topoisomerase II activity. Hence, this was a good first step in the purification protocol. A large proportion of the protein which co-eluted with topoisomerase II activity from the hydroxylapatite column did not bind to the phosphocellulose P11 column (Figure 11). However, topoisomerase II activity then eluted off this column at a similar potassium phosphate concentration to the bulk of the remaining protein. This may include a number of other DNA-binding proteins which are known to associate with the anionic phosphocellulose P11 matrix due to its similar charge properties to DNA. Coomassie staining of peak fractions from each subsequent column suggested that three FPLC purification steps were necessary to obtain a highly purified preparation.

An interesting observation on the FPLC MonoQ anion exchange column was the separation of a minor peak of decatenation activity eluting at approximately 250mM NaCl. The major peak of decatenation activity eluted at approximately 300mM NaCl. In order to increase purity, only the major peak was usually taken onto the next column. However, on one occasion following FPLC MonoQ chromatography, the activity from

both the minor and main peak was followed separately down one more column, FPLC MonoS, and the specific activity and yield of this preparation with the four chromatographic columns used was determined (Table 4). Protein deteminations were done by the method of Bradford (1976), whilst activities were assayed by carrying out decatenation reactions on a series of dilutions of each peak fraction. One unit of enzyme activity was defined as the amount of topoisomerase II required to completely decatenate 1μg of kDNA in 10 minutes at 30°C.

The total activity of the crude nuclear extract is likely to be higher than that shown in Table 4 as the aliquot of this extract retained for these measurements had to be dialysed overnight due to the presence of 1M NaCl. Topoisomerase II activity could not be measured in such high salt. Hence, it is likely that some activity was lost during this dialysis period. This would explain why the yield appears to go up after the hydroxylapatite chromatography step. The amount of protein in the crude nuclear extract also appears to give a smaller than expected value considering the amount of protein in the hydroxylapatite peak and the observation that the bulk of protein appeared to wash straight through this column. Again, it is possible that the dialysis affected this measurement, perhaps through some precipitation of proteins on reducing the salt concentration.

Allowing for the fact that the values for the crude nuclear extract may have been affected by dialysis, there is an increase in specific activity at each step of the purification procedure. The split of activity on FPLC MonoQ into two peaks explains why there is not such an increase in specific activity at this step. It is interesting that there is only approximately twice as much activity in the main peak as prepeak emphasizing the importance of both of these separate peaks. Apparently, the least successful chromatographic step was the phosphocellulose P11 column where there was nearly a three-fold loss of yield for only a minor increase in specific activity. However, the profile shown in Figure 11 suggests that this column purified the topoisomerase II activity away from a large bulk of protein and thus the surprisingly small increase in specific activity most likely results from an overall loss of active topoisomerase II protein at this stage.

To assess the purity of a preparation made using all five chromatographic steps, the fraction with peak activity from the final FPLC Mono S column was electrophoresed on a 7.5% SDS-polyacrylamide gel and stained with Coomassie blue (Figure 12). The peak fraction

contained a predominant protein with an apparent molecular weight of 170kDa. From 3 x 10^{10} HeLa cells, approximately 100μg of topoisomerase II protein were purified.

4.2.2 Identification of the Purified Protein as the α Isozyme of Human Topoisomerase II.

Western blotting of the active fractions from the FPLC Mono Q column (Fraction IV) and FPLC Mono S column (Fraction VI) with topoisomerase IIα and β isozyme-specific antisera (Negri *et al.*, 1992), indicated that the α and β isozymes co-purified through the hydroxylapatite, phosphocellulose and FPLC Mono Q columns (up to Fraction IV) (Figure 13). However the peak activity from the final FPLC Mono S column (Fraction VI) contained solely topoisomerase IIα protein (Figure 13). Detection of the bands on the Western blots was done by the Enhanced Chemiluminescence technique which is extremely sensitive; topoisomerase IIβ could not be detected in this final fraction (Fraction VI) even on very long exposures. Co-purification of the two isozymes over hydroxylapatite and FPLC Mono Q, but separation on FPLC Mono S, was also reported by Drake *et al.* (1987), when two forms of the enzyme were first distinguished. Preferential degradation due to the greater lability of the β isozyme could also explain the preferential purification of the α isozyme.

The two peaks which separated on the FPLC MonoQ column were both blotted onto nitrocellulose and probed with anti-topoisomerase IIα and β monoclonal antibodies. Detection using the ECL technique showed that both peaks contained both topoisomerase IIα and β proteins, demonstrating that the appearence of two peaks did not represent resolution of the two human topoisomerase II isozymes. (data not shown).

4.2.3 Co-purification of a Kinase which Phosphorylates the Topoisomerase IIα Protein.

To test whether the purified topoisomerase IIα preparation contained any kinase activity, it was incubated with [γ^{32}P]-ATP, separated by SDS-PAGE and autoradiographed. A phosphorylated band of 170kDa was seen, suggesting that the topoisomerase IIα protein had been radiolabelled (Figure 14, lane 1). To confirm that this was due to phosphorylation of the protein, rather than simply ATP-binding, the

reaction was repeated at both 4°C and 30°C. The topoisomerase IIα protein was only labelled in the reaction carried out at 30°C, suggesting that it was the result of an active enzymatic phosphorylation reaction, and not simple ATP binding which should be reasonably temperature-insensitive (data not shown).

The eukaryotic topoisomerase II has never been shown to have autophosphorylation activity, nor does it contain any of the conserved kinase domain motifs present in all protein kinases sequenced so far (Hanks and Quinn, 1991). The conclusion then is that this preparation contains a protein kinase activity that can phosphorylate the human topoisomerase IIα protein.

4.2.4 The Biochemical Properties of the Co-purifying Kinase Suggest it is Casein Kinase II.

In order to try and identify the co-purifying kinase, the preparation was again incubated with [γ^{32}P]-ATP, this time in the presence of inhibitors of potentially relevant kinases (Figure 14). The phosphorylation of topoisomerase IIα was almost completely inhibited by heparin which specifically inhibits casein kinase II (Hathaway *et al.*, 1980), but was unaffected by PKi, a peptide inhibitor of PKA, and H-7, an isoquinolinesulfonamide inhibitor of cyclic nucleotide-dependent protein kinases and protein kinase C (Hidaka *et al.*, 1984). This suggested that it might be casein kinase II (CK II) that was co-purifying with human topoisomerase IIα.

Two previous reports of a kinase co-purifying with topoisomerase II, in *Drosophila* (Sander *et al.*, 1984) and in mouse (Saijo *et al.*, 1990), both showed the kinase to be heparin-sensitive but with a lower sensitivity than expected for the CK II from those organisms. Hence, these authors argued against the co-purifying kinase being a classical CK II, but perhaps a related casein kinase family member.

When purified mammalian CK II was added to the human topoisomerase IIα preparation there was a much more efficient phosphorylation of the 170kDa protein (confirmed in chapter 5). The degree of heparin sensitivity of the phosphorylation attributable to the co-purifying kinase was compared with that of purified mammalian CK II. This was done by carrying out a series of topoisomerase IIα phosphorylation reactions with different heparin concentrations, in the absence or presence of purified CK II (Figure 15). The autoradiograph

was scanned by densitometry and the level of topoisomerase IIα phosphorylation plotted against heparin concentration (Figure 16). From this the concentration of heparin needed to inhibit 50% of the phosphorylation was measured as 1.2μg/ml for the co-purifying kinase and 1.8μg/ml for purified mammalian CK II. Hence the co-purifying CK II had a comparable heparin-sensitivity to CK II.

Another characteristic of CK II is its ability to use GTP, instead of ATP, as the phosphate donor. The phosphorylation of the purified topoisomerase IIα protein was carried out in the presence of [γ^{32}P]-GTP either in the absence or presence of purified CK II (Figure 17). Clearly the co-purifying kinase was able to use GTP to phosphorylate topoisomerase IIα protein, and this phosphorylation was increased by the addition of purified CK II. These biochemical data support the notion that the co-purifying kinase is CK II.

4.2.5 One-dimensional Phosphopeptide Mapping also Suggests the Co-purifying Kinase is Casein Kinase II.

The purified human topoisomerase IIα protein was phosphorylated either by incubation with [γ^{32}P]-ATP alone, i.e. by the co-purifying kinase, or by the addition of purified CK II. The phosphorylated proteins were gel purified, and then subjected to one-dimensional phosphopeptide analysis following partial cleavage with S. *aureus* V8 protease. An autoradiograph of a typical cleavage pattern is shown in Figure 18. The pattern of phosphopeptides is highly similar in that all the phosphopeptides generated by the co-purified kinase are also present after phosphorylation by purified CK II. There does appear to be a difference in the efficiency with which different phosphopeptides are phosphorylated, in that the two peptides most heavily phosphorylated by the co-purifying kinase, at approximately 23kDa and 20kDa, are not the peptides most heavily phosphorylated by purified CK II.

4.3 Discussion.

The α isozyme of human topoisomerase II has been specifically purified to near-homogeneity. Using cultured HeLa cells as a source approximately 100μg of protein was obtained from 3 x 10^{10} cells. This yield is relatively low considering that topoisomerase II is a moderately abundant protein in all proliferating cells, and HeLa cells are known to

have a particularly high level of expression of this protein. However, it probably reflects the low stability of mammalian topoisomerase II protein that has been observed by other workers. For instance, Drake *et al.* (1987) reported a low fold purification of topoisomerase II protein from human P388 amsacrine-resistant cells, which they attributed to the very low half-life of the protein at 4°C (a matter of only several days), especially when in dilute solution. They obtained a similar yield of several hundred micrograms from 2×10^{10} cells.

In the original purification of HeLa cell topoisomerase II by Miller *et al.* (1981), it was shown that freezing of the cells prior to purification further reduced the stability of the protein, perhaps as a result of the release of proteases from ruptured lysosomes. They found that when using fresh cells the enzyme stability rose dramatically, and hence in our preparations fresh cells were always used.

The ability to purify the α isozyme of topoisomerase II, free of any β isozyme, was crucial for the study of the phosphorylation of the individual isozymes of topoisomerase II. The western blot data using α- and β-specific monoclonal antibodies showed that Fraction IV of the preparation (i.e. after FPLC MonoQ) contained both isozymes, whilst Fraction VI (i.e. after the final column, FPLC MonoS) contained exclusively topoisomerase IIα. It is possible that the α and β isozymes were sufficiently separated either on FPLC phenylsepharose or FPLC MonoS to prevent overlap of the two peaks of activity. Indeed, Drake *et al.* (1987) were able to separate the two isozymes by chromatography on MonoS. However, it is equally likely that during the last two purification steps topoisomerase IIβ was degraded, as there was no second peak of topoisomerase II activity off FPLC MonoS corresponding to the β isozyme.

Interestingly, assay of different fractions coming off the FPLC MonoQ column showed that decatenation activity did appear to resolve into two peaks, with a lower amount of activity preceding the bulk of activity. Both of these peaks contained both topoisomerase IIα and β isozyme as identified using the monoclonal antibodies. Hence these two peaks did not represent separation of the two isozymes. Boege *et al.* (1991) also separated human topoisomerase II over FPLC MonoQ into two peaks which both contained the α and β isozyme. They showed that both fractions contained similar amounts of each isozyme, but differed markedly with respect to their pH optima for activity, and their sensitivity to m-AMSA and orthovanadate. They proposed that epigenetic modifications such as phosphorylation, acylation or isoprenylation of

topoisomerase II may explain the different properties of these two subtypes. Of these modifications only phosphorylation has been demonstrated for topoisomerase II, and it is possible that these two peaks represent different phosphorylation states of topoisomerase II.

Both peaks were kept and further purified separately over the final two columns. Both contained only detectable topoisomerase IIα protein after the final FPLC MonoS column. However, due to lack of time the two fractions have not yet been studied in detail to investigate whether they do indeed represent differently phosphorylated species of topoisomerase IIα.

Previous attempts to purify eukaryotic topoisomerases have sometimes yielded preparations containing copurifying protein kinases. This has been the case for both topoisomerase I (Pommier *et al.*, 1990) and topoisomerase II from *Drosophila* (Sander *et al.*, 1984), mouse (Saijo *et al.*, 1990) and yeast (Cardenas *et al.*,1992). In the case of topoisomerase II, the *Drosophila* and mouse preparations contained copurifying kinases of very similar enzymatic properties, which were characteristic of the casein kinase family. The kinases were heparin sensitive, and not stimulated by Ca^{2+} or cyclic nucleotides. However several criteria argued against them being CK II. The heparin concentration required for 50% inhibition of the kinase activity was much higher than that previously reported for CK II (Hathaway *et al.*, 1980). Little inhibition was observed in the presence of competing GTP for the mouse copurifying kinase. Likewise for the *Drosophila* kinase the ionic strength optimum and substrate preference were different to that of the previously identified *Drosophila* CK II. Cardenas *et al.* (1992) however, claim that the kinase co-purifying with the yeast topoisomerase II is in fact CK II.

We have also identified a co-purifying kinase activity in our preparation of topoisomerase IIα, which can phosphorylate the topoisomerase IIα protein. This is the first report of a co-purifying kinase with human topoisomerase II. The degree of heparin sensitivity of this kinase and its efficiency of using GTP as a phosphate donor suggest that it is human CK II.

Furthermore, one dimensional phosphopeptide maps generated following partial V8 protease digestion were similar between purified topoisomerase IIα phosphorylated *in vitro* either with the co-purifying kinase alone, or with additional purified mammalian CK II. Each phosphopeptide produced by the co-purifying kinase was phosphorylated

to a much greater extent upon the addition of CK II. There did appear to be a difference in the efficiency of phosphorylation of different phosphopeptides. Comparison with the pattern of phosphopeptides generated from topoisomerase IIα phosphorylated *in vivo* suggested that those sites favoured *in vivo* were also the favoured sites for the co-purifying kinase. Alternatively, addition of purified CK II caused other weakly phosphorylated peptides to become as strongly phosphorylated. The similarity in the sizes of the phosphopeptides is further strong evidence that the co-purifying kinase is indeed CK II.

Other kinases that phosphorylate topoisomerase IIα give quite different phosphopeptide patterns after partial V8 cleavage (see chapters 6 and 7). The differences in target preference reflected in the intensities of the different phosphopeptides could be due to the co-purifying kinase being human, whilst the purified CK II is rabbit. Alternatively the CK II which has co-purified with topoisomerase IIα may be modified in a different way from the purified CK II; or it may simply be the result of having a vastly different amount of CK II present. It could also reflect a difference in the exact state of the purified topoisomerase IIα compared to the enzyme *in vivo*.

An antibody to the α catalytic subunit of CK II was shown to blot a band of approximately 40kDa from crude nuclear extracts of HeLa cells (data not shown). However, it did not show up a similar sized band in the preparation of purified human topoisomerase IIα protein. This is perhaps not that surprising, as the very low level of phosphorylation of topoisomerase IIα by this co-purifying kinase means that there can only be a trace amount of kinase present, which is probably below the level of detection for this antibody. It has not been proven that this kinase is authentic human CK II and the possibility remains that it is a closely related member of the casein kinase family.

The chromatographic properties of CK II on the columns used here are quite different from those of topoisomerase II (Hathaway *et al.*, 1980). Hence, it would not be expected to co-purify with topoisomerase IIα by coincidence. Whilst we have not formally shown an interaction between the co-purifying kinase and topoisomerase IIα, it is highly suggestive that, if the kinase is CK II, its presence in the preparation is the result of physical association between the two proteins. This would be strong evidence for the involvement of CK II in the regulation of topoisomerase IIα protein in human cells.

Purification Fraction	Protein (mg)	Volume (ml)	Specific Activity[b] (units/mg)	Total Activity (units)	Yield (%)
Crude Nuclear Extract	144	320	1600	2.3×10^5	100
Hydroxylapatite	59	130	5100	3.0×10^5	130
Phosphocellulose P11	17.5	90	7100	1.2×10^5	52
FPLC MonoQ[a]					
Prepeak	1.34	8	8900	1.2×10^4	5.2
Main Peak	3.96	11	5200	2.1×10^4	9.1
FPLC MonoS [c]					
Prepeak	0.06	0.6	12500	750	0.3
Main Peak	0.084	0.6	17900	1500	0.7

Table 4 Purification of topoisomerase II activity from HeLa cells. Figures were calculated from a purification preparation employing four columns: hydroxyapatite, phosphocellulose P11, FPLC MonoQ and FPLC MonoS. [a] The decatenation activity from the FPLC MonoQ column separated into two peaks which were followed independently. [b] Activity was defined as 1 unit = Amount of topoisomerase II required to decatenate 1µg kDNA in 10 minutes at 30°C. [c] Fractions were kept separate following the final FPLC MonoS column, hence the values given are for the fraction with highest activity.

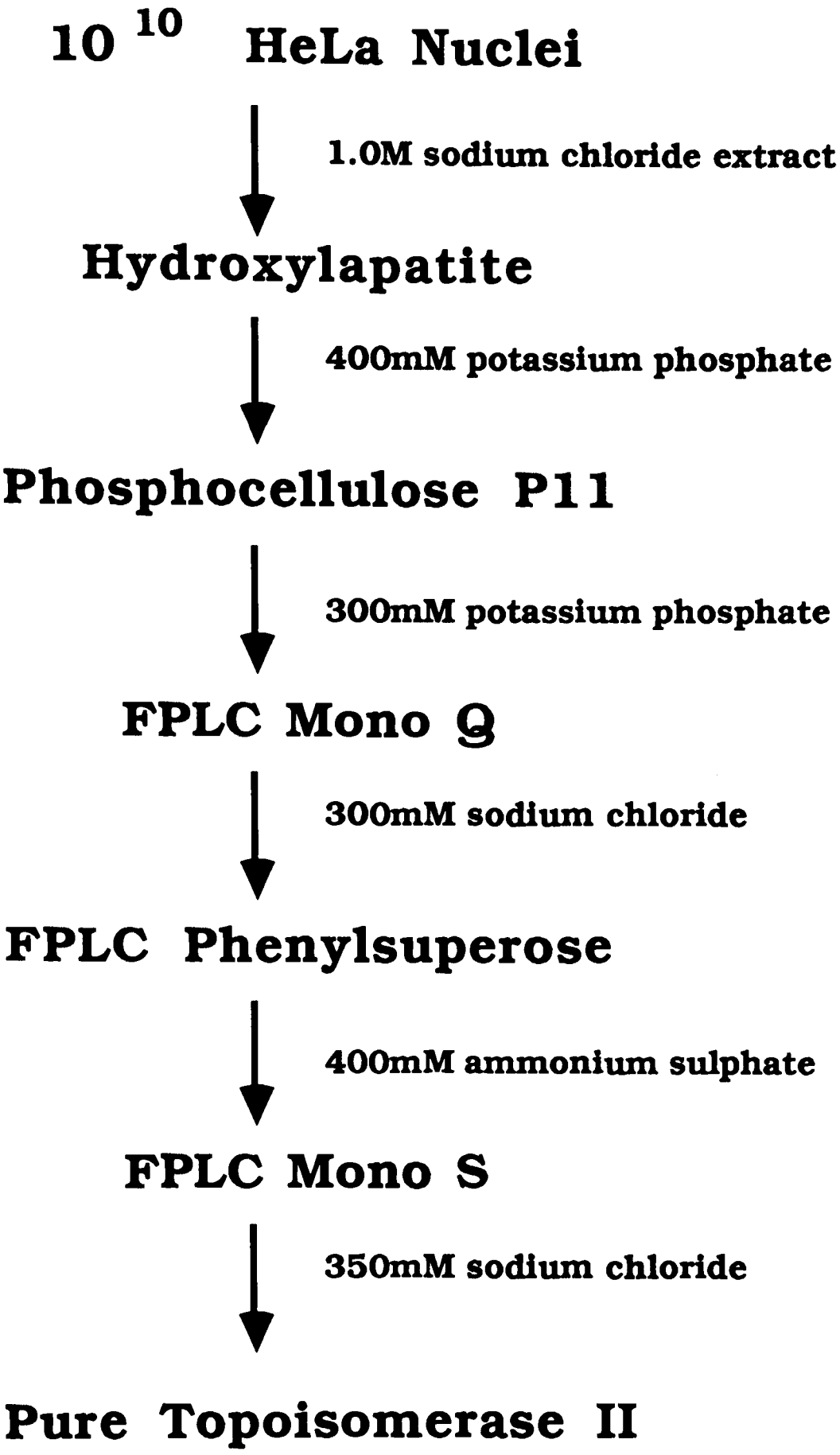
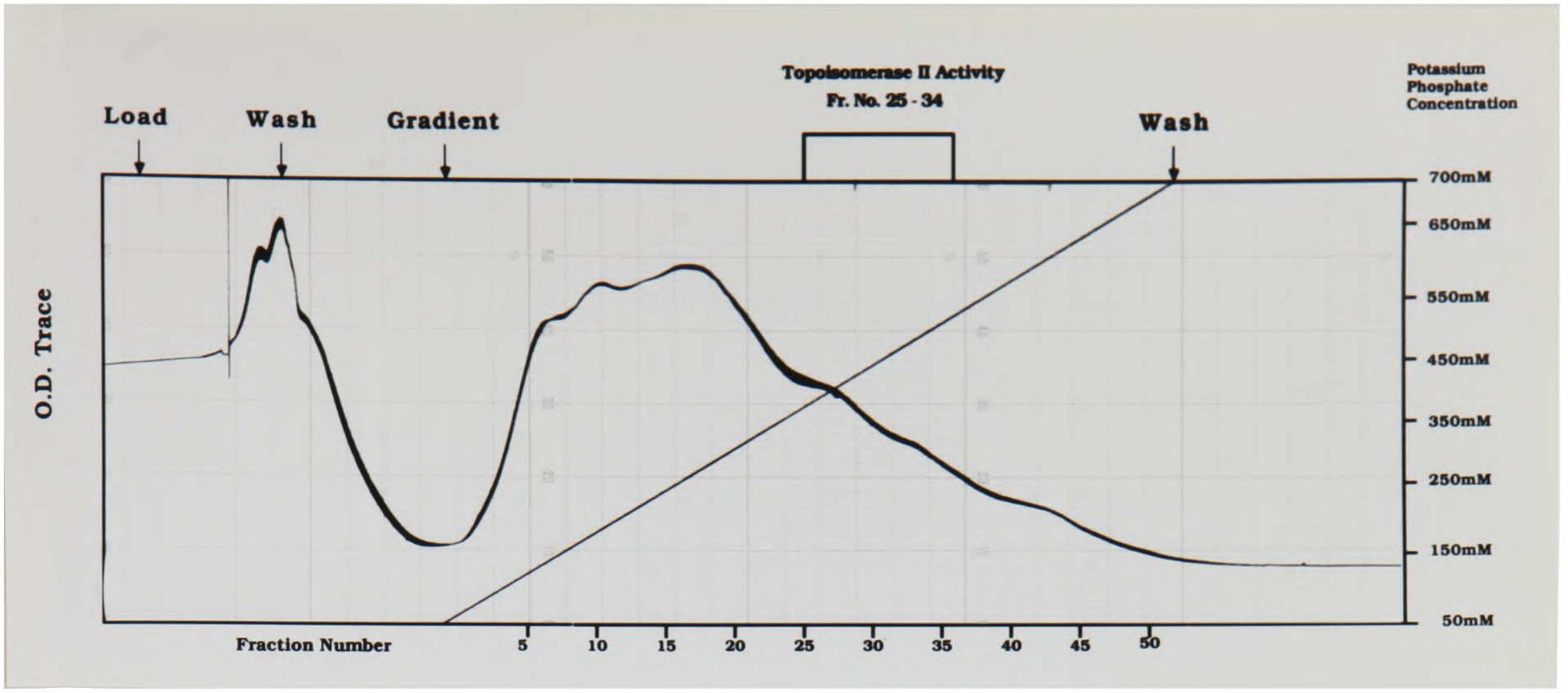


Figure 9 Protocol for purification of human topoisomerase II protein. The flow diagram shows the columns used in the purification procedure and the salt concentration at which topoisomerase II decatenation activity eluted off each column.



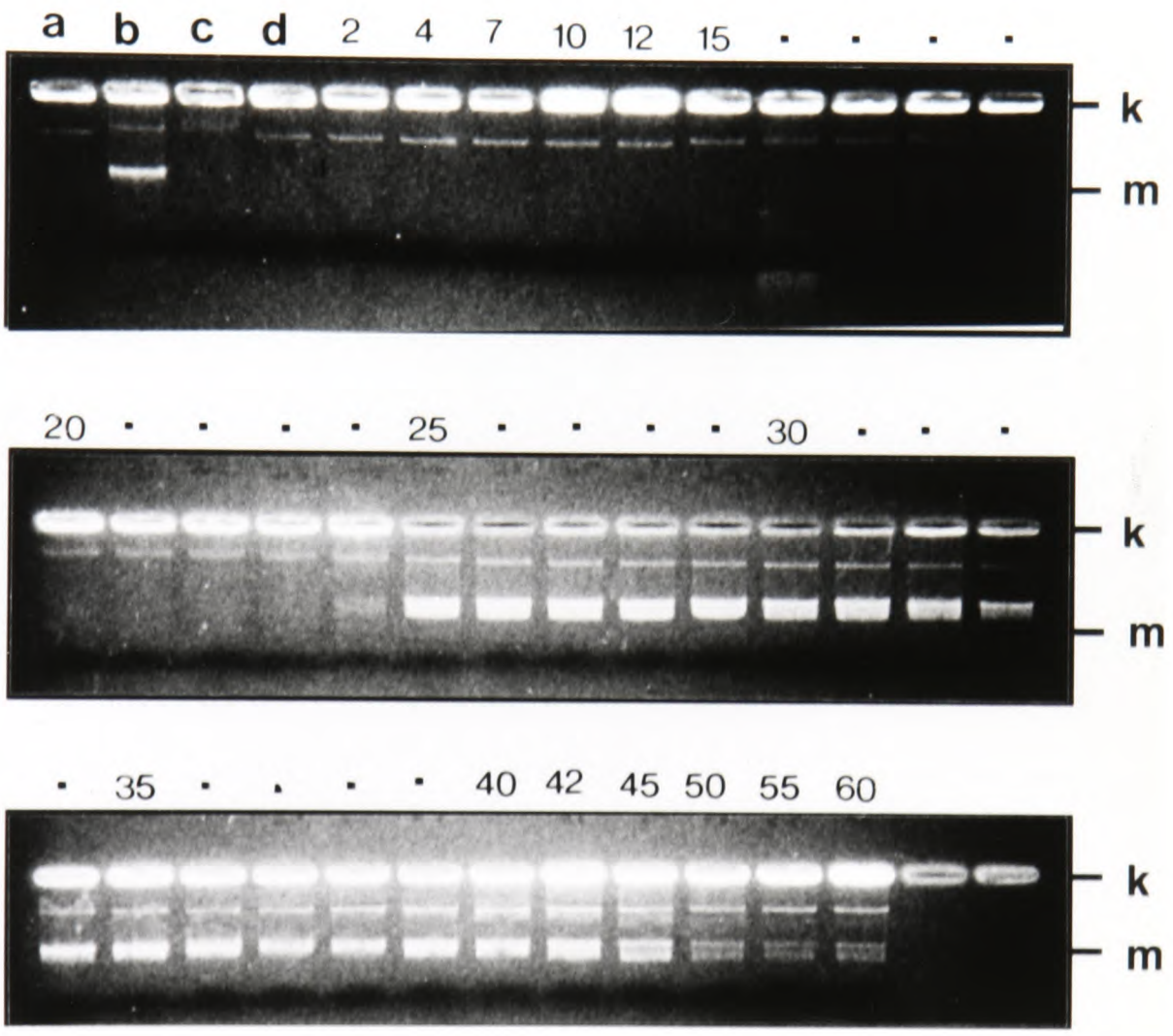
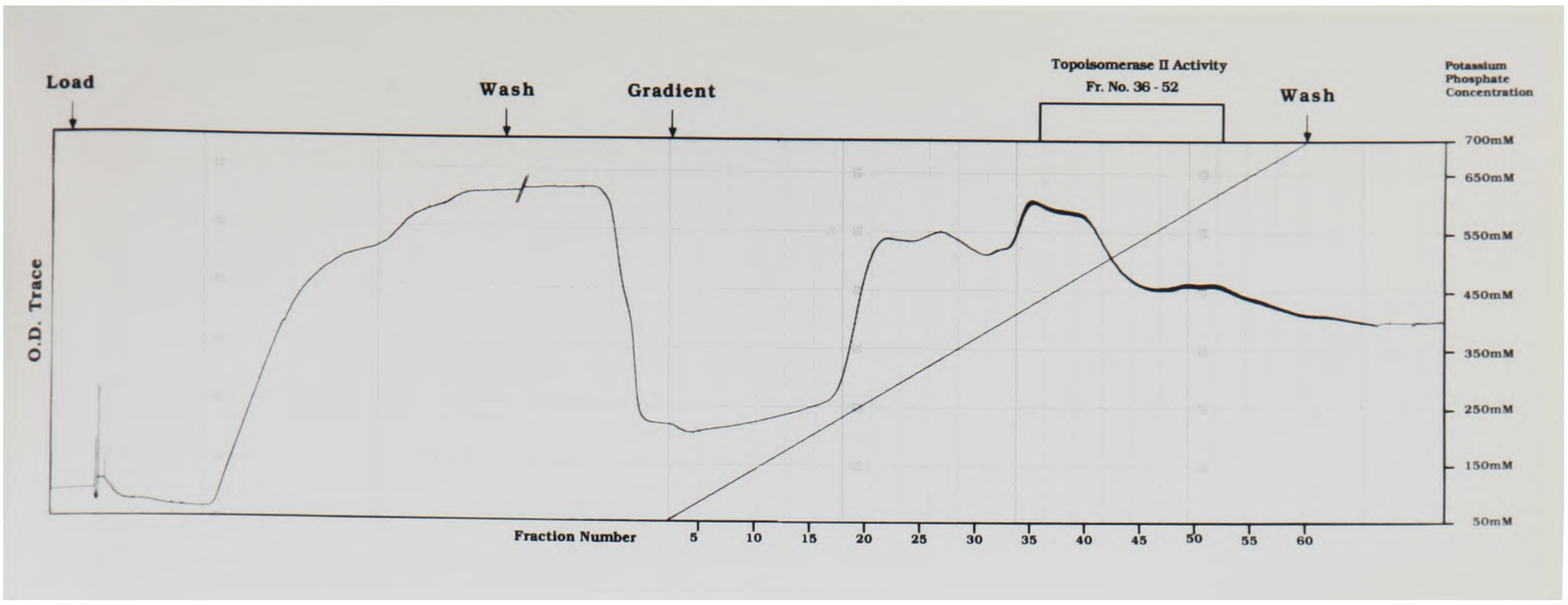


Figure 10 Purification of topoisomerase II activity by hydroxylapatite chromatography. The top panel shows the protein concentration eluting off the column as measured directly by an O.D. monitor connected to the fraction collector and the position of topoisomerase II activity as measured by a decatenation assay shown in the bottom panel. The point at which the crude nuclear extract was loaded onto the column, the column washed with 50mM potassium phosphate, the gradient started, and the column washed with 700mM potassium phosphate are shown sequentially on top of the trace. A decatenation assay (bottom panel) was carried out with no topoisomerase II (a), a control crude nuclear extract (b), the sample loaded on to the column (c), the low-salt wash-through from the column (d) and the fractions collected during the gradient. Corresponding fraction numbers are indicated on both panels. The position of the catenated kDNA (k) and the minicircles released by decatenation (m) are indicated.



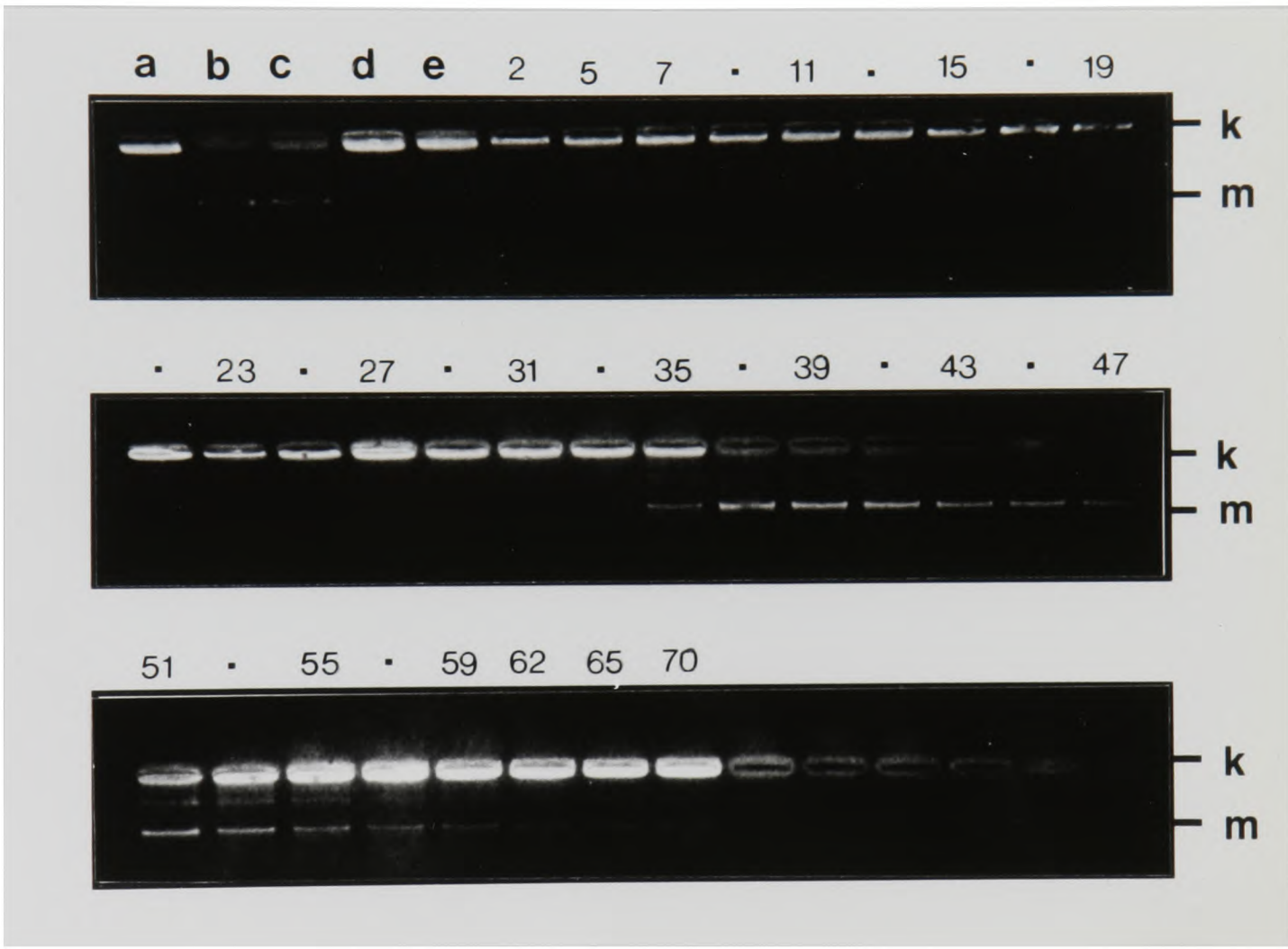


Figure 11 Purification of topoisomerase II activity by phosphocellulose chromatography. See the legend for Figure 10. The wash-through from this column was collected as two separate pools representing the wash-through collected during loading of the hydroxylapatite peak (d) and the wash-through collected during low-salt wash of the column prior to running the gradient (e).

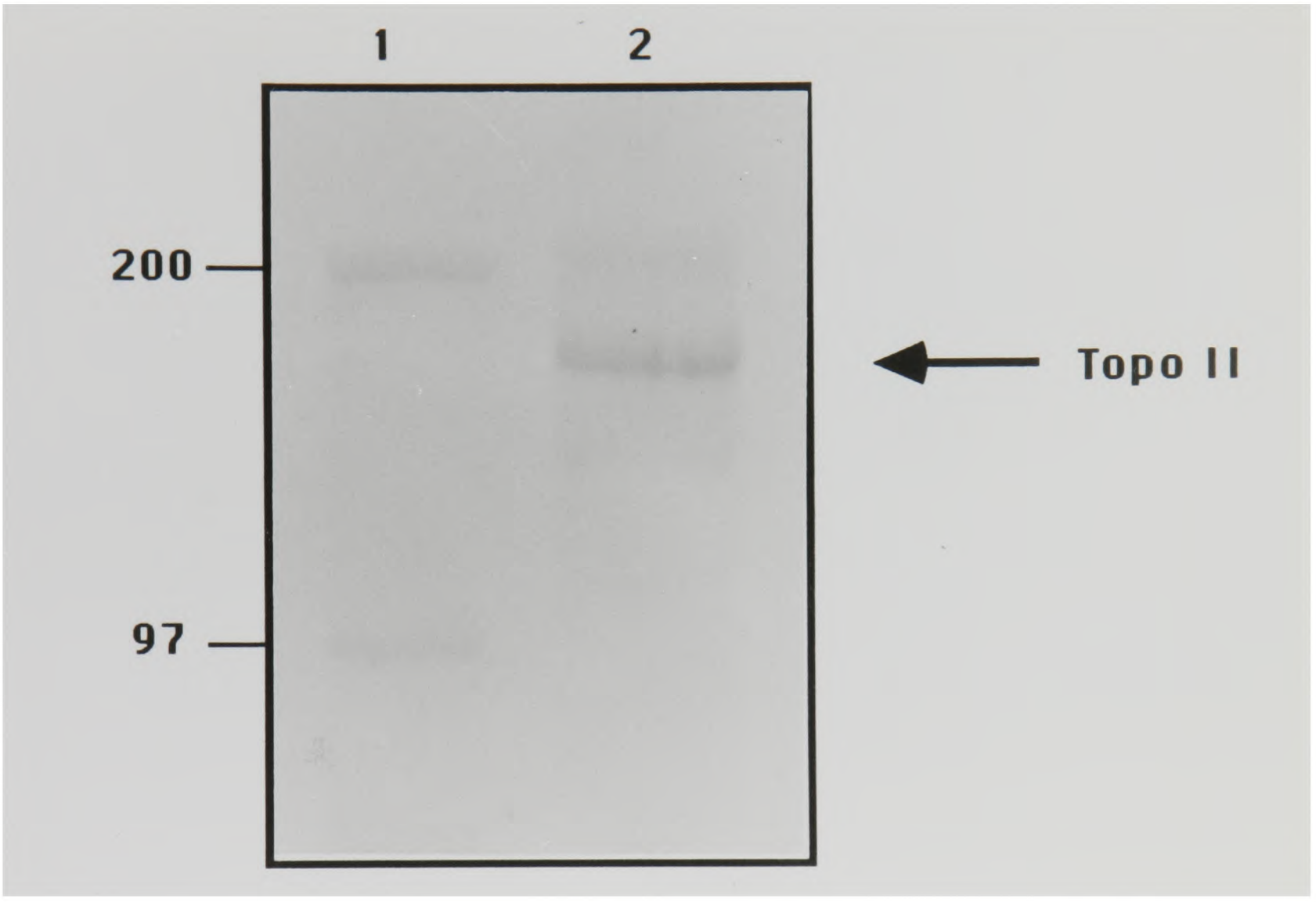


Figure 12 Purified human topoisomerase II protein. Fraction VI from the FPLC MonoS column (lane 2) was electrophoresed alongside protein molecular weight markers (lane 1) on a 7.5% SDS-polyacrylamide gel. The gel was stained with Coomassie blue. The positions of the topoisomerase II protein and of the molecular weight markers are indicated.

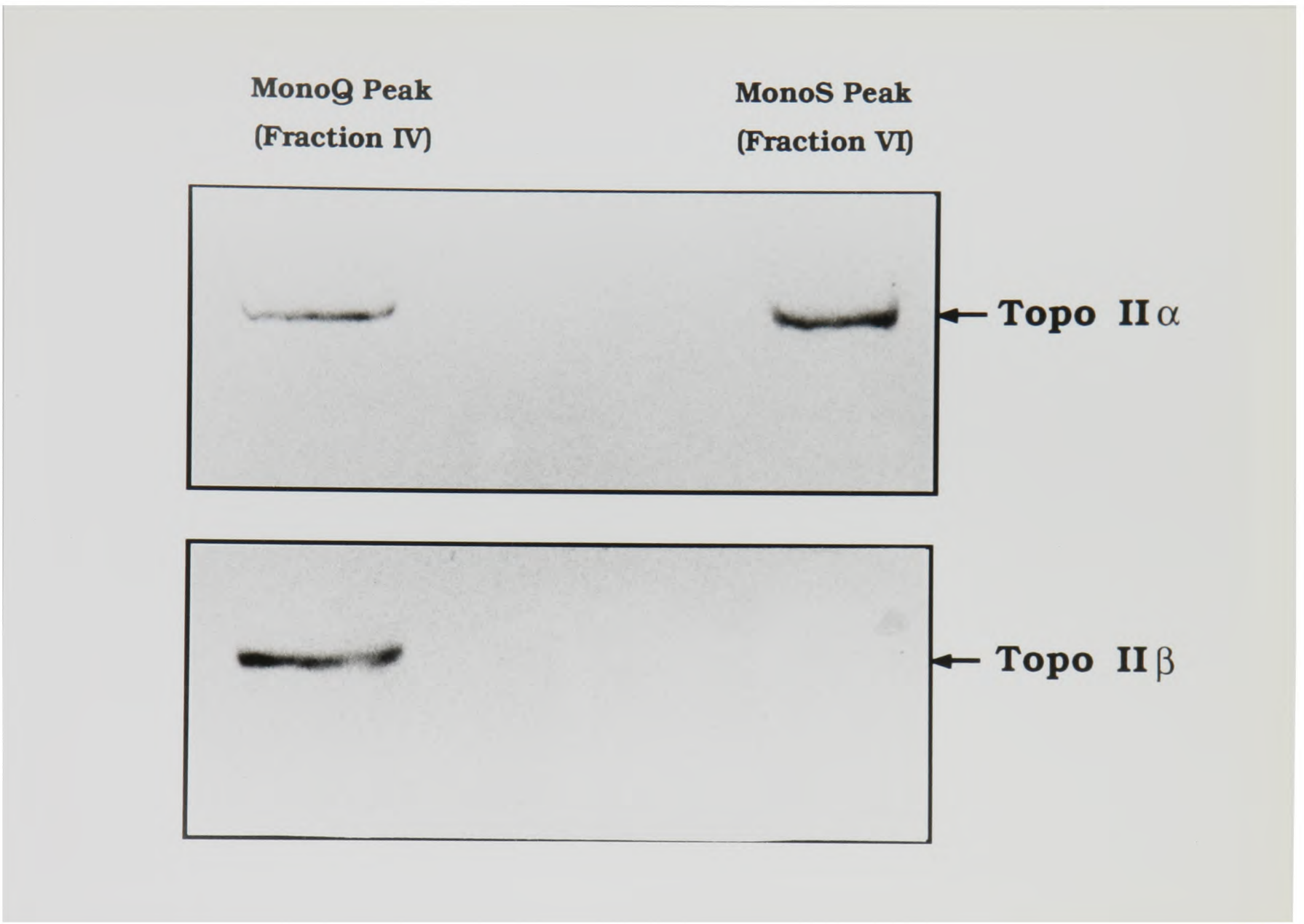


Figure 13 The purified human topoisomerase II activity contains exclusively the α isozyme. The peak decatenation activities from the FPLC MonoQ column (Fraction IV) and the FPLC MonoS column (Fraction VI) were run on 7.5% SDS-polyacrylamide gels and then blotted onto nitrocellulose. The filters were probed with monoclonal antibodies specific for either the α (top panel) or the β (bottom panel) isozyme of human topoisomerase II.

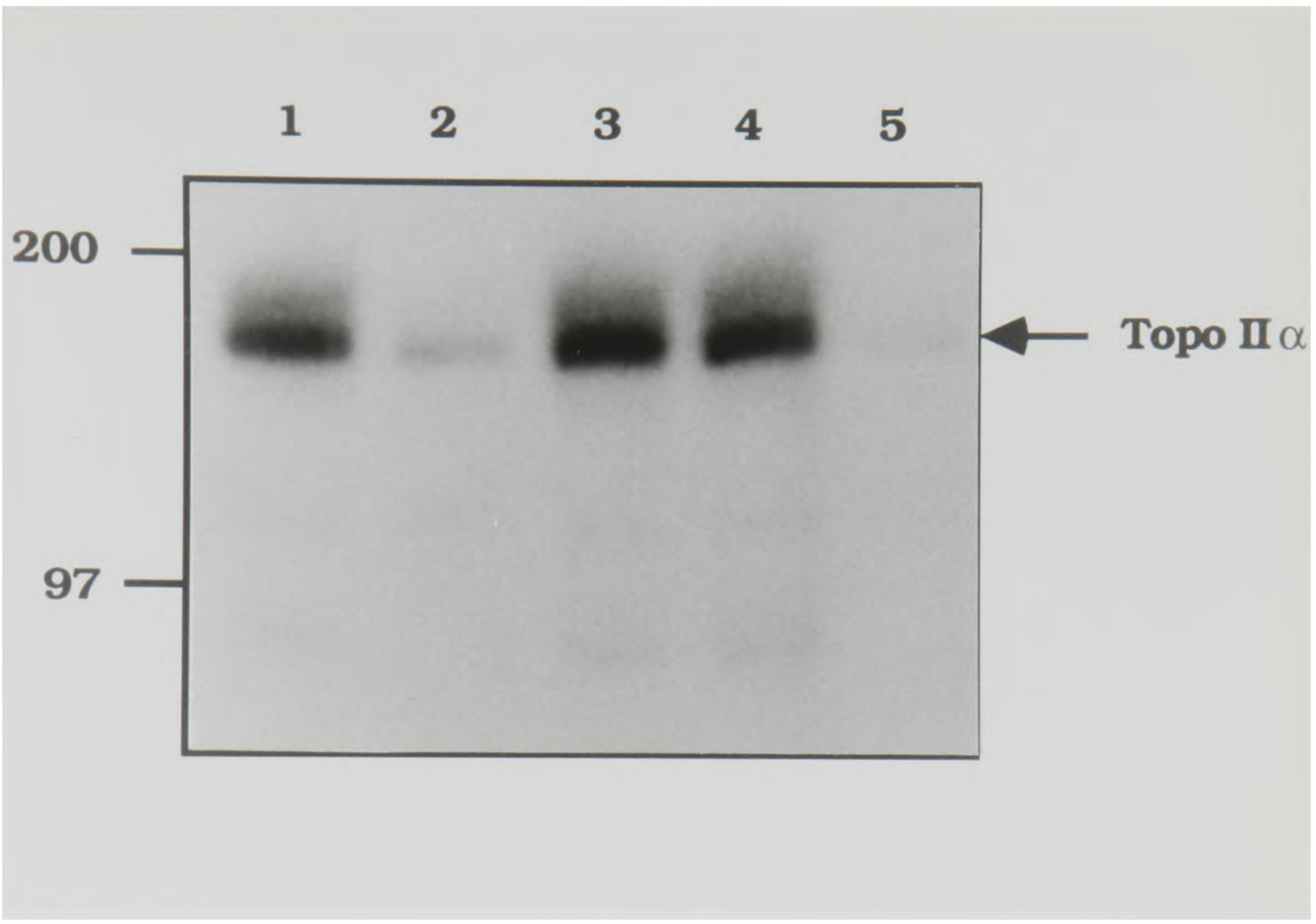


Figure 14 The purified human topoisomerase IIα preparation contains a heparin-sensitive kinase which phosphorylates the topoisomerase IIα protein. The purified human topoisomerase IIα was incubated in the presence of [γ^{32}P]-ATP in the absence or presence of known protein kinase inhibitors, and then run out on a 7.5% SDS-polyacrylamide gel, dried and autoradiographed.The position of molecular weight standards (kDa) are shown on the left, and the position of the topoisomerase IIα protein is indicated on the right. The reaction was carried out with no inhibitor (lane 1), 10μg/ml heparin (lane 2), 10μM PKA inhibitor peptide (lane3), 20μM H7 (lane 4) or all three inhibitors (lane 5).

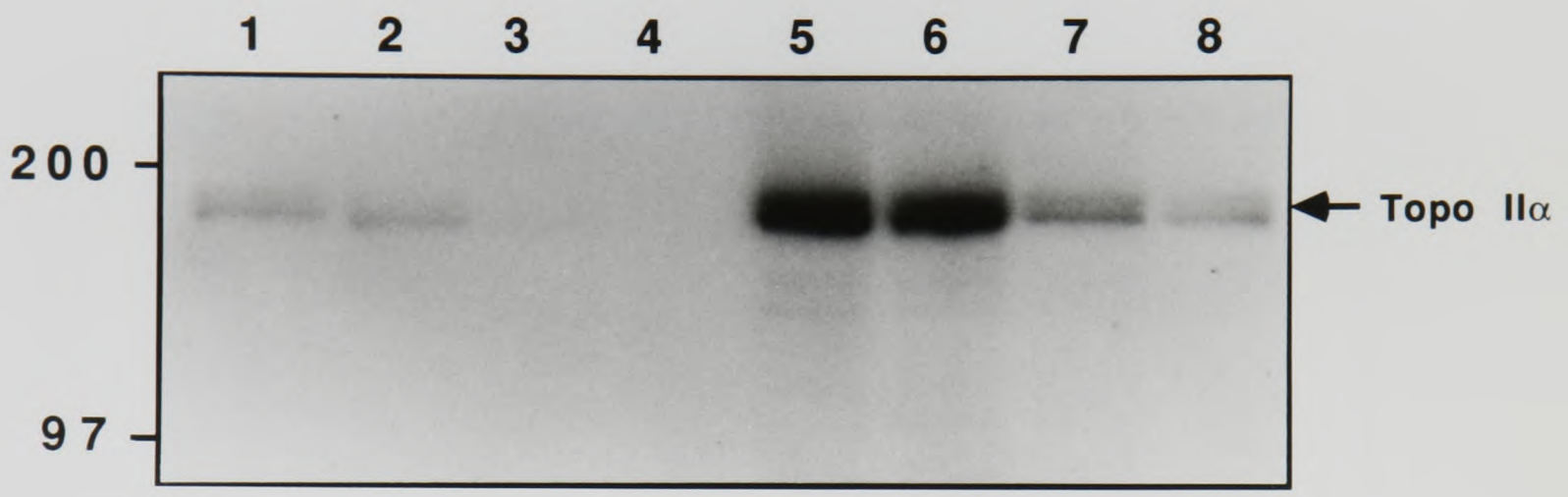


Figure 15 Heparin sensitivity of the co-purifying kinase compared to purified mammalian casein kinase II. Purified human topoisomerase IIα protein was incubated in the presence of [γ^{32}P]-ATP either without (lanes 1-4) or with (lanes 5-8) purified mammalian casein kinase II. The samples were run out on a 7.5% SDS-polyacrylamide gel, dried and autoradiographed. The reactions contained heparin at the concentration of 0μg/ml(lanes 1 and 5), 0.5μg/ml (lanes 2 and 6), 2μg/ml (lanes 3 and 7) or 10μg/ml (lanes 4 and 8). The position of molecular weight standards (kDa) are shown on the left and the topoisomerase IIα protein shown on the right.

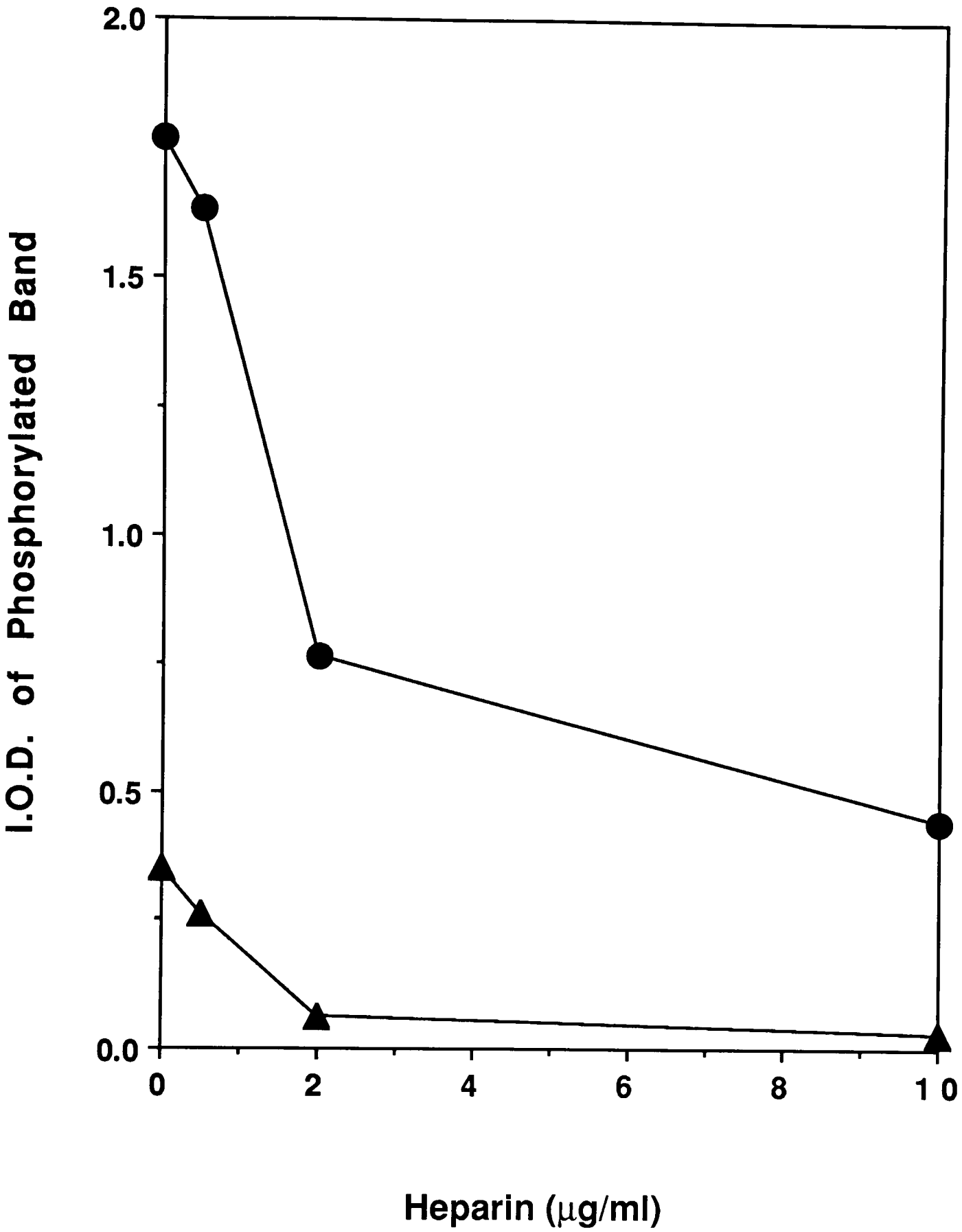


Figure 16 The co-purifying kinase and CK II have comparable heparin sensitivities. The autoradiogram shown in Figure 15 was scanned by densitometry and the band intensity of the topoisomerase IIα protein phosphorylated in the absence (▲) or presence (●) of purified mammalian CK II plotted as I.O.D. units (integrated optical density) against heparin concentration.

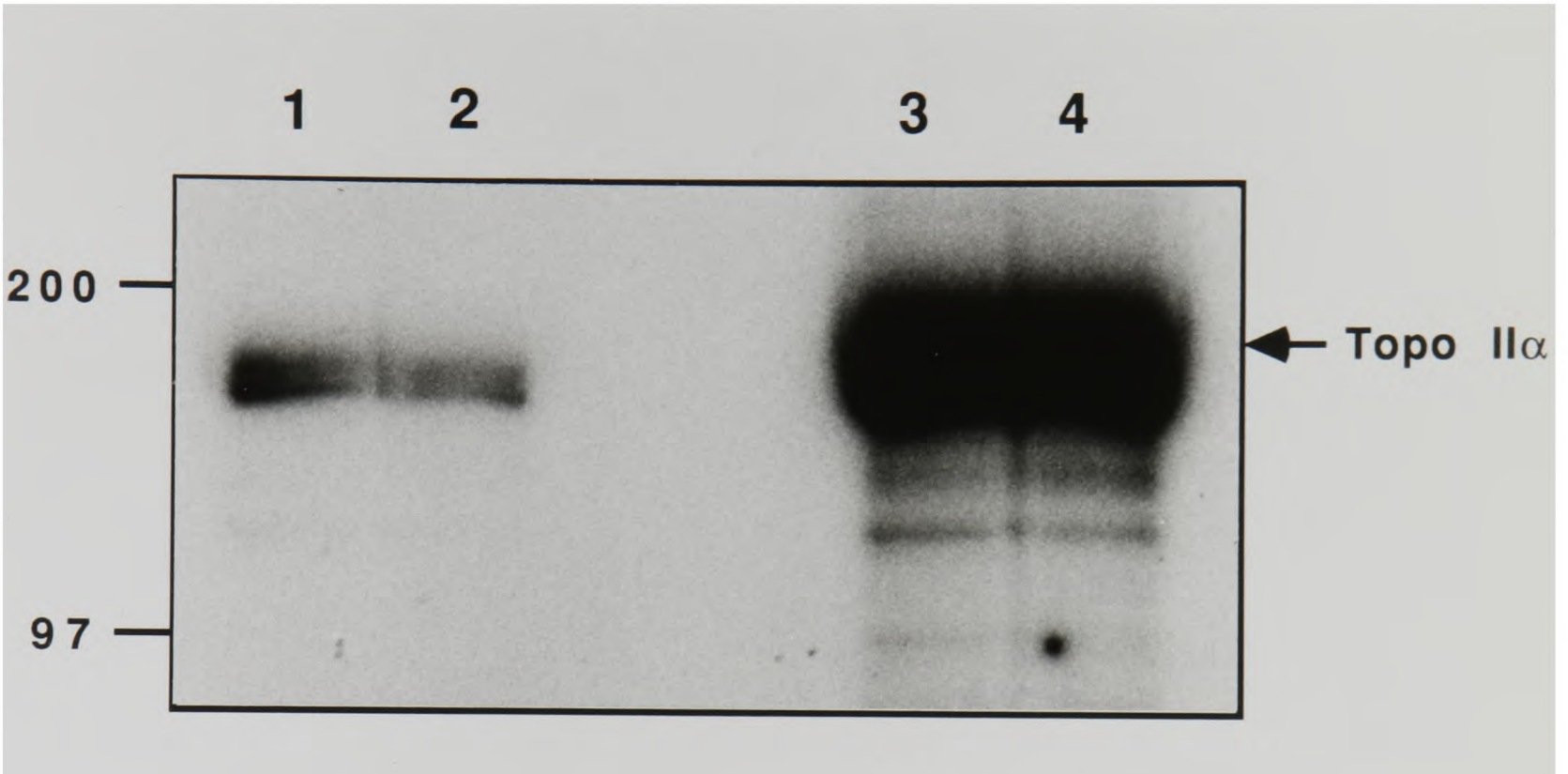


Figure 17 The co-purifying kinase and CK II can phosphorylate topoisomerase IIα using GTP as the phosphate donor. Duplicate samples of purified human topoisomerase IIα protein were incubated with [γ^{32}P]-GTP in the absence (lanes 1,2) or presence (lanes 3,4) of purified mammalian CK II. The samples were then electrophoresed on a 7.5% SDS-polyacrylamide gel, the gel dried and autoradiographed. The position of molecular weight standards (kDa) are shown on the left and the topoisomerase IIα protein shown on the right.

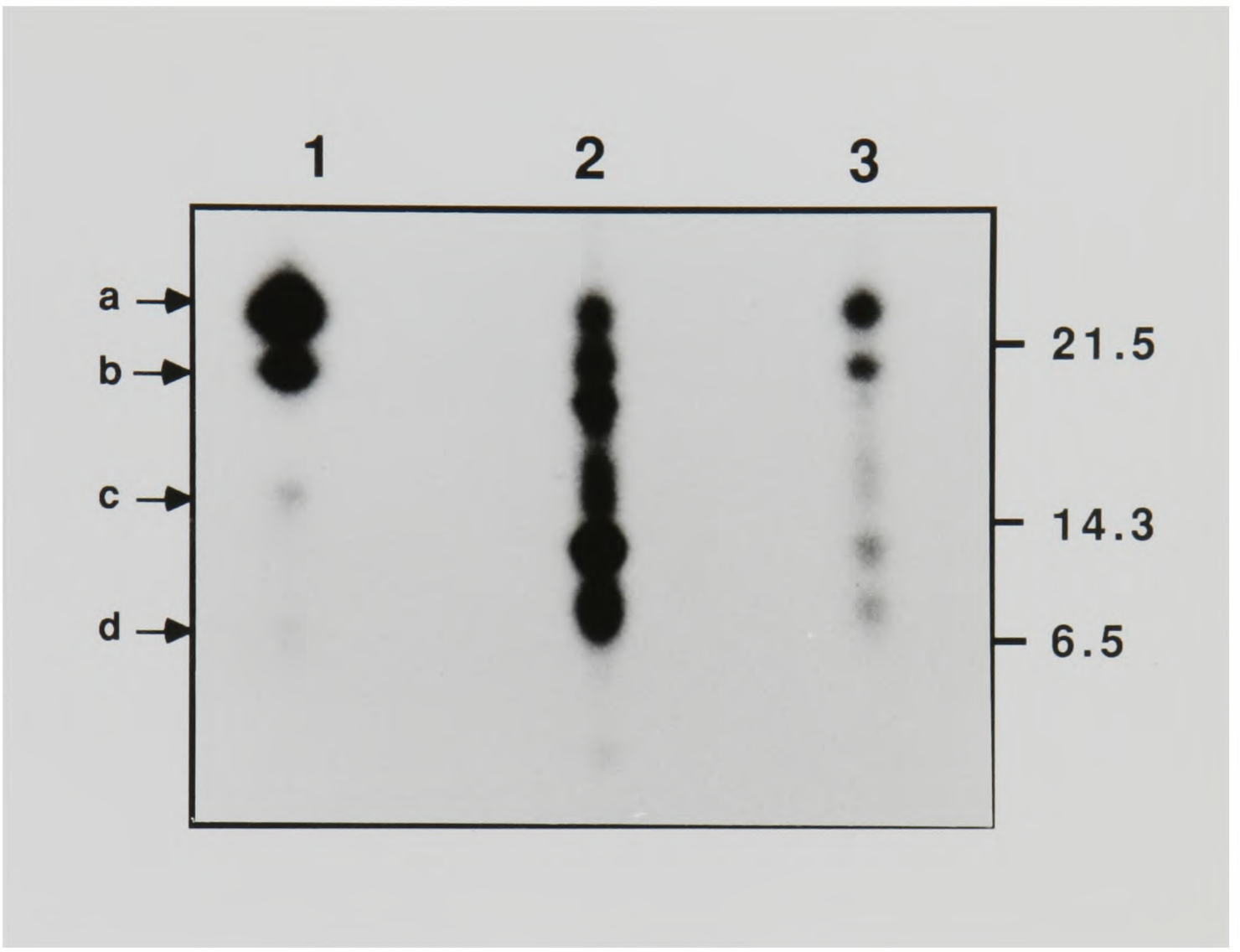


Figure 18 One-dimensional V8 phosphopeptide maps of *in vivo* and *in vitro* phosphorylated topoisomerase IIα protein. Topoisomerase IIα was labelled *in vivo* with ^{32}P-orthophosphate (lane 1), or phosphorylated *in vitro* with [γ^{32}P]-ATP in the presence (lane 2) or absence (lane 3) of purified CK II. Samples were processed for one-dimensional phosphopeptide mapping as described in section 2.12. The position of molecular weight standards are shown on the right (kDa). The 4 phosphopeptides present in the *in vivo* labelled sample (lane 1) have been indicated with arrows on the left (a, b, c, d).

Chapter 5 PHOSPHORYLATION OF HUMAN TOPOISOMERASE IIα PROTEIN BY CASEIN KINASE II.

5.1 Introduction.

Topoisomerase II has been shown to be a phosphoprotein in a variety of eukaryotic organisms, including yeast (Cardenas *et al.*, 1992), *Drosophila* (Ackerman *et al.*, 1988), sponge (Rottman *et al.*, 1987), chicken (Heck *et al.*, 1989), mouse (Saijo *et al.*, 1990), and human (Kroll and Rowe, 1992). Phosphorylation was shown to be either exclusively or predominantly on serine residues, with some reports demonstrating a minor proportion of phosphothreonine. However, none of the studies in vertebrates have distinguished between the two isozymes of topoisomerase II, which exist in these species. Hence it was not known whether one or both isozymes are phosphoproteins *in vivo*.

As was described in section 1.11, casein kinase II has been shown to be a key regulator of topoisomerase II in lower eukaryotes. Casein kinase II (CK II) is a serine/threonine protein kinase found in all eukaryotic cells (reviewed in Tuazon and Traugh, 1991). The holoenzyme is a heterotetramer of the structure $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$, where the α and α' subunits provide the catalytic activity, and the β subunit plays a regulatory role. The recent sequencing of CK II cDNA clones from several different species revealed a high degree of conservation of all the subunits during evolution.

Gene disruption experiments in *S. cervisiae* demonstrated that expression of at least one catalytic subunit of CK II was essential for cell viability (Padmanabha *et al.*, 1990). Cells mutant for both catalytic subunits arrest preferentially at non-permissive temperatures as large budded cells, characteristic of a G_2-M arrest point (Gasser *et al.*, 1992). This arrest point may be due in part to the requirement of CK II to activate topoisomerase II, which in turn is required for the proper traversal of mitosis.

CK II has a number of unique properties amongst protein kinases that characterize its activity; these include its ability to use GTP as a phosphate donor instead of ATP, and its high degree of sensitivity to heparin (Hathaway *et al.*, 1980). Its name comes from its ability to use the acidic protein casein as a strong *in vitro* substrate, and indeed the kinase has been shown to have a very strict requirement for multiple acidic residues around the target phosphorylation site (Kuenzel *et al.*, 1987).

Until recently, little was known about the physiological substrates of CK II, but a number of proteins, particularly involved in DNA metabolism, have now been identified as *in vivo* substrates for CK II. These include RNA polymerases (Stetler and Rose, 1982), some transcription factors (Marais *et al.*, 1992; Voit *et al.*, 1992; Lin *et al.*, 1992), the tumour suppressor gene product p53 (Meek *et al.*, 1990), DNA ligase I (Prigent *et al.*, 1992), and DNA topoisomerases I and II (Durban *et al.*, 1985; Ackerman *et al.*, 1988). Particular interest has been paid to the phosphorylation of many nuclear oncoproteins by CK II; for instance, *c-myc*, *v-myc*, *c-myb*, *c-fos*, *c-jun*, the adenovirus E1a protein and SV40 Large-T antigen are all phosphorylated by CK II (reviewed in Krebs *et al.*, 1988).

Whether there is a common function behind all these phosphorylations is not clear. The most striking results are those which suggest that CK II phosphorylation is absolutely essential for activity of an enzyme or transcription factor, as has been proposed for DNA ligase I (Prigent *et al.*, 1992). These authors propose that CK II phosphorylation of the C-terminal domain leads to a conformational change in the protein making the active site of the enzyme accessible. Mutating the potential CK II sites in the serum response factor (SRF) considerably reduced the binding of SRF to the *c-fos* serum response element (Janknecht *et al.*, 1992), suggesting that in some substrates CK II phosphorylation has an effect on DNA binding efficiency.

Alternatively, CK II phosphorylation might regulate nuclear-cytoplasmic protein transport as shown for the SV40 Large T-antigen (Rihs *et al.*, 1991). Here, multiple CK II phosphorylation sites exist in close proximity to the nuclear localization sequence (NLS) of the T-antigen, and phosphorylation of at least one of them is essential for transport to the nucleus. Putative CK II sites exist close to the predicted NLS in other proteins, including adenovirus E1a protein, p53 and the human and mouse *c-rel* oncoproteins. In the case of yeast topoisomerase II, phosphorylation by CK II does not seem to be a prerequisite for nuclear targeting, since in the CK II ts mutants at non-permissive temperatures, topoisomerase II is properly localised to the nucleus (Gasser *et al.*, 1992).

The fact that most of the substrates for CK II appear to be nuclear proteins points to a requirement for CK II activity in the nucleus. The question is whether CK II is found throughout the cell, or does some degree of control exist through compartmentalization of this kinase.

Initially, conflicting results were reported on the subcellular localization of CK II, with some groups reporting CK II activity to be predominantly cytoplasmic (Edelman *et al.*, 1987), whilst others emphasised its presence in the nucleus (Filhol *et al.*, 1990). A recent report used indirect immunofluoresence and cell fractionation studies to clearly demonstrate that, in chicken and human cells, CK II is located predominantly in the cell nucleus (Krek *et al.*, 1992).

The phosphorylation by CK II of a number of transcription factors involved in the early gene response, points to this kinase playing an important role in signal transduction. This in turn begs the question of what regulates CK II activity. This has proved to be something of a conundrum. It has been reported that CK II activity is significantly elevated during early embryogenesis (Schneider *et al.*, 1986), upon differentiation of 3T3-L1 cells (Sommercorn and Krebs, 1987), following serum stimulation of quiescent WI38 human lung fibroblasts (Carrol and Marshak, 1989), and in solid human tumours and rapidly proliferating non-neoplastic tissue (Prowald *et al.*, 1984; Munstermann *et al.*, 1990). Furthermore, CK II activity has been shown to be regulated in response to insulin, insulin-like growth factor 1 and epidermal growth factor (Sommercorn *et al.*, 1987; Klarlund and Czech, 1988; Ackerman and Osheroff, 1989). These results suggest a general response to proliferation and postulate that CK II plays an important role in cell growth.

However, as yet, no second messenger has been defined which directly modifies CK II. Evidence suggests that regulation is, at least in part, due to covalent modification, and not just due to protein synthesis or translocation of the protein within the cell (Krebs *et al.*, 1988). One report showed that the CK II regulatory β subunit was phosphorylated *in vitro* by the cell cycle-regulated $p34^{cdc2}$ kinase, causing a 2- to 5-fold increase in enzyme activity, and it is possible that this may be involved in a cascade leading to multiple target phosphorylations at mitosis (Mulner-Lorillon *et al.*, 1990). However, there is no evidence as yet, that CK II activity *in vivo* is cell cycle-regulated. Interestingly, Krek *et al.* (1992) showed that the CK II subunits became redistributed throughout the cell during mitosis, and that there was particular association of a fraction of CK II with elements of the mitotic spindle. This relocalization could, in theory, lead to cell cycle-specific phosphorylation of substrates by CK II, without having a cell cycle-related change in the specific activity of the enzyme.

The identification of topoisomerase II from *Drosophila* as a substrate for CK II, and the demonstration that this phosphorylation leads to a modest change in topoisomerase II activity was discussed in section 1.11. Whilst other kinases, particularly protein kinase C, have also been shown to phosphorylate topoisomerase II, evidence from lower eukaryotes points to CK II as playing a major role in phosphorylation and activation of topoisomerase II *in vivo*.

Having specifically purified the α isozyme of human topoisomerase II, the aim was to identify whether this too was a substrate for CK II *in vivo*, and to look at the effect of any phosphorylation on the activity of the human topoisomerase IIα isozyme *in vitro*. Furthermore, we hoped to gain some information on the potential target sites for phosphorylation within the protein, so as to begin to build a model of how CK II might modulate the enzyme activity. The demonstration that CK II could co-purify through several chromatographic steps with human topoisomerase IIα already suggested a functional interaction *in vivo*.

5.2 Results.

5.2.1 Human Topoisomerase IIα is a Phosphoprotein *in vivo*.

Nuclear proteins were prepared from HeLa cells grown in the presence of ^{32}P-orthophosphate or ^{35}S-methionine. Labelled topoisomerase IIα protein was immunoprecipitated using the CRB specific antiserum, and the precipitate processed by SDS-PAGE and autoradiography. A ^{32}P-labelled protein, immunoprecipitated by this antiserum, had a mobility identical to that of a protein immunoprecipitated from control cells labelled with ^{35}S-methionine (Figure 19). The apparent molecular weight of this protein was 170kDa. This indicates that topoisomerase IIα is a phosphoprotein in HeLa cells.

5.2.2 Phosphoamino Acid Analysis of Human Topoisomerase IIα Protein *In Vivo*.

To identify the phosphorylated amino acid residues in the topoisomerase IIα protein, *in vivo* labelled protein was purified from an SDS-polyacrylamide gel, acid-hydrolysed and the amino acids separated by two-dimensional electrophoresis. The position of the unlabelled phosphorylated residues, pSer, pThr and pTyr, were determined by

ninhydrin staining of the plate. The majority of the ^{32}P-label migrated with authentic phosphoserine, but a small proportion (approximately 5%) of phosphothreonine was also evident (Figure 20). No phosphotyrosine was detected, even after long autoradiographic exposures.

5.2.3 Preparation of Human Topoisomerase IIα Protein lacking a Co-purifying Kinase Activity.

As described in chapter 4, a kinase with properties characteristic of CK II co-purified with human topoisomerase IIα protein on several occasions. This kinase was able to phosphorylate the purified topoisomerase IIα protein, suggesting that human topoisomerase IIα is a substrate for CK II. Another preparation of human topoisomerase IIα protein was purified apparently free of kinase activity. This was confirmed by the absence of any labelled band at around 170kDa on an autoradiograph (even after long exposures), following incubation of the purified preparation with [$\gamma^{32}P$]-ATP and subsequent electrophoresis (data not shown). This preparation was used to confirm that human topoisomerase IIα protein was phosphorylated *in vitro* by CK II. Indeed this preparation was used in all subsequent studies on phosphorylation and activity measurements presented in this chapter.

5.2.4 Human Topoisomerase IIα Protein is a Substrate *in vitro* for Casein Kinase II.

CK II, purified from rabbit skeletal muscle (as described by Woodgett, 1989), was kindly provided by Dr. J. R. Woodgett (Ludwig Institute, London) and assayed for activity against casein (Figure 21); the activity was shown to be heparin-sensitive. To test whether the kinase-free preparation of human topoisomerase IIα protein could act as a substrate for mammalian CK II, it was incubated with [$\gamma^{32}P$]-ATP either in the absence or presence of purified CK II. The reaction products were separated on a 7.5% SDS-polyacrylamide gel, which was dried and exposed to X-ray film. Figure 21 shows an autoradiograph of the dried gel indicating that human topoisomerase IIα is indeed phosphorylated by mammalian CK II *in vitro*. This phosphorylation was also confirmed to be heparin-sensitive (Figure 21).

Further evidence that topoisomerase IIα is a substrate for CK II *in vitro* was demonstrated by the ability of purified CK II to phosphorylate

human topoisomerase IIα protein that had been immunoprecipitated using the CRB antibody (data not shown).

5.2.5 Phosphoamino Acid Analysis of Topoisomerase IIα Protein Phosphorylated *in vitro* by Casein Kinase II.

Purified human topoisomerase IIα protein was phosphorylated by purified mammalian CK II *in vitro*, and the reaction products subjected to SDS-PAGE on a 7.5% gel. The position of the labelled topoisomerase IIα protein on the dried gel was identified by autoradiography, cut out and subjected to acid hydrolysis, and the amino acids separated by two-dimensional electrophoresis. CK II phosphorylation of topoisomerase IIα protein *in vitro* was exclusively on serine residues (Figure 22), with no phosphothreonine or phosphotyrosine even after long exposures. This differed from the phosphoamino acid analysis of *in vivo* phosphorylated human topoisomerase IIα, where a small proportion of phosphothreonine was detected.

5.2.6 Two-dimensional Phosphopeptide Maps from Human Topoisomerase IIα Protein Phosphorylated *in vivo* and *in vitro* by Casein Kinase II.

Evidence that a kinase phosphorylates a specific protein substrate *in vivo* can be obtained by demonstrating that the kinase phosphorylates sites within the protein *in vitro* that are also phosphorylated *in vivo*. Thus far no phosphorylation sites in any eukaryotic topoisomerase II protein have been mapped precisely, and the most direct method to compare *in vivo* with *in vitro* phosphorylation is two-dimensional phosphopeptide mapping. In this technique the phosphorylated protein is digested to completion with a protease, such as trypsin, and the peptides separated in two dimensions by electrophoresis and chromatography. The positions of the phosphopeptides can then be detected by autoradiography. Complete digestion with proteases which cleave frequently in the protein such as those mentioned, means that the peptides generated will usually be fairly short. Hence their migration in both dimensions will be heavily dependent on their amino-acid composition. Thus, the pattern of phosphopeptides resulting from phosphorylation by one kinase is generally very different from the pattern resulting from phosphorylation

by another kinase, and the kinases that are relevant *in vivo* can be identified.

Here, topoisomerase IIα protein was phosphorylated either *in vitro* using purified CK II and [γ^{32}P]-ATP, or *in vivo* by metabolically labeling cells with ^{32}P-orthophosphate. The phosphorylated proteins were then run out on a 7.5% SDS-polyacrylamide gel, the gel dried and the labelled band detected by autoradiography. The band was cut out, and digested with trypsin as described in section 2.13. Two-dimensional maps were generated and are shown in Figure 23.

The recovery of topoisomerase II protein was poor, usually being of the order of only 10-20%. This meant that the phosphorylated topoisomerase IIα protein needed to have a very high level of ^{32}P incorporation in order to detect the radiolabelled spots on phosphopeptide mapping. For this reason, there was also not enough material to do a 'mix' of *in vivo* and *in vitro* samples, which would have been useful in confirming co-migration of phosphopeptides.

Digestion of *in vivo* labelled topoisomerase IIα protein produced a complex pattern of phosphopeptides, comprising at least twelve species that could be resolved. Of these, six had a mobility closely matching phosphopeptides derived from the purified topoisomerase IIα protein phosphorylated *in vitro* by CK II (Figure 23). This suggested that CK II was responsible for part, but not all, of the phosphorylation of human topoisomerase IIα protein *in vivo*.

The efficiency of phosphorylation of the peptides which co-migrated in *in vivo* and *in vitro* experiments was not the same (Figure 23): spots 1 and 3 being the major phosphopeptides *in vivo*, and spot 2 being the major phosphopeptide *in vitro*. Furthermore, phosphorylation of topoisomerase IIα by CK II *in vitro* generated other spots which were not seen at all *in vivo* (Figure 23).

5.2.7 Phosphorylation of the C-terminal Domain of Human Topoisomerase II *in vivo* and *in vitro* by Casein Kinase II.

A number of good potential target sites for CK II lie within the C-terminal domain of the human topoisomerase IIα protein, including the best fit target site which is just 7 residues from the C-terminus itself. A combination of one dimensional phosphopeptide mapping and western blotting using an antibody raised to the C-terminus of human topoisomerase IIα was used to investigate whether the C-terminal domain

is phosphorylated in human topoisomerase IIα protein *in vivo* and by CK II *in vitro*.

Human topoisomerase IIα protein, labelled *in vivo* with ^{32}P-orthophosphate, was run out on a 7.5% SDS-polyacrylamide gel, the gel was autoradiographed without drying, and the labelled gel slice cut out. This gel slice was then inserted into the well of a 17.5% SDS-polyacrylamide gel and subjected to partial V8 proteolysis during running of the gel, as described by Cleveland *et al.* (1977). Following electrophoresis, the gel was dried, fixed and autoradiographed (Figure 24).

The pattern of phosphopeptides produced by partial V8 proteolysis was highly reproducible, suggesting that the protease was cutting distinct protease-sensitive sites. *In vivo* labelled human topoisomerase IIα yielded two major phosphopeptides of molecular weight 23kDa and 20kDa (designated 'a' and 'b' respectively in Figure 24), and two minor ones of molecular weight 15kDa and 7kDa (designated 'c' and 'd' respectively in Figure 24).

A phosphopeptide map was also generated under the same conditions using purified human topoisomerase IIα phosphorylated *in vitro* by purified mammalian CK II. Comparison of the maps showed that the two major phosphopeptides seen *in vivo*, and perhaps also the two minor spots, comigrated with spots seen after phosphorylation with CK II *in vitro*. However at least three other phosphopeptides were generated after *in vitro* phosphorylation. These results support those of the two-dimensional tryptic maps, whereby CK II seems able to phosphorylate the same peptides *in vitro*, that are phosphorylated *in vivo*, but that other sites become phosphorylated as well *in vitro* which are not phosphorylated *in vivo*.

Topoisomerase IIα protein was also immunoprecipitated from unlabelled HeLa cells, and processed in the same way. After electrophoresis on the 17.5% gel, the gel was blotted onto nitrocellulose, and incubated with the CRB polyclonal antiserum, which had been raised to a peptide closely related to the C-terminus of human topoisomerase IIα protein. ECL detection revealed that the 20kDa phosphopeptide (labelled 'b'), seen *in vivo* and *in vitro* comigrated with a peptide detected using this antibody (Figure 24).

This indicates that the C-terminal 20kDa of human topoisomerase IIα is phosphorylated *in vivo*, and is a site for CK II phosphorylation. Yeast topoisomerase II protein has also been shown to be phosphorylated at

sites close to the C-terminus by CK II (Cardenas *et al.*, 1992). However, the data presented here suggests that another major site of phosphorylation *in vivo* is not located at the extreme C-terminus of the protein.

5.2.8 Potential Casein Kinase II Phosphorylation Sites in the C-terminal Region of Human Topoisomerase IIα Protein.

CK II phosphorylates serine or threonine residues in an acidic local environment. More precise substrate specificity determinants for CK II sites have been deduced from studies with synthetic peptides (Kuenzel *et al.*, 1987; Kennelly and Krebs, 1991). The best substrates are those in which multiple acidic residues are present on both sides of the target serine or threonine. Acidic residues on the N-terminal side enhance the kinetic constants but are not absolutely necessary; acidic residues on the C-terminal side are absolutely required, and can be aspartate, glutamate or phosphorylated serine. An acidic residue at the (+3) site, i.e. the third residue on the C-terminal side, appears to be particularly good in conferring a CK II site.

CK II phosphorylates human topoisomerase IIα protein on serine residues, and has at least one strong target site in the C-terminal 20kDa. Table 5 shows the sequence and location of six serine-containing peptides from within this region which lie in potential CK II consensus sites. As discussed in section 1.4, the C-terminal domain of human topoisomerase IIα protein is highly charged and contains many serine and threonine potential target sites for several kinases. Indeed, the C-terminus of *S. cerevisiae* topoisomerase II also appears to be phosphorylated *in vivo* by CK II (Cardenas *et al.*, 1992).

5.2.9 Activation of Topoisomerase IIα Protein by Casein Kinase II Phosphorylation *in vitro*.

Using the purified human topoisomerase IIα protein, the specific activity of topoisomerase IIα phosphorylated *in vitro* by CK II was compared to that of untreated enzyme in a decatenation assay (Figure 25). Samples of purified human topoisomerase IIα protein were either untreated or phosphorylated with purified CK II before starting the decatenation reaction. Aliquots were then removed from this reaction at time intervals and stopped with DNA loading buffer. Phosphorylation by

CK II led to a 10-fold enhancement in the initial rate of decatenation catalysed by the topoisomerase IIα protein (Figure 26). The initial rate of reaction of topoisomerase IIα was similarly measured in a plasmid relaxation assay (Figure 27). The initial rate of relaxation was enhanced by 2- to 3-fold following CK II phosphorylation (Figure 28).

5.2.10 Dephosphorylation of Purified Human Topoisomerase IIα Protein *in vitro* Markedly Reduces its Enzymatic Activity.

The demonstration that human topoisomerase IIα is a phosphoprotein *in vivo*, means that it is highly likely that the purified protein will already be at least partially phosphorylated. As phosphorylation by CK II increases the specific activity of the purified protein *in vitro*, the effect of dephosphorylating the purified enzyme using potato acid phosphatase (PAP) was measured.

In order to confirm that PAP is able to dephosphorylate the residues that are phosphorylated by CK II, its activity against CK II-phosphorylated casein was measured (Figure 29). Incubation for 15 minutes at 30 units/ml of PAP, in the presence of 10μg/ml heparin to inhibit the CK II, led to an approximate 90% reduction in phosphorylation (Figure 30).

Using conditions shown to reduce phosphorylation by 90%, the purified topoisomerase IIα protein was then dephosphorylated *in vitro* using PAP and the decatenation activity compared to that of the untreated enzyme. Phosphatase treatment appeared to markedly reduce its specific activity. To confirm that this reduction in activity was the direct result of dephosphorylation, reactions were repeated following treatment of the topoisomerase IIα protein either with heat-inactivated PAP (incubated at 90°C for 10 minutes), or with native PAP together with 10mM sodium phosphate, which was shown to completely inhibit PAP (data not shown). Under these conditions, the activity of the topoisomerase IIα protein was very similar to that of the untreated enzyme (Figure 31). PAP-treated topoisomerase IIα enzyme showed an 8-fold reduced initial rate of reaction compared to enzyme treated with heat-inactivated PAP (Figure 32).

Topoisomerase IIα protein that had either been incubated with PAP or PAP buffer alone, as described above, was also electrophoresed on a 6% SDS-polyacrylamide gel and immunoblotted with the topoisomerase IIα-specific monoclonal antibody, 6F3. The positions of the untreated and

dephosphorylated protein were detected using the enhanced chemiluminescence protocol (Figure 33). Treatment with PAP caused a significant increase in the mobility of the topoisomerase IIα protein on SDS-PAGE compared to protein incubated with PAP buffer alone. This is further evidence that the purified protein is already partially phosphorylated.

5.2.11 Reactivation of Dephosphorylated Human Topoisomerase IIα Protein by Casein Kinase II.

A general phosphatase such as PAP should show little or no specificity for phosphate groups added by any particular kinase. In order to determine whether the CK II phosphorylation is critical for activity, the effect of rephosphorylating the dephosphorylated topoisomerase IIα protein with CK II was assessed. Purified topoisomerase IIα protein was incubated with PAP buffer alone or with 30 units/ml PAP at 30°C for 10 minutes. 10mM sodium phosphate was then added to each sample to inhibit the PAP and either CK II buffer alone or 1 unit of CK II added to samples which had been PAP-treated. CK II buffer was added to the topoisomerase IIα protein which had been incubated with PAP buffer alone. Following a further 10 minute incubation of all samples at 30°C, a decatenation assay was carried out as before (described in 5.2.9) and samples electrophoresed on a 1% agarose gel (Figure 34).

The initial rate of reaction of dephophorylated topoisomerase IIα protein which had not been rephosphorylated was reduced by 4- to 5-fold compared to that of untreated topoisomerase IIα (Figure 35). This difference is slightly less than that shown earlier (Figure 32) but then the treatment with PAP was for 15 minutes in that case compared to just 10 minutes in this experiment. Prolonged incubation of purified topoisomerase IIα protein at 30°C led to a reduction in decatenation activity, presumably through degradation; hence, longer incubations with PAP and then CK II were not feasible. The initial rate of reaction of the dephosphorylated topoisomerase IIα protein was restored to a level 3- to 4-fold above that of untreated enzyme by rephosphorylation with CK II (Figure 35). Again this value is lower than that obtained previously (Figure 26), but this could be due to residual PAP activty still being present or the reduced topoisomerase IIα activity following the longer incubation time at 30°C prior to the decatenation assay.

5.3 Discussion.

Post-translational modification of proteins by phosphorylation is one of the most important means by which enzymatic activity is regulated in human cells (Krebs & Beavo, 1979; Hunter, 1987). This modification can lead to inhibition or stimulation of enzymatic activity, or to an altered affinity for a target protein or DNA. These changes are generally mediated either through allosteric conformational changes or through simple electrostatic charge effects.

Topoisomerase IIα has been shown here to be a phosphoprotein in human HeLa cells in culture. This result is consistent with the findings of Kroll and Rowe (1991) who demonstrated that HeLa cell topoisomerase II protein is phosphorylated *in vivo* without distinguishing between the topoisomerase IIα and β isozymes. The results of *in vivo* phosphoamino acid analysis on topoisomerase IIα are similar but not identical to those of Kroll and Rowe (1991) on topoisomerase II, in that the acceptor residue was predominately phosphoserine, but with a small proportion of phosphothreonine, while Kroll and Rowe found exclusively phosphoserine. All studies on eukaryotic topoisomerase II where phosphoamino acid analysis has been done show serine as the major phosphorylated residue. However, some phosphothreonine has been shown *in vivo* in yeast (Cardenas et al., 1992), quail (Schroder et al., 1989), and *Drosophila* (Sander *et al.*, 1984), although sometimes very long exposures are needed to see it.

Phosphoamino acid analysis on *in vitro* phosphorylated human topoisomerase IIα protein showed that CK II phosphorylates topoisomerase IIα exclusively on serine residues. Hence it appears that the small proportion of phosphothreonine seen *in vivo* must be attributable to a kinase other than CK II. It is possible that the threonine phosphorylation only occurs under conditions which specifically stimulate this other kinase (or kinases), and are not essential to the basal activity of the topoisomerase II enzyme. This may explain why some investigators see exclusively phosphoserine even on long exposures.

Two dimensional phosphopeptide mapping indicated that topoisomerase IIα protein is phosphorylated at multiple sites *in vivo*. At least 12 separate phosphopeptides could be distinguished. Although CK II-mediated phosphorylation accounted for a proportion of these phosphopeptides, it seems likely that at least one other kinase phosphorylates topoisomerase IIα protein *in vivo*. This confirms the

suggestion from the phosphoamino acid analysis where CK II phosphorylated topoisomerase IIα protein exclusively on serine residues *in vitro*, but *in vivo* some phosphothreonine was also seen.

For some comigrating phosphopeptides there was an obvious discrepancy in the efficiency of phosphorylation between *in vivo* and CK II phosphorylated topoisomerase IIα. These observations are likely to be explained by the difference between the topoisomerase IIα protein in its natural cellular environment, and in its highly purified state. It may reflect the fact that *in vivo* there are likely to be more constraints over which sites get phosphorylated, perhaps as a result of interactions with other proteins or DNA, or a slightly altered conformation *in vivo* as a result of multiple post-translational modifications.

Mobility of phosphopeptides in the chromatographic dimension relies on the hydrophobicity of the phosphopeptide, with the more hydrophobic peptides migrating further. Mobility in the electrophoretic dimension depends upon the charge of the phosphopeptide, which in turn is determined by both the presence of charged residues in the peptide, and the number of phosphates (i.e. phosphorylated amino acids) in the peptide. So phosphopeptides lying on a diagonal line from negative electrophoretic and low chromatographic mobilities to positive electrophoretic and high chromatographic mobilities (i.e. bottom-left to top-right in the maps shown here), could represent single peptides with multiple target sites which become sequentially more phosphorylated. Indeed, this was observed in two-dimensional phosphopeptide maps of overexpressed yeast topoisomerase II (Cardenas *et al.*, 1992). However, no such pattern is present with human topoisomerase IIα protein, and this suggests significant differences in the distribution of phosphorylation sites in topoisomerase II in yeast compared to topoisomerase IIα in humans.

The activity of the purified topoisomerase IIα protein could be markedly inhibited by dephosphorylation, whilst phosphorylation by CK II activated the purified enzyme. This implies that the topoisomerase IIα protein was isolated in a partially phosphorylated state. A change in the mobility of the topoisomerase IIα protein on SDS-PAGE following dephosphorylation is consistent with this suggestion, although it remained to be shown that rephosphorylation could shift the protein back to the original mobility. It is possible that the enzyme is wholly inactive in the absence of phosphorylation, although this has not formally been shown.

CK II phosphorylation enhanced the decatenation activity of purified topoisomerase IIα by 10-fold but only enhanced the relaxation activity by 2- to 3-fold. This could be due to conditions not being optimized for the plasmid relaxation experiment. However, it is also possible that CK II phosphorylation preferentially enhances the decatenation activity of topoisomerase IIα such as is required for chromosome segregation.

Despite the apparently complex nature of topoisomerase IIα protein phosphorylation *in vivo*, the data are consistent with CK II having a key role in regulating topoisomerase II activity and thus the topological state of nuclear DNA in human cells. The dephosphorylated topoisomerase IIα enzyme could be reactivated by CK II to the extent that it exhibited an activity above that of the purified, but untreated, enzyme, implicating CK II as a key regulator of topoisomerase IIα enzyme activity *in vivo*. It is even possible that topoisomerase IIα protein requires prior phosphorylation by CK II for activity, as appears to be the case for another nuclear protein, DNA ligase I (Prigent *et al.*, 1992). If that were the case, further control of activity *in vivo* may be mediated either by other kinases, or by the action of phosphatases.

The yeast topoisomerase II enzyme is phosphorylated *in vivo* by CK II at sites clustered near the C-terminus of the protein (Cardenas *et al.*, 1992). We have shown that this region of the human topoisomerase IIα protein is similarly modified. C-terminal peptides in the topoisomerase IIα protein sequence which include serine residues and match the consensus for CK II phosphorylation sites derived by Kennelly and Krebs (1991) are shown in Table 5. Of these, 3 are in a conserved location in the C-terminal domain of the yeast enzyme. It is noteworthy that the strongest match to the CK II consensus site, which lies within 15 residues of the C-terminus, is not only conserved in the yeast enzyme but also in the human topoisomerase IIβ protein (Figure 3, chapter 1). However, the observation that topoisomerase IIα protein immunoprecipitated with a polyclonal antiserum to a C-terminal peptide can still be phosphorylated by CK II means that it is likely that some sites of CK II phosphorylation are not at the extreme C-terminus.

This C-terminal domain is unique to eukaryotic topoisomerase II enzymes, unlike the highly conserved ATPase and breakage/reunion domains, making it unlikely that it serves any direct catalytic function. The data presented here, and those of Cardenas *et al.* (1992), are consistent with a regulatory role for the C-terminal domain. It is

interesting to note that although yeast and human topoisomerase II enzymes may be regulated in a similar manner by CK II, it is the putative regulatory C-terminal domain which has diverged significantly between yeast and humans. Thus, there may be significant differences in the effects of phosphorylation between the yeast and human topoisomerase II enzymes, particularly as yeast, in contrast to mammalian cells, appear to express only one topoisomerase II isozyme.

In the *in vivo* studies on yeast topoisomerase II phosphorylation, the yeast protein was overexpressed from an inducible plasmid in yeast cells, as labelling studies were too weak using the endogenous level of protein (Cardenas *et al.*, 1992). Despite the demonstration that this overexpressed protein is not abnormally located within the cell, care must be taken in interpretating these results as it is possible that phosphorylation of certain sites may be influenced by the intracellular level of the enzyme. For instance, if phosphorylation is normally required to stimulate the activity at mitosis, then having an increased level of topoisomerase II protein in the cell due to overexpression may fulfill this requirement without the need for phosphorylation; hence this phosphorylation might not occur. We have been able to study *in vivo* phosphorylation purely on the endogenous topoisomerase IIα protein.

How CK II phosphorylation might be altering the activity and biological function of human topoisomerase IIα has still to be addressed and some of the possible mechanisms are discussed in chapter 8.

Residue Numbers	Sequence
1295-1308	D**S**E**S**DR**SS**DE**S**NFD**
1328-1341	DLD**S**DEDF**S**DFDE
1375-1382	V**S**DLEADD
1468-1479	ST**S**DD**S**DS**S**NFE
1492-1497	GE**S**DDF**
1522-1528	EE**S**DEDD**

Table 5 Putative CK II sites in the C-terminal domain of human topoisomerase IIα protein. The one letter amino acid code is used and the residue numbers refer to the position in the human topoisomerase IIα protein sequence (see Figure 3, chapter 1). Potential serine acceptor sites are in bold-faced type. Double asterisks denote sites present in a conserved location in the *S. cerevisiae* topoisomerase II sequence (Cardenas *et al.*, 1992).

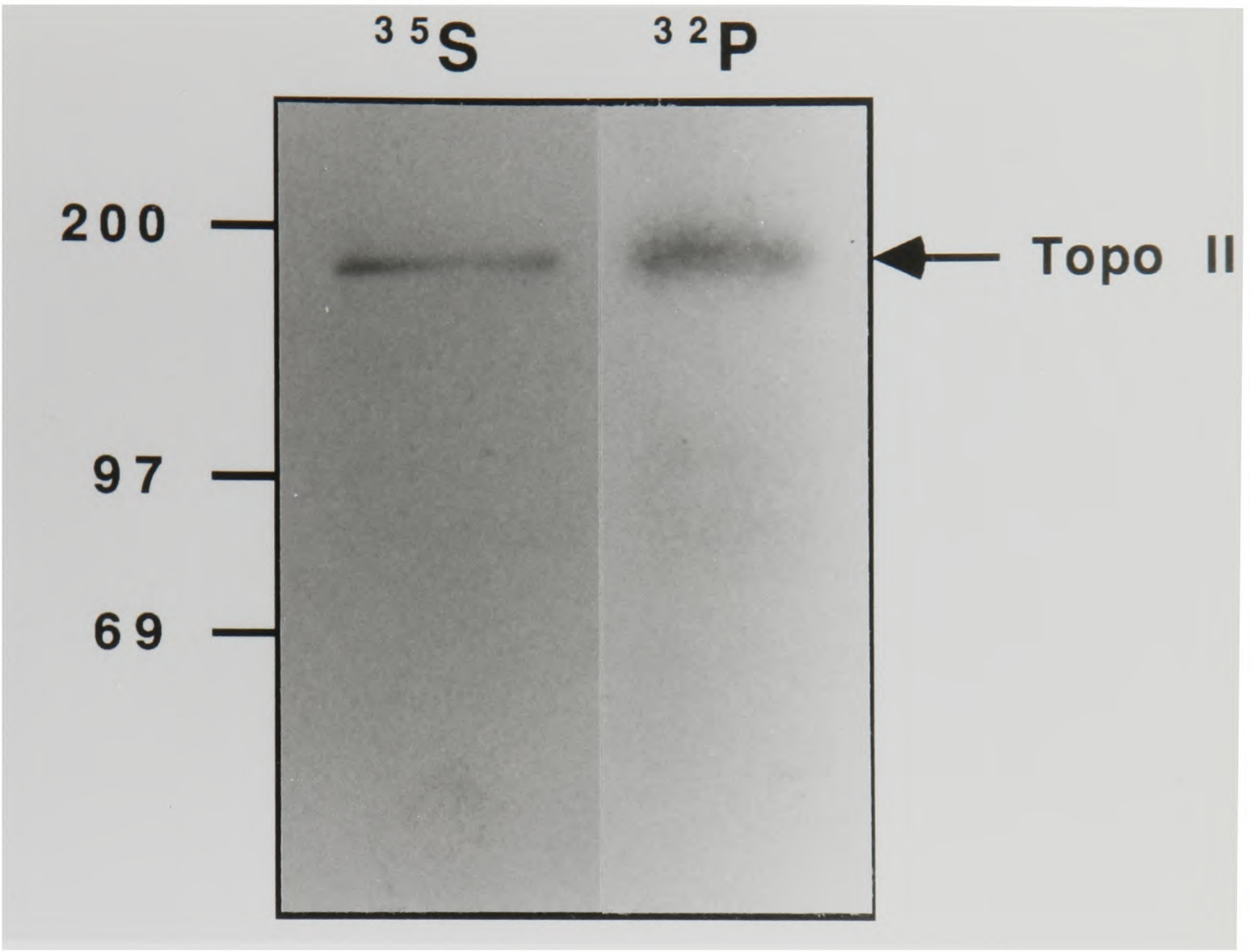


Figure 19 Topoisomerase IIα is a phosphoprotein *in vivo*. The α isozyme of topoisomerase II was immunoprecipitated from nuclear extracts of HeLa cells metabolically labelled with either ^{35}S-methionine (left lane) or ^{32}P-orthophosphate (right lane). The position of the 170kDa topoisomerase IIα protein is indicated. Molecular weight markers are shown on the left.

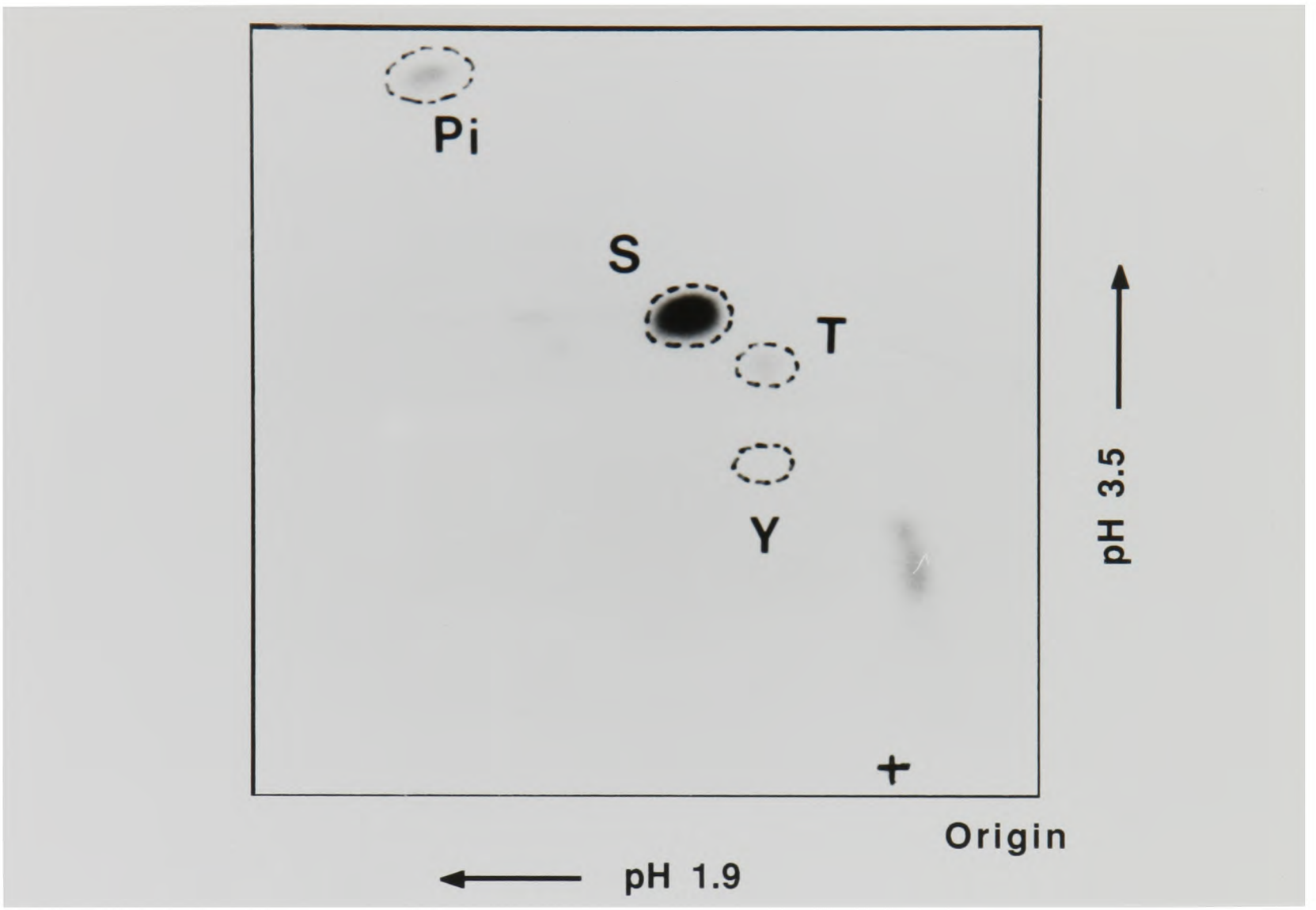
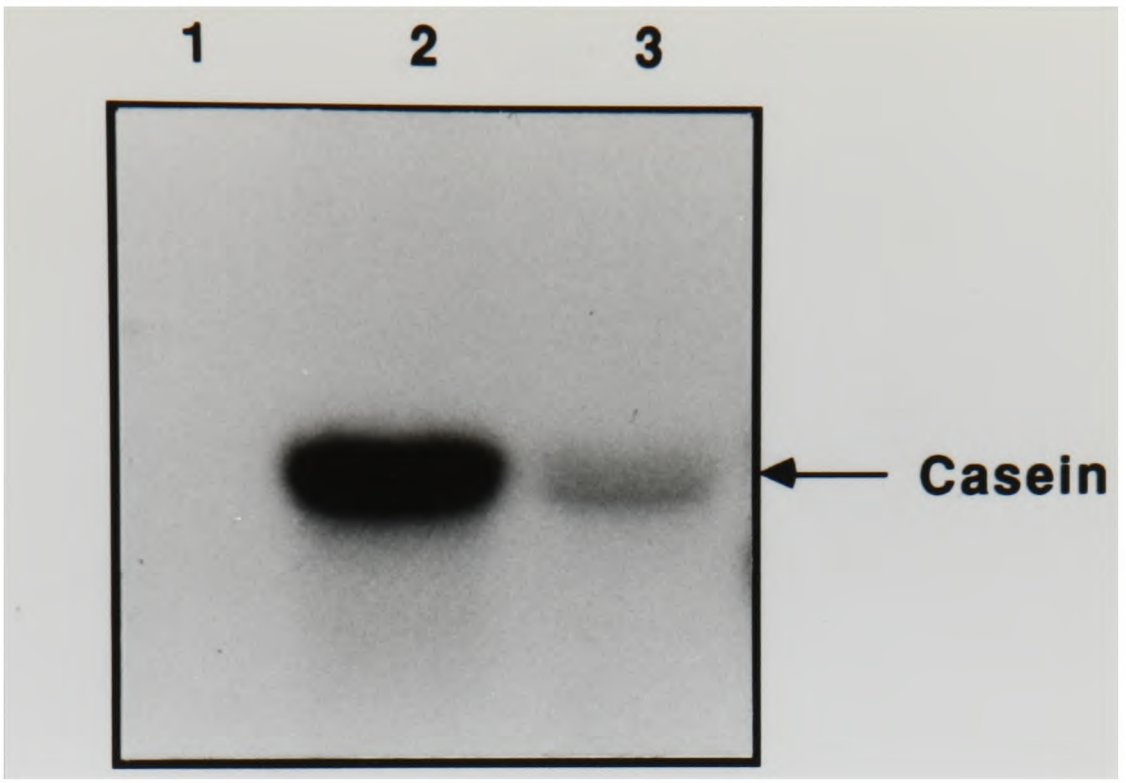


Figure 20 Phosphoamino-acid analysis of *in vivo* ^{32}P-labelled human topoisomerase IIα protein. Phosphorylated topoisomerase IIα was hydrolysed in 5.7M HCl at 110°C for 1 hour. The hydrolysate was mixed with non-radioactive phosphorylated amino acid standards and analysed by two-dimensional thin-layer electrophoresis. An autoradiogram of the thin-layer plate is shown. Dashed circles indicate the positions of the standards as visualized by ninhydrin staining. Phosphoserine (S); phosphothreonine (T); phosphotyrosine (Y); inorganic phosphate (Pi).

(a)



(b)

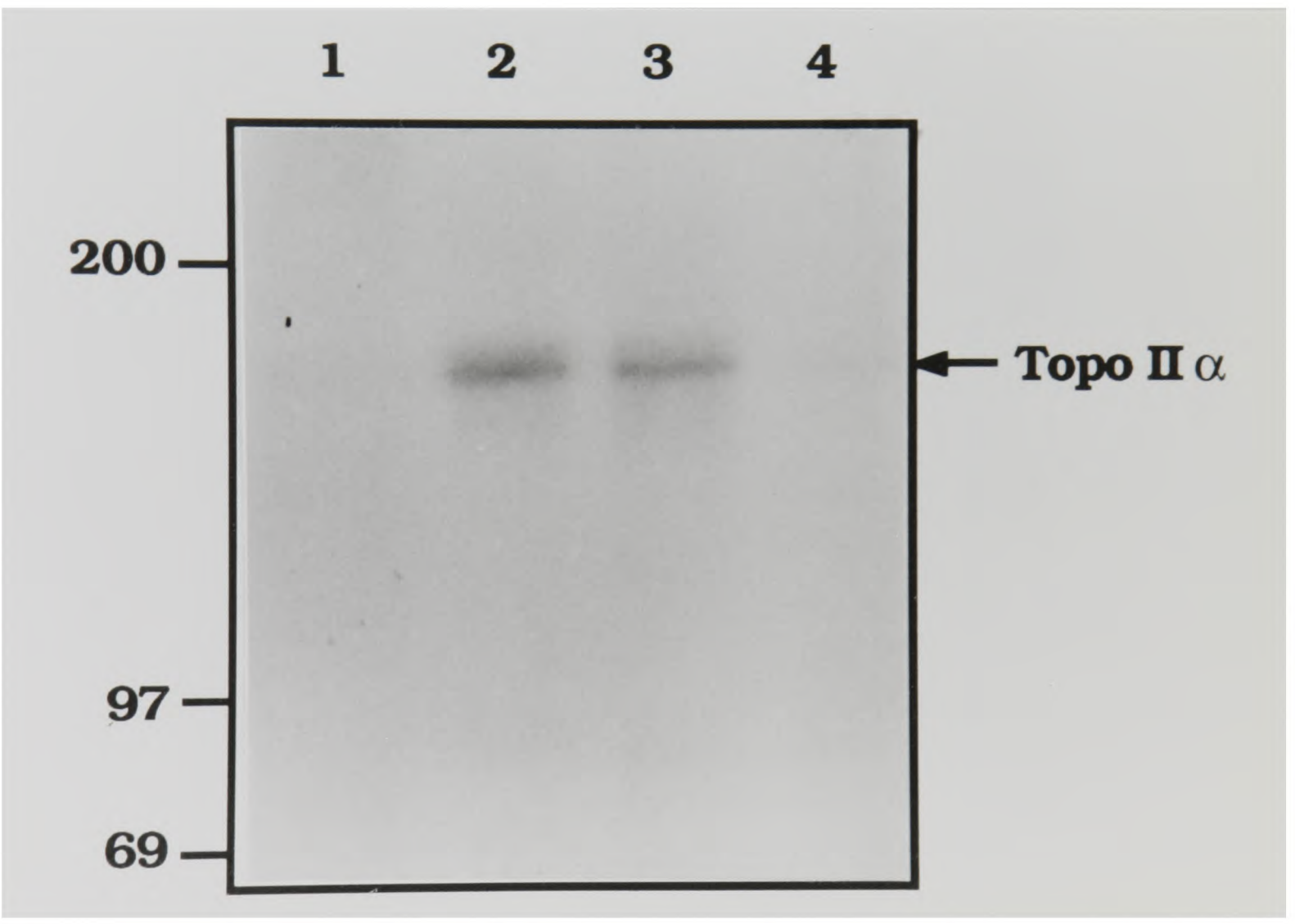


Figure 21 The purified human topoisomerase IIα protein is a substrate for purified mammalian casein kinase II. (a) Purified mammalian CK II was shown to be active by incubating alone (lane 1), or with casein in the absence (lane 2) or presence of 10μg/ml heparin (lane 3). (b) Topoisomerase IIα protein was incubated in the absence (lane 1) or presence (lanes 2-4) of purified CK II. The effect on phosphorylation of co-incubation with heparin at 1μg/ml (lane 3) or 10μg/ml (lane 4) is also shown.

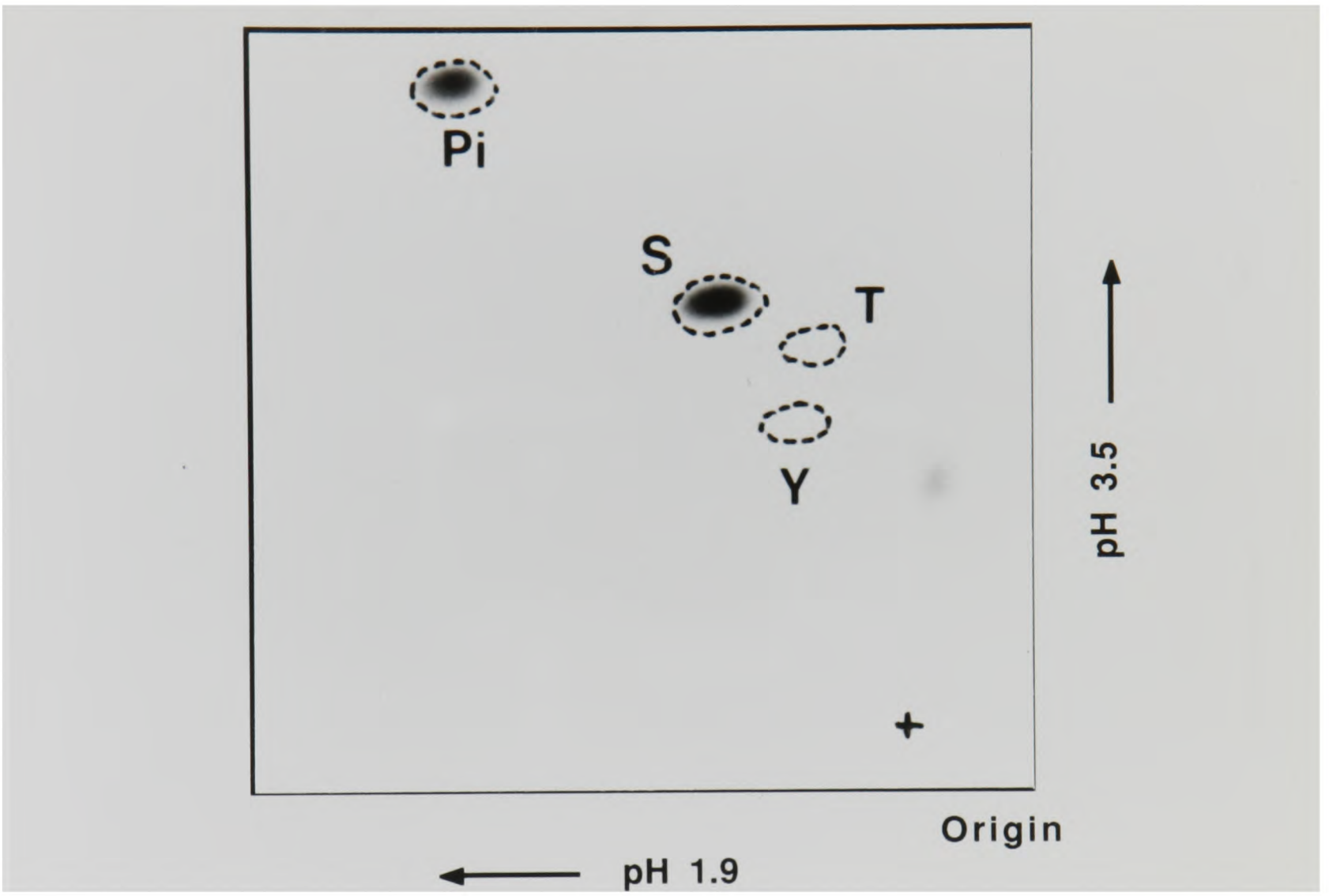


Figure 22 Phosphoamino-acid analysis of purified human topoisomerase IIα phosphorylated by purified mammalian casein kinase II *in vitro*. Purified human topoisomerase IIα protein was incubated in the presence of purified mammalian casein kinase II and [γ^{32}P]-ATP, run out on a 7.5% SDS-polyacrylamide gel and then treated in the same way as the *in vivo* sample (Figure 20). An autoradiogram of the thin-layer plate is shown. Dashed circles indicate the positions of the standards: phosphoserine (S); phosphothreonine (T); phosphotyrosine (Y); inorganic phosphate (Pi).

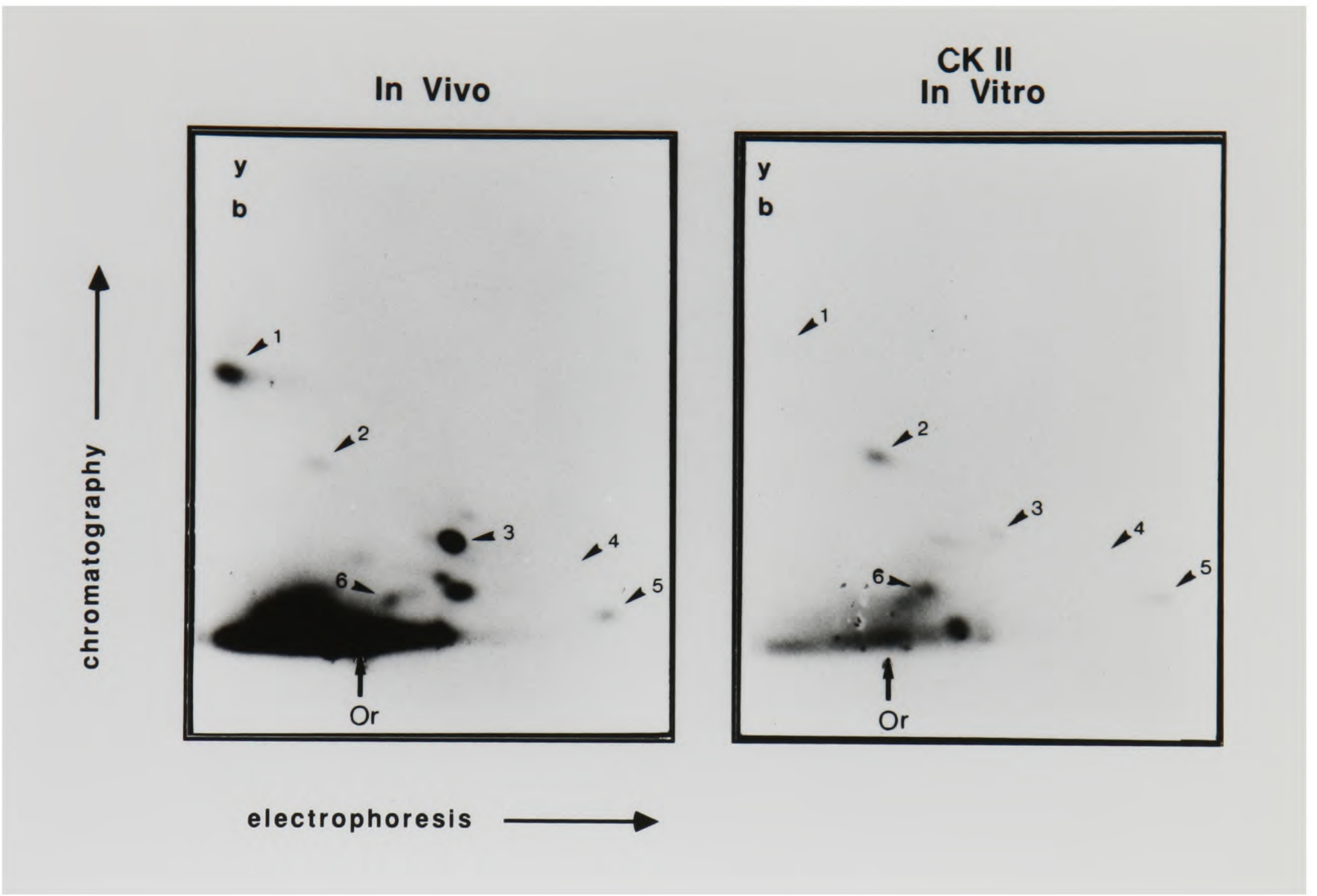


Figure 23 Two dimensional tryptic phosphopeptide maps of topoisomerase IIα protein phosphorylated *in vivo* and with CK II *in vitro*. Peptides were separated by high voltage electrophoresis followed by chromatography. Phosphopeptides were visualised by autoradiography. The positions of the origin (Or) and the two chromatographic markers, DNP-lysine (y) and xylene cyanol (b), are indicated. The numbered arrows indicate phosphopeptides present in the *in vivo* labelled sample which co-migrated with peptides phosphorylated *in vitro* by CK II.

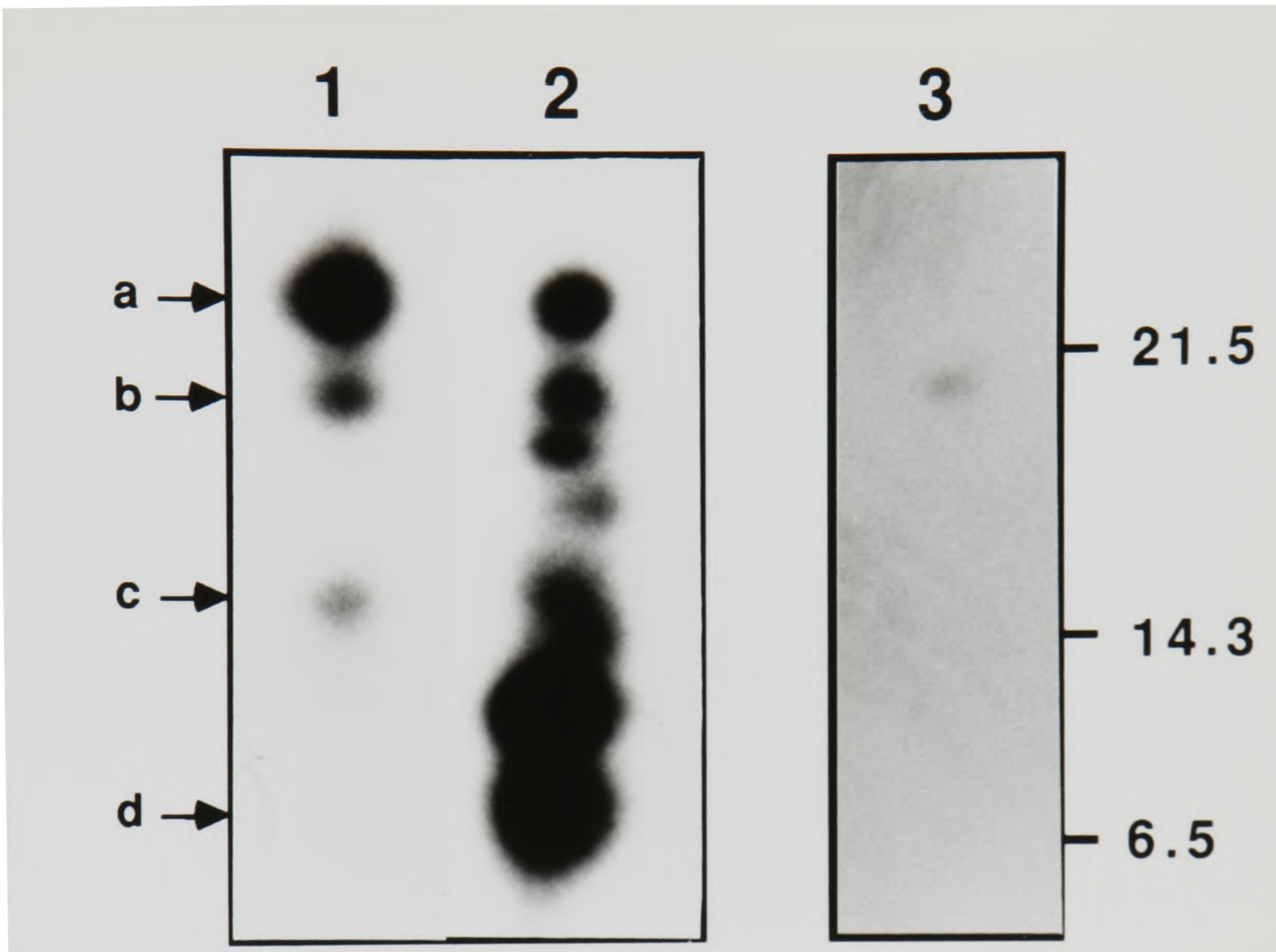


Figure 24 Topoisomerase IIα protein is phosphorylated *in vivo* by CK II on its C-terminal domain. Topoisomerase IIα protein was labelled *in vivo* with ^{32}P-orthophosphate (lane 1) or phosphorylated *in vitro* with purified CK II (lane 2). Phosphopeptides were identified using the one dimensional electrophoresis method of Cleveland *et al.* (1977). The positions of molecular weight standards are shown on the right. The 4 major phosphopeptides present in the *in vivo* labelled sample are designated 'a-d' and indicated by arrows. In parallel, topoisomerase IIα protein was immunoprecipitated from cells and similarly processed. The cleaved peptides were immunoblotted using the CRB antibody raised to the C-terminal region of topoisomerase IIα protein (lane 3).

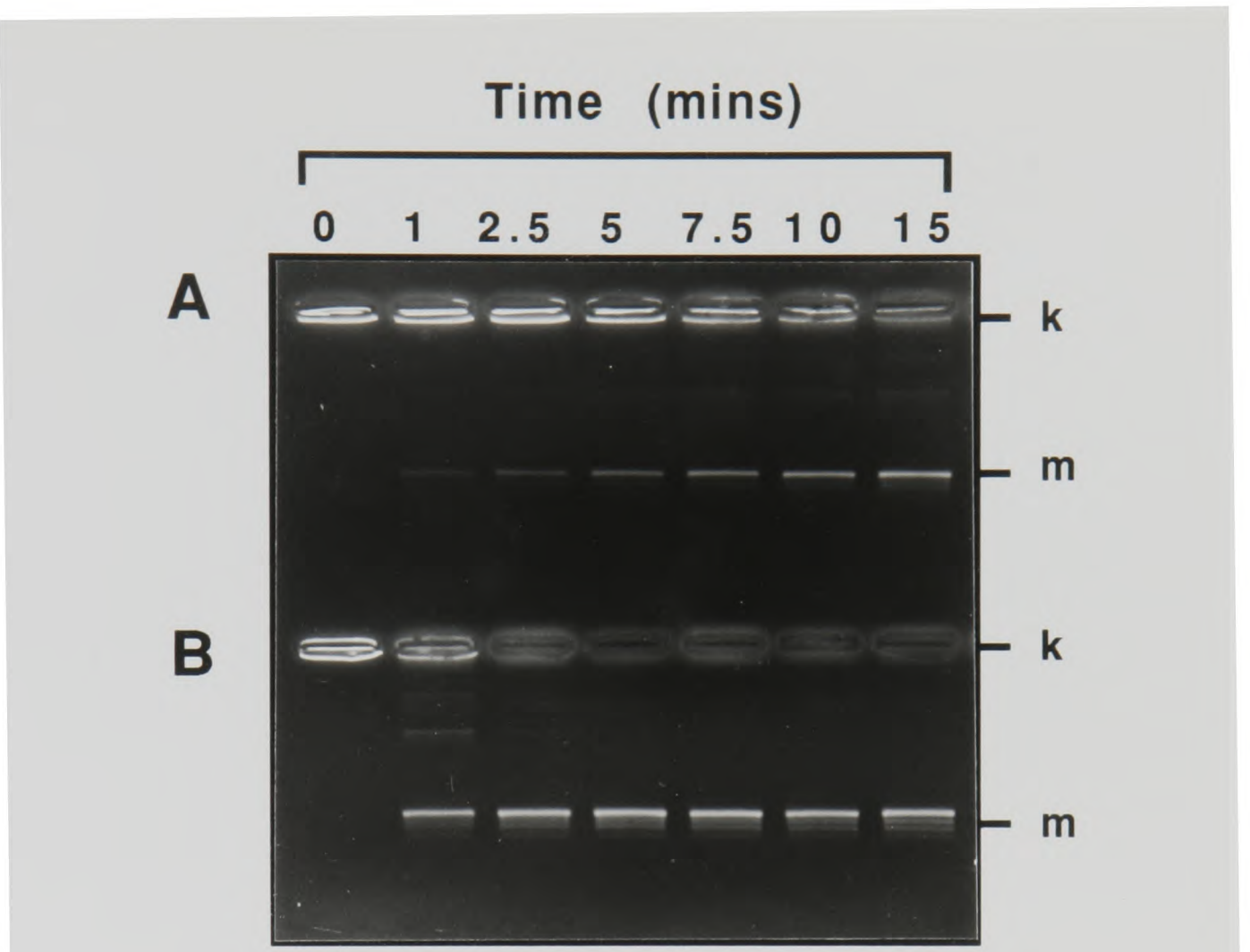


Figure 25 Decatenation activity of untreated and CK II phosphorylated human topoisomerase IIα protein. The decatenation activity was measured as the rate of release of minicircles (m) from kinetoplast DNA (k) with time. Before carrying out the decatenation reaction the topoisomerase IIα was incubated at 30°C for 10 minutes in the absence (A, top panel) or presence (B, bottom panel) of purified mammalian CK II. Samples were taken and stopped at time T= 0, 1, 2.5, 5, 7.5, 10 and 15 minutes and run on a 1% agarose gel. The gel was stained with ethidium bromide and photographed under UV-illumination.

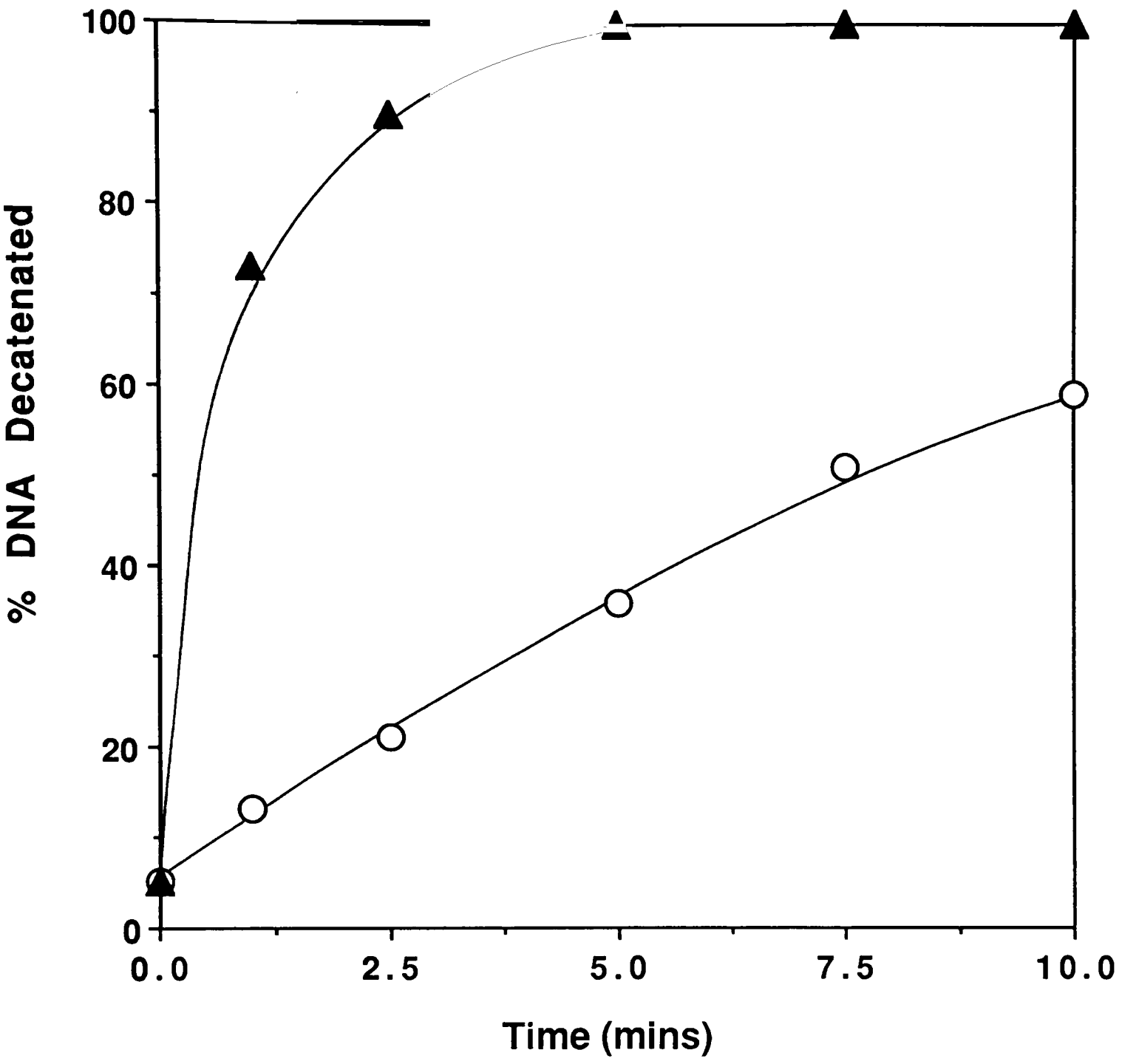


Figure 26 Phosphorylation of human topoisomerase IIα by CK II *in vitro* leads to an increase in the specific decatenation activity of the enzyme. The photograph shown in Figure 25 was scanned by densitometry and the % of DNA decatenated plotted against time. Activity is shown in the absence (○) or presence (▲) of purified casein kinase II.

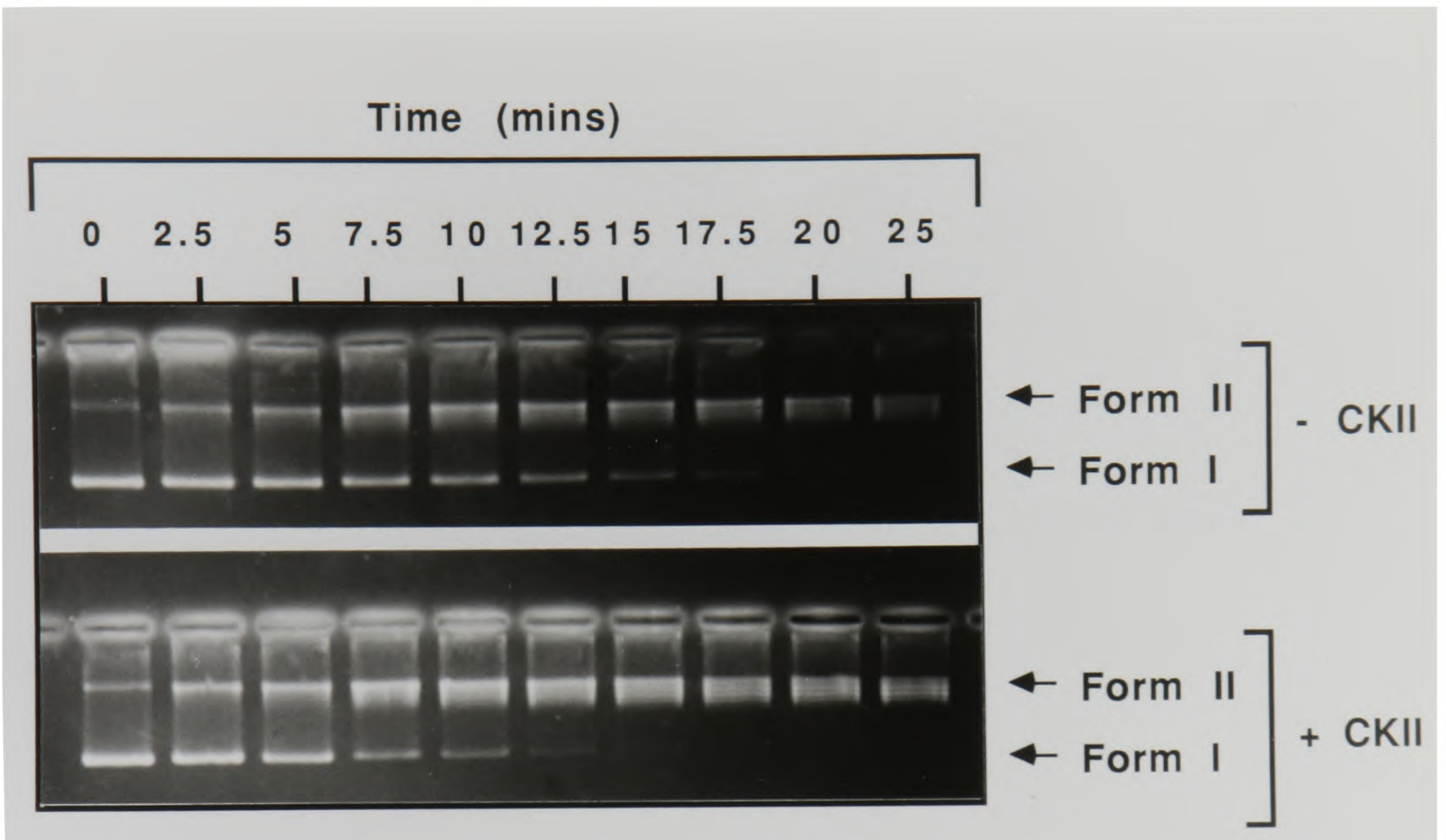


Figure 27 Relaxation activity of untreated and CK II phosphorylated human topoisomerase IIα protein. The relaxation activity was measured as the rate of relaxation of a supercoiled plasmid substrate from Form I (supercoiled) to Form II (relaxed) DNA with time. Before carrying out the relaxation reaction the topoisomerase IIα protein was incubated at 30°C for 10 minutes in the absence (top panel) or presence (bottom panel) of purified mammalian CK II. Samples were taken and stopped at time T= 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 and 25 minutes and electrophoresed on a 1% agarose gel. The gel was stained with ethidium bromide and photographed under UV-illumination.

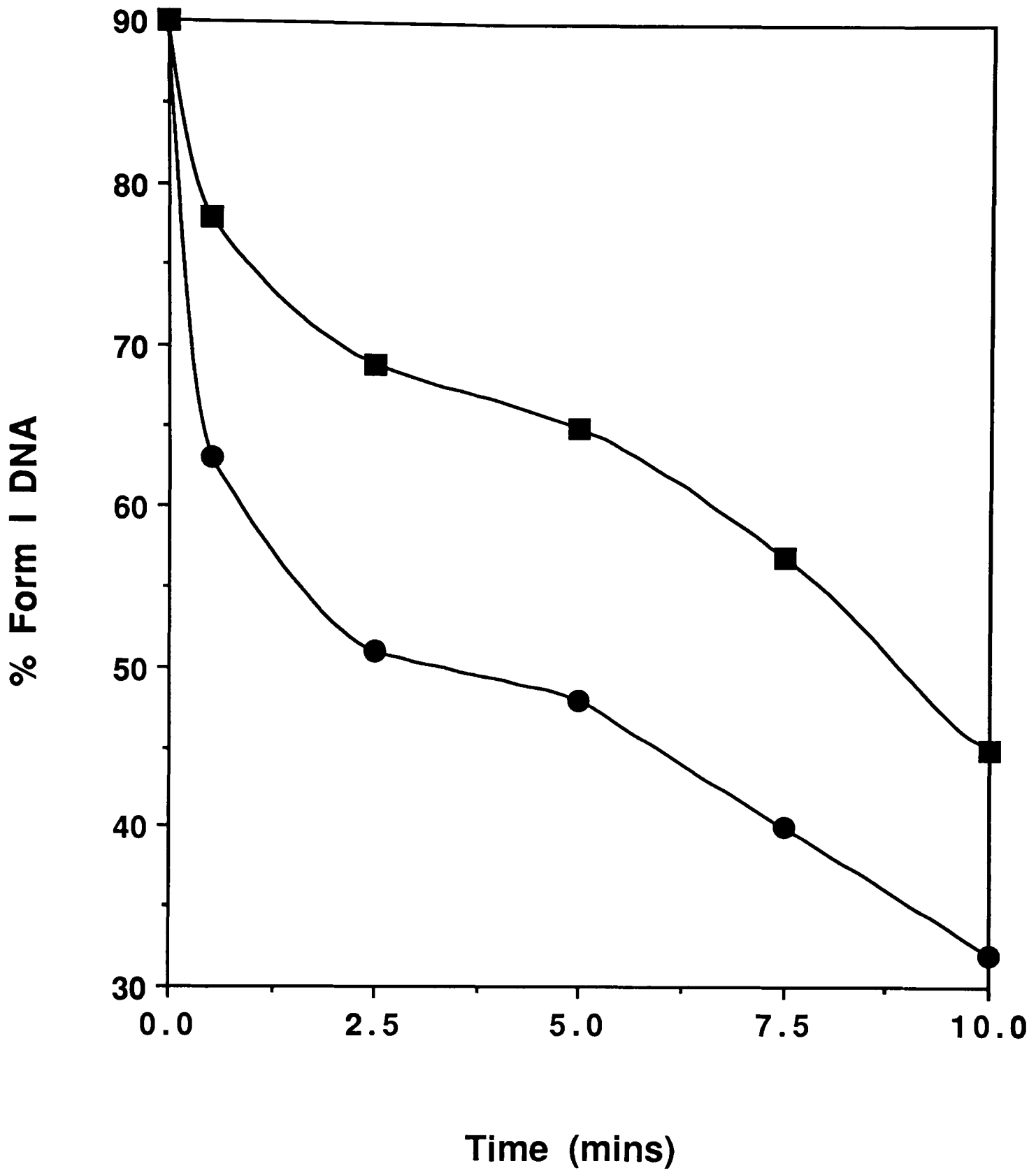


Figure 28 Phosphorylation of human topoisomerase IIα by CK II *in vitro* leads to an increase in the specific relaxation activity of the enzyme. The photograph shown in Figure 27 was scanned by densitometry and the % of Form I (supercoiled) DNA plotted against time. Activity is shown in the absence (■) or presence (●) of purified casein kinase II.

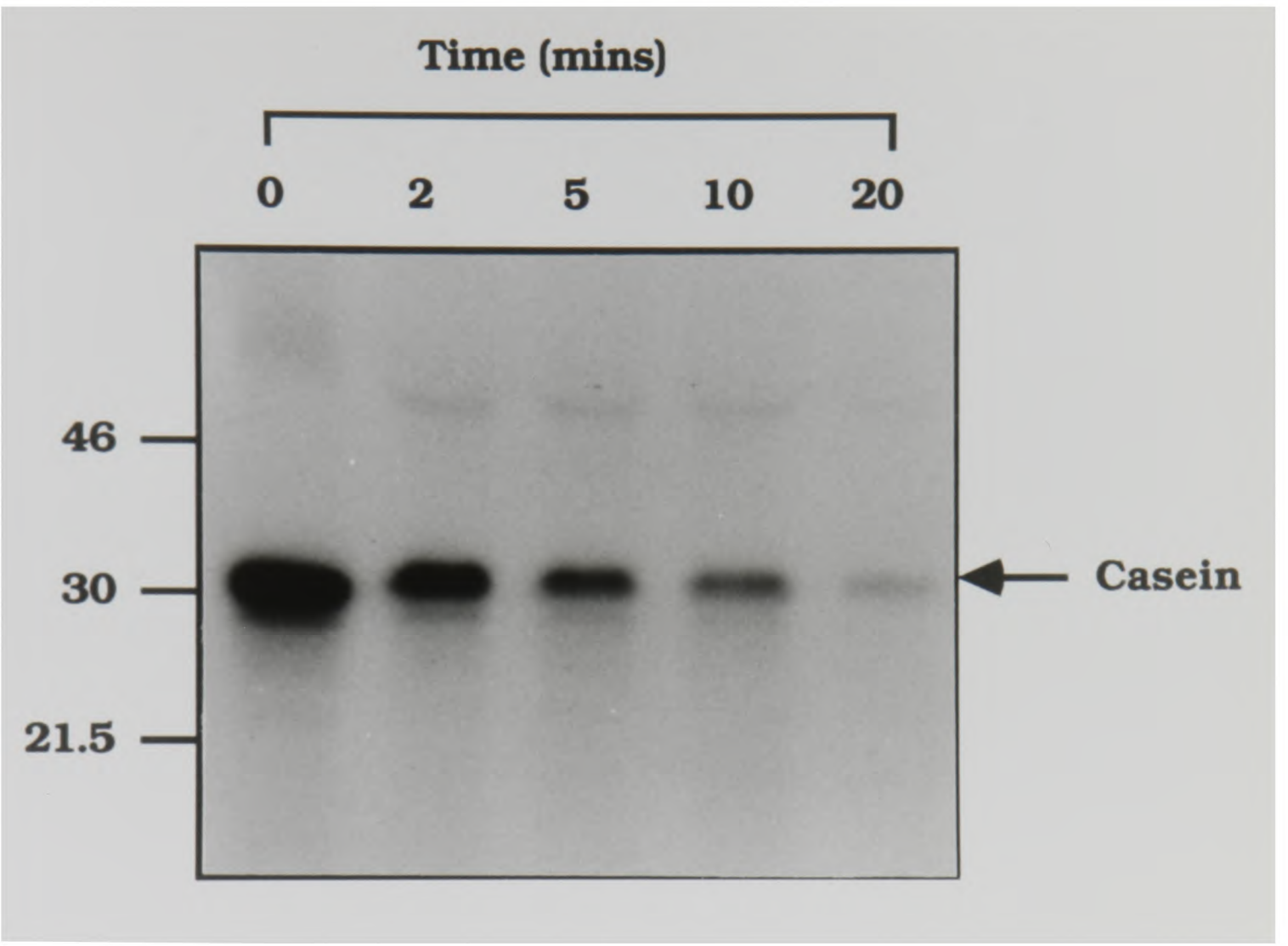


Figure 29 Dephosphorylation of phosphorylated casein by potato acid phosphatase (PAP). Casein was phosphorylated by CK II in the presence of [γ^{32}P]-ATP for 10 minutes at 30°C and then the CK II inhibited with 10μg/ml heparin. PAP was then added to the phosphorylated casein and the reaction stopped by boiling in SDS-loading buffer after 0, 2, 5, 10 or 20 minutes. The samples were separated on a 12% SDS-polyacrylamide gel, and the gel dried and autradiographed. Molecular weight markers are shown on the left, and the position of the phosphorylated casein on the right.

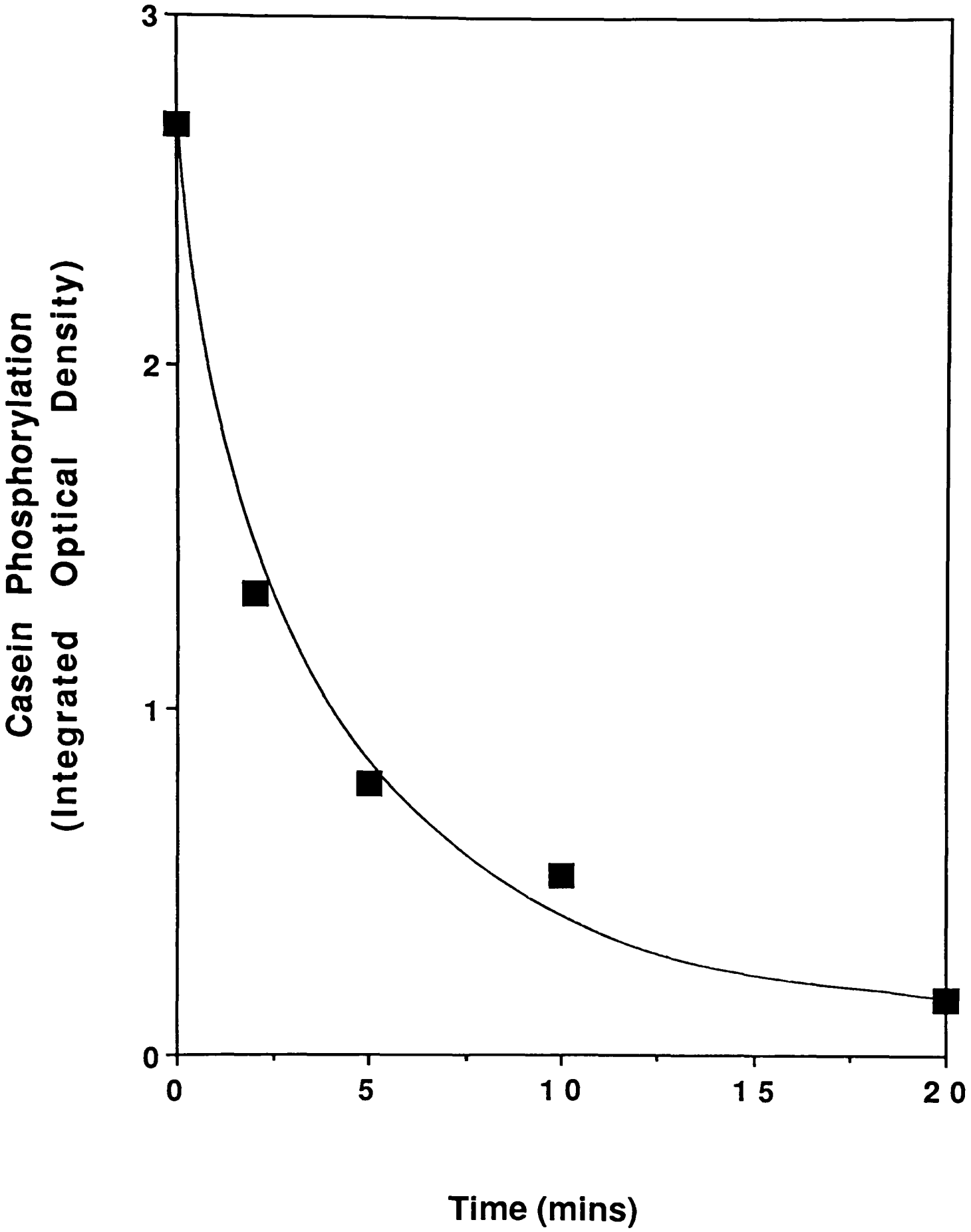


Figure 30 Potato acid phosphatase activity. The autoradiograph from Figure 29 was scanned by densitometry, and the level of phosphorylation of casein plotted against time after addition of potato acid phosphatase together with heparin to inhibit the CK II (■).

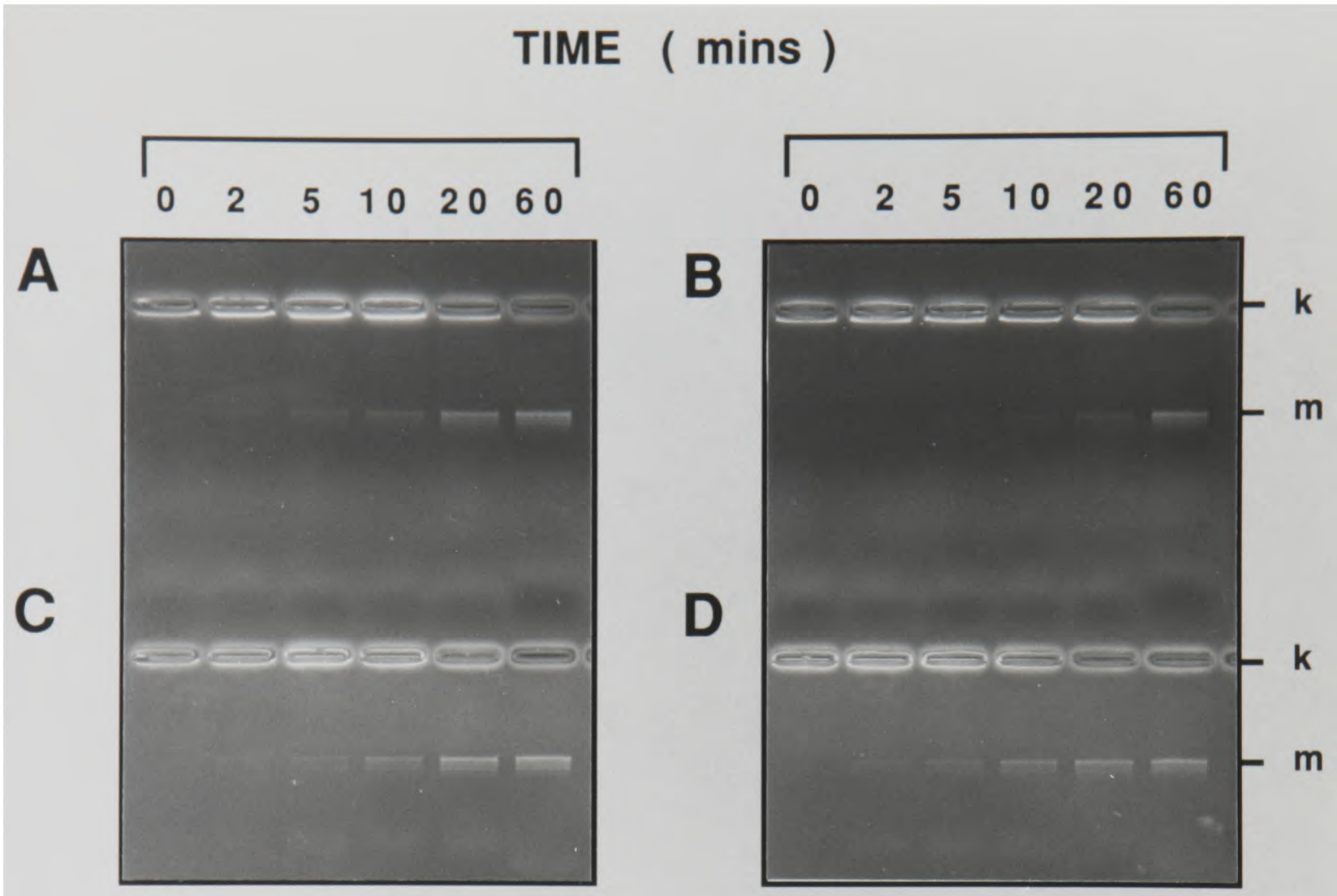


Figure 31 Decatenation activity of untreated and PAP treated human topoisomerase IIα protein. The decatenation activity was measured as the rate of release of minicircles (m) from kinetoplast DNA (k) with time. Before carrying out the decatenation reaction the topoisomerase IIα protein was incubated at 30°C for 10 minutes in the presence of (A) no PAP, (B) PAP, (C) PAP which has been preheated to 90°C for 10 minutes, or (D) PAP together with 10mM sodium phosphate. Samples were taken and stopped at time T= 0, 2, 5, 10, 20 and 60 minutes and run on a 1% agarose gel. The gel was stained with ethidium bromide and photographed under UV-illumination.

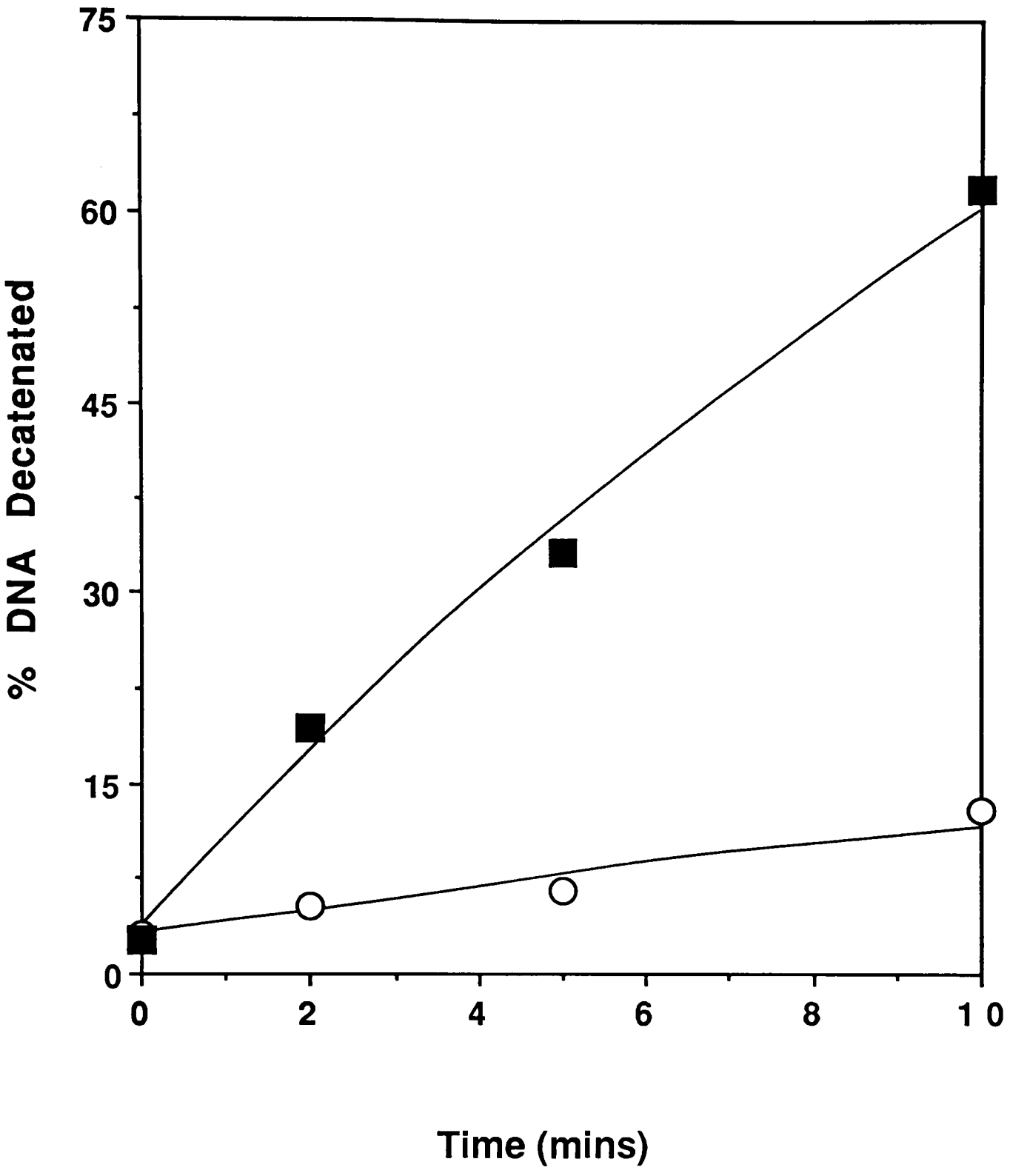


Figure 32 Dephosphorylation of human topoisomerase IIα by PAP *in vitro* leads to an decrease in the specific activity of the enzyme. Panels (B) and (C) from the photograph shown in Figure 31 was scanned by densitometry and the % of DNA decatenated plotted against time. Activity is shown either in the the presence of untreated PAP (○) or heat-inactivated PAP (■).

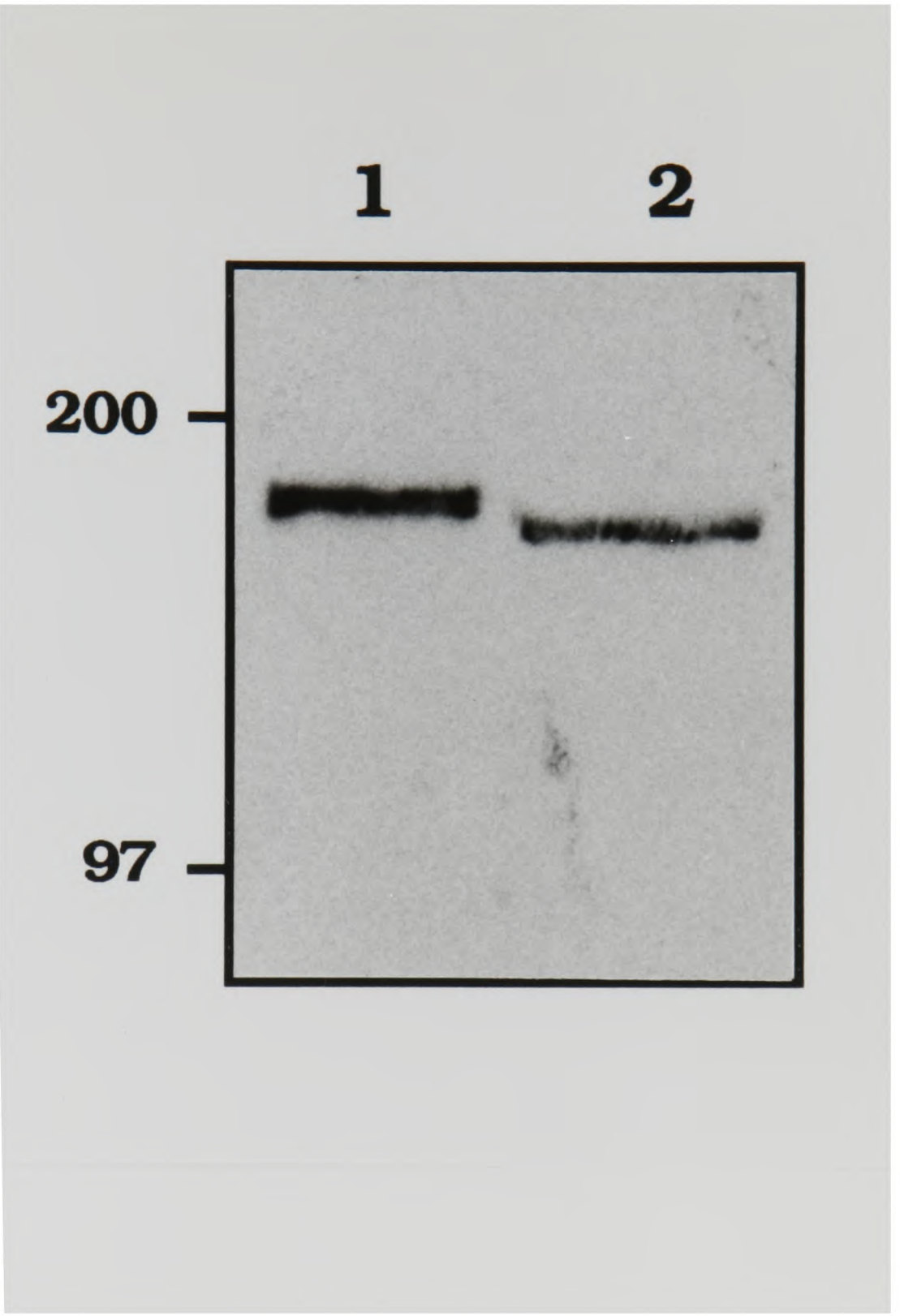


Figure 33 Increase in relative mobility on SDS-PAGE of topoisomerase IIα protein following dephosphorylation. Purified topoisomerase IIα was incubated alone (lane 1) or with PAP (lane 2) for 10 minutes at 30°C. Samples were then separated by 6% SDS-PAGE and blotted onto nitrocellulose membrane. The blot was then incubated with the α-specific monoclonal antibody, 6F3, and detected by ECL. Positions of molecular weight markers (kDa) are shown on the left.

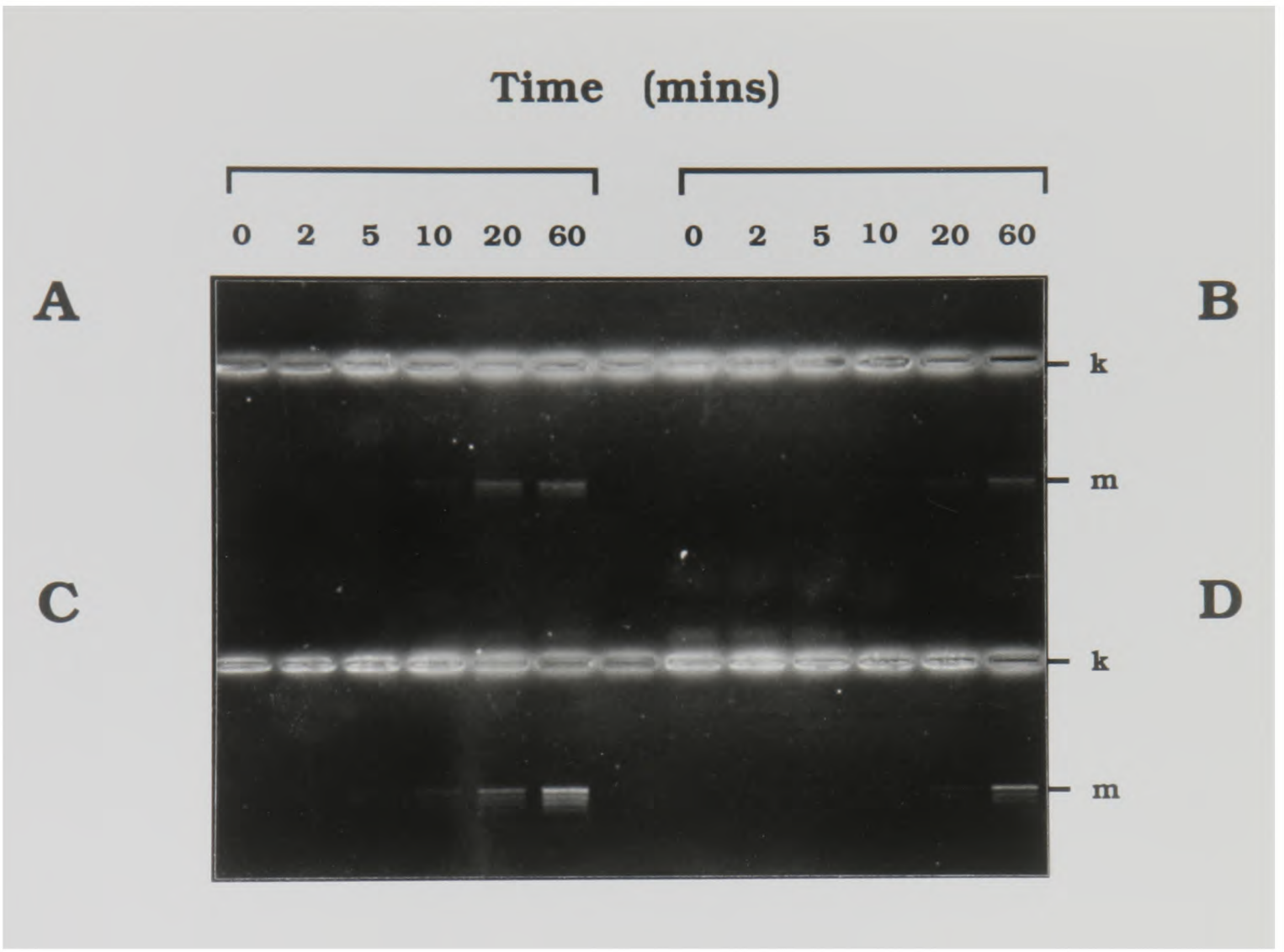


Figure 34 Reactivation of dephosphorylated topoisomerase IIα protein by casein kinase II, but not PKA. The decatenation activity was measured as the rate of release of minicircles (m) from kinetoplast DNA (k) with time. Before carrying out the decatenation reaction the topoisomerase IIα protein was incubated at 30°C for 10 minutes in the absence (A) or presence (B, C and D) of PAP. Samples were then given a further 10 minute incubation at 30°C with no kinase (A and B), CK II (C) or PKA catalytic subunit (D). Samples were taken and stopped at time T= 0, 2, 5, 10, 20 and 60 minutes and run on a 1% agarose gel. The gel was stained with ethidium bromide and photographed under UV-illumination.

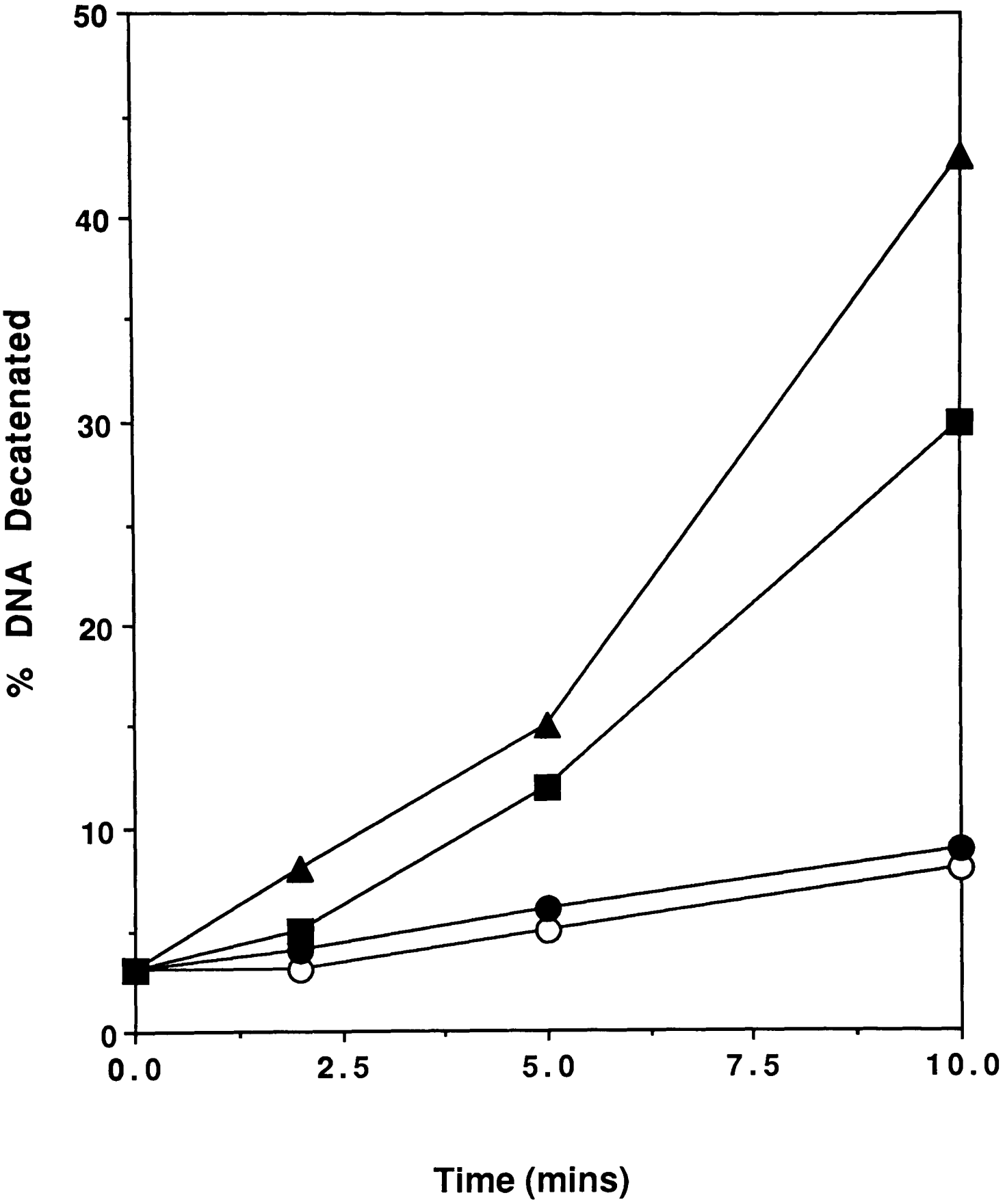


Figure 35 Activity of dephosphorylated topoisomerase IIα protein following rephosphorylation by CK II, but not PKA, is above that of untreated enzyme. Panels A, B, C and D from Figure 34 were scanned by densitometry and the % DNA decatenated plotted against time. Topoisomerase IIα following no treatment (■), dephosphorylation by PAP (○), and rephosphorylation by CK II (▲) or PKA (●) following PAP treatment.

Chapter 6 PHOSPHORYLATION OF HUMAN TOPOISOMERASE II PROTEINS BY PROTEIN KINASE A.

6.1 Introduction.

A number of observations described in the previous chapter, demonstrate that whilst CK II appears to account for some of the phosphorylation of human topoisomerase IIα protein seen *in vivo*, it does not account for it all. For instance, two-dimensional tryptic mapping clearly shows that CK II cannot account for all the phosphopeptides generated *in vivo* and phosphoamino acid analysis shows that CK II phosphorylates topoisomerase IIα exclusively on serine *in vitro*, and yet some phosphothreonine is seen *in vivo*. Hence, we set out to identify which kinases were responsible for this additional phosphorylation seen *in vivo*.

Previous work on the phosphorylation of topoisomerase II in lower eukaryotes suggested that it can act as a target for multiple kinases *in vitro* (Sayhoun *et al.*, 1986; Cardenas *et al.*, 1992). Two aspects concerning the biological regulation of topoisomerase II phosphorylation in mammalian cells are of particular interest. One is the cell cycle dependent phosphorylation of topoisomerase II which may play a crucial role in regulating the mitotic functions of topoisomerase II (discussed more fully in chapter 7). The other is whether the phosphorylation state of topoisomerase II changes in response to various extracellular signals, for example growth stimulation or induction of differentiation. This may be required if the cell is going to switch to a more or less active state with a concomitant increase or decrease in DNA metabolism. It is possible that this is linked to the changes in phosphorylation of topoisomerase II seen in some drug resistant cell lines (Takano *et al.*, 1991; Fernandes *et al.*, 1990).

The cellular function of phosphorylation of human topoisomerase IIα protein by CK II has not yet been identified. However, the general lack of regulation of CK II activity in the cell suggests that it is unlikely to be the primary regulator of changes in phosphorylation in response to external signals. The best characterized protein kinases that mediate signal transduction are protein kinase C (discussed in chapter 7) and protein kinase A.

Many hormonal signals stimulate a rise in the intracelluler level of the second messenger, cyclic AMP, via the plasma membrane associated

enzyme adenylate cyclase. cAMP then carries out its function as a second messenger via two parallel pathways. The first is the direct activation of gene transcription through the binding of cAMP to transcription factors, which in turn bind to cAMP response elements (CRE) in the promoters of various genes (Roesler *et al.*, 1988). The second is through the binding of cAMP to the regulatory subunit of the ubiquitous serine/threonine kinase, PKA or cAMP-dependent protein kinase (reviewed in Edelman *et al.*, 1987; Taylor, 1989; Cho-Chung, 1990).

The PKA holoenzyme is an R_2C_2 tetramer composed of two regulatory subunits (R), and two catalytic subunits (C). There appear to be two classes of PKA designated type I and type II, which contain different regulatory subunits, RI and RII respectively, but which share a common catalytic subunit. Both R subunits each contain two tandem cAMP-binding sites, in their carboxy-terminal domains; however, they differ in their amino-terminal domains, where the RI subunit has a high affinity ATP binding site, and the RII subunit has a site for autophosphorylation by the C-subunit. The recent elucidation of the high-resolution crystal structure of the PKA C-subunit was a major achievement, and now provides a template for viewing the conserved catalytic core of all eukaryotic protein kinases (Knighton *et al.*, 1991).

The major function of the R subunits is to bind to and inhibit the C-subunits in the absence of cAMP. Upon binding of cAMP by the regulatory subunits, the holoenzyme dissociates into an R_2 dimer and two active C subunits, which then phosphorylate their downstream targets. Even within the R and C subunit class, there exist several unique and antigenically distinct gene products. Some of these appear to be expressed in a developmentally-regulated or tissue-specific manner, and this suggests that the different PKA isozymes may have distinct roles in different physiological processes.

A heat stable protein inhibitor, PKI, has been identified in cells which may function to suppress PKA activity stimulated by basal levels of cAMP. The R subunits and PKI all contain segments of sequence that closely resemble the sequences of preferred substrates and thus appear to act as pseudosubstrate inhibitors.

A report by Costa *et al.* (1975) suggested that the activity of type I and type II PKAs in CHO cells was cell cycle-dependent, with type I activity being highest at mitosis, and type II activity being highest at the G_1/S boundary. This suggested some differential role of the two major types of PKA in cell cycle progression. However, PKA has previously been

reported to be a negative regulator of early mitotic cycles of a variety of organisms (Maller and Krebs, 1977). Inhibition of PKA by microinjection of PKI, the inhibitor protein of PKA, or R subunits induces maturation in amphibians (Maller and Krebs, 1977) and results in rapid and pronounced chromatin condensation at all phases of the cell cycle in rat embryo fibroblasts (Lamb *et al.*, 1991).

Both the R and C subunits of PKA can be multiply phosphorylated by several different kinases. Of particular interest to this study, was the observation that the RII subunit is phosphorylated *in vivo* on two sites that can be phosphorylated by CK II *in vitro* (Carmichael *et al.*, 1982; Hemmings *et al.*, 1982). The function of these particular phosphorylations are not known, but suggest a possible coordination between the CK II and PKA regulatory pathways.

PKG, or cGMP-dependent kinase, is 50-fold more sensitive to activation by cGMP than by cAMP. This kinase has been purified from a number of sources but its physiological role is still not well understood (reviewed in Edelman *et al.*, 1987). It is a homodimer of approximately 76kDa in which the catalytic domain is contained within the C-terminal region of each subunit whilst the remainder of the sequence encodes an inhibitory domain, two cGMP binding sites with different binding affinities and a dimerization domain. It has been proposed that the inhibitory region of one subunit inhibits the catalytic site of the other subunit in an antiparallel fashion. Hence, each PKG monomer fulfills the function of an R_1C_1 PKA dimer. It appears that PKG is primarily cytosolic in many tissues, although association with the particulate fraction has also been reported (Edelman *et al.*, 1987).

PKA and PKG exhibit a similar substrate specificity towards peptides and proteins *in vitro*. Both enzymes require multiple basic residues positioned amino terminal to the phosphorylation site and both prefer serine residues as phosphate acceptors. According to Kennelly and Krebs (1991) the core consensus sequence most frequently recognised by PKA is Arg-Arg/Lys-X-Ser; for PKG, it is $(Arg/Lys)_{2-3}$-X-Ser. Although little different, a basic residue immediately C-terminal and possibly a proline residue immediately N-terminal to the phosphorylation site appear to be positive determinants for PKG, whereas these residues are neutral or negative determinants for PKA.

Hence, due to their implication in signal transduction and cell cycle control, both of these previously characterized cyclic-nucleotide

dependent protein kinases were tested as potential kinases of human topoisomerase IIα protein.

At this point, preliminary studies were also initiated into the regulation by phosphorylation of the human topoisomerase IIβ protein, due to the availability of β-specific immunoprecipitating antibodies. There are no reports so far in the literature relating to the post-translational modification of this isozyme. It is highly likely that the pattern of regulation is going to be significantly different from that of the human α isozyme because of its different expression pattern and its low degree of homology in the proposed regulatory C-terminal domain.

6.2 Results.

6.2.1 Human Topoisomerase IIα Protein is Phosphorylated by Protein Kinase A, but not by Protein Kinase G.

Phosphorylation of casein was used to demonstrate activity of the catalytic subunit of PKA and PKi, an inhibitory peptide for PKA derived from the natural inibitory protein PKI, was shown to completely inhibit this phosphorylation at 5μM (Figure 36). A heptapeptide, RKRSRAE, was used to demonstrate the activity of PKG (Figure 36). Purified human topoisomerase IIα protein was then incubated with either the catalytic subunit of PKA or the α isozyme of PKG in the presence of [γ^{32}P]-ATP and the proteins separated by SDS-PAGE. Figure 37 shows that incubation of topoisomerase IIα with PKA resulted in specific phosphorylation of a 170kDa protein. This reaction could be inhibited by PKi but not by a non-specific peptide. In contrast, no phosphorylation of the topoisomerase IIα protein by PKG could be demonstrated.

6.2.2 Phosphopeptide Mapping demonstrates that Protein Kinase A Phosphorylates Human Topoisomerase IIα Protein *in vivo*.

Topoisomerase IIα protein was either metabolically labelled in cultured HeLa cells by growth in medium containing ^{32}P-orthophosphate or phosphorylated *in vitro* by PKA. The phosphorylated proteins were electroblotted from an SDS-polyacrylamide gel to Immobilon membrane and the position of the topoisomerase IIα protein identified by autoradiography. The proteins were eluted from the membrane, digested with trypsin and the resulting peptides separated by electrophoresis

(first dimension) and chromatography (second dimension). Digestion of the *in vivo* labelled topoisomerase IIα protein produced the complex pattern of phosphopeptides described in chapter 5. The topoisomerase IIα protein phosphorylated *in vitro* by PKA catalytic subunit also showed a complex array of phosphopeptides, five of which co-migrated with phosphopeptides generated by *in vivo* labelling (Figure 38).

As was seen for CK II, phosphorylation of topoisomerase IIα by PKA also generated some phosphopeptides *in vitro* which were not seen *in vivo*. This is likely to be caused by tighter restrictions on the phosphorylation of topoisomerase IIα protein *in vivo* as a result of other post-translational modifications *in vivo* and multiple interactions with other proteins and DNA (see section 5.3).

6.2.3 Protein Kinase A may also Phosphorylate Human Topoisomerase IIα Protein within the C-terminal Region.

One dimensional mapping of phosphopeptides generated by partial V8 protease cleavage (according to the method of Cleveland *et al.*, 1977) revealed a pattern that was markedly different following PKA phosphorylation from that generated by CK II or the heparin-sensitive co-purifying kinase (Figure 39). Although the phosphopeptides are not that well resolved, it is possible that the stongly phosphorylated peptide at around 20-21kDa co-migrates with the phosphopeptide labelled 'b' in Figure 24 (chapter 5), which was suggested to come from the extreme C-terminus of topoisomerase IIα. Clearly there are many other phosphopeptides which may represent other regions within the topoisomerase IIα protein.

A peptide was synthesized based on the sequence of one very good potential PKA phosphorylation site from within the C-terminal 20kDa. The sequence of the 14mer peptide was AKTKNRRKRKPSTS and comes from residues 1457-1470 of the human topoisomerase IIα protein. The peptide was tested in an *in vitro* kinase assay with both PKA and CK II. PKA, but not CK II, could efficiently phosphorylate this peptide (Figure 40).

6.2.4 Phosphorylation by Protein Kinase A Stimulates Topoisomerase IIα Activity *in vitro*.

The effect of phosphorylation by PKA on the activity of the topoisomerase IIα protein was compared with that of untreated topoisomerase IIα protein in both plasmid relaxation (Figure 41) and decatenation (Figure 43) assays. For both assays, purified topoisomerase IIα protein was pre-incubated at 30°C for 10 minutes with kinase buffer alone or 10 units of PKA catalytic subunit. Supercoiled plasmid or kDNA reaction mix was then added and aliquots taken out and stopped at given time intervals, before running on a 1% agarose gel. Decatenation assays were also carried out with purified topoisomerase IIα protein that had been pre-incubated with PKA together with the inhibitor peptide, PKi, and also with CK II together with PKi for comparison.

Figure 42 shows that the PKA-phosphorylated topoisomerase IIα protein had a 1.5- to 2-fold increased initial rate of plasmid relaxation activity compared to untreated topoisomerase IIα. The initial rate of decatenation of PKA-phosphorylated topoisomerase IIα protein was similarly enhanced (Figure 44). Pre-incubation with PKA and the inhibitor, PKi, blocked the increase in the initial rate of reaction due to the PKA phosphorylation, increasing the evidence that activation was indeed due to PKA phosphorylation and not some contaminant. The CK II phosphorylated topoisomerase IIα protein had an even greater initial rate of reaction, as expected from the greater degree of activation demonstrated in chapter 5. This was not apparently blocked by the PKi peptide.

6.2.5 Protein Kinase A Cannot Reactivate Dephosphorylated Topoisomerase IIα.

Dephosphorylation of the purified topoisomerase IIα protein with potato acid phosphatase resulted in a marked reduction in the rate of decatenation catalysed by this enzyme (Figure 32, chapter 5). To assess whether the dephosphorylated topoisomerase IIα protein could be re-activated by PKA, as well as by CK II (see chapter 5), the dephosphorylated enzyme was rephosphorylated *in vitro* by PKA and the decatenation activity of the modified enzyme assessed. The result (Figures 34 and 35, chapter 5) shows that while CK II could reactivate the dephosphorylated topoisomerase IIα protein to an extent that it was

more active than the untreated, purified topoisomerase IIα protein, phosphorylation by PKA produced no significant reactivation of the enzyme.

6.2.6 Co-operation Between CK II and PKA Phosphorylations.

In order to determine whether the effects on topoisomerase IIα activity of CK II and PKA phosphorylation are additive or not, decatenation activity was compared following phosphorylation with each kinase alone or in combination. The purified human topoisomerase IIα protein was pre-incubated at 30°C for 10 minutes alone, with purified CK II, the catalytic subunit of PKA , or CK II and PKA together (Figure 45). Figure 46 shows that CK II and PKA phosphorylation stimulate the activity by approximately 5-and 2-fold respectively. However, in combination there is an approximate 10-fold stimulation of activity.

6.2.7 Human Topoisomerase IIβ is a Phosphoprotein *in vivo*.

Nuclear proteins were prepared from exponentially growing HeLa cells in the presence of ^{32}P-orthophosphate or ^{35}S-methionine. Labelled topoisomerase IIβ proteinwas immunoprecipitated using the β-specific monoclonal antibody, 8D7, and the precipitate processed by SDS-PAGE and autoradiography. A ^{32}P-labelled protein of molecular weight 150kDa immunoprecipitated with this antibody co-migrated with a protein band labelled with ^{35}S-methionine. (Figure 47). The earlier results showing that under these preparation conditions topoisomerase IIβ is isolated as a stable 150kDa fragment (chapter 3), indicates that the phosphorylated band is indeed topoisomerase IIβ. This also shows that the 30kDa fragment cleaved off either *in vivo*, or during preparation cannot contain all the potential phosphorylation sites within the topoisomerase IIβ protein.

6.2.8 Protein Kinase A and Casein Kinase II Phosphorylate the Stable 150kDa Fragment of Human Topoisomerase IIβ *in vitro*.

The stable 150kDa fragment of human topoisomerase IIβ that can be immunoprecipitated by the β-specific monoclonal antibody, 8D7, was tested as a substrate for three of the kinases shown to phosphorylate human topoisomerase IIα isozyme *in vitro*. Following crude nuclear

extraction from HeLa cells and immunoprecipitation with either no antibody or 8D7, the immunoprecipitates were added to standard kinase reactions with CK II, the catalytic subunit of PKA or PKC-β1 (Figure 48). Results showed that a 150kDa protein was phosphorylated by the catalytic subunit of PKA and, to a lesser extent, CK II, following immunoprecipitation with 8D7. This band was not present in immunoprecipitates carried out with no antibody or in PKC-β1 immunoprecipitates.

It is possible that major sites of phosphorylation are on the 30kDa fragment that has been cleaved off, particularly as the N- and C-terminal appear to contain the majority of protein kinase consensus target sites in both topoisomerase IIα and β. However, it appears that the 150kDa fragment of topoisomerase IIβ is a substrate for PKA and CK II *in vitro*.

6.3 Discussion.

These experiments demonstrate that the human topoisomerase IIα isozyme is a substrate for the catalytic subunit of PKA, as well as CK II, confirming that it can act as a substrate for more than one kinase *in vitro*. Furthermore, comparison of two-dimensional tryptic phosphopeptide maps of *in vivo* phosphorylated topoisomerase IIα with PKA-phosphorylated topoisomerase IIα *in vitro* suggest that PKA phosphorylates human topoisomerase IIα protein *in vivo*. Most of the phosphopeptides generated from *in vivo* labelled topoisomerase IIα, which were not accounted for by CK II phosphorylation, co-migrated with phosphopeptides generated by PKA phosphorylation. Whilst this is strong evidence for the involvement of PKA phosphorylation *in vivo*, further mapping experiments need to be carried out on *in vivo* labelled cells following stimulation with PKA agonists, such as forskolin or 8-Br-cAMP. These agents, which elevate the level of cAMP in cells and hence stimulate PKA *in vivo*, should increase the proportion of phosphorylation of the phosphopeptides thought to be generated by PKA phosphorylation.

The best consensus for a PKA recognition site is RR/KXS as described in section 6.1. However, RR/KXT and RXS/T are also recognised by PKA (Kennelly and Krebs, 1991). Table 6 shows all the sequences within the human topoisomerase IIα protein which match these consensus sequences. Two of these sequences fall in the first 120 residues of the protein where there are no good CK II consensus sequences. Shiozaki and Yanagida (1991) suggested that the N-terminus

of yeast topoisomerase II was a target for phosphorylation as well as the C-terminus. Within the C-terminal 20kDa of the human topoisomerase IIα protein there at least three matches, including one which has a very highly basic stretch just upstream of the potential serine acceptor residue. This latter site was incorporated into a synthetic peptide and shown to be very efficiently phosphorylated by PKA, demonstrating its potential as a PKA phosphorylation site.

PKA phosphorylation, like CK II phosphorylation, led to an increase in both the decatenation and plasmid relaxation activity of the enzyme. The degree of activation, which was similar for both assays, was considerably less than for CK II and of the order of 1.5- to 2-fold. However, this was a highly reproducible increase in activity and it was possible that a proportion of the purified topoisomerase IIα protein was already phosphorylated on its PKA sites, hence masking a greater activation change.

Therefore, the effect on decatenation activity of rephosphorylating the dephosphorylated topoisomerase IIα isozyme with PKA was also measured. Unlike CK II, the catalytic subunit of PKA did not appear to be capable of restoring the activity to the dephosphorylated protein. This could be explained in various ways. Firstly, that PAP does not dephosphorylate the sites phosphorylated by PKA and hence there is no scope for rephosphorylation. Secondly, that PKA phosphorylation of the protein requires the prior phosphorylation by another kinase such as CK II. Hence, alone PKA could not reactivate the enzyme as it couldn't phosphorylate the dephosphorylated protein. Thirdly, that phosphorylation by PKA does not require prior phosphorylation by CK II, whereas the ability to activate the enzyme does. In other words, modulation of activity by PKA phosphorylation requires certain other phosphorylations to already be in place.

The second possibility mentioned was that PKA phosphorylation required prior phosphorylation by CK II. This pattern of phosphorylation, where one kinase can only act after another, has been termed heirarchical phosphorylation and is discussed in more detail in section 8.1. As PKA recognises phosphoacceptor residues in a basic environment, it is not immediately obvious how the addition of a negatively charged phosphate by CK II phosphorylation would lead to a stronger recognition site for PKA. Clearly though the structure of phosphorylation sites is more complex than this and one example has already been described of CK II phosphorylation stimulating PKA phosphorylation (Girault, 1989). DARPP-

32 is phosphorylated by CK II on serine residues which are 8 and 11 amino-acids upstream of the stimulated PKA phosphorylation site. Interestingly, the best fit PKA site in topoisomerase IIα protein is at residue 1468 in the C-terminal region and this has been shown to be strongly phosphorylated by PKA when synthesized as a peptide. Three out of the next seven residues downstream of 1468 are all in potential CK II recognition sites (see Table 5, chapter 5). Further evidence that PKA and CK II could be phosphorylating sites which are close together come from the tryptic phosphopeptide maps where one phosphopeptide generated *in vivo* comigrates with phosphopeptides present when purified topoisomerase IIα protein was phosphorylated *in vitro* by both CK II and PKA (labelled phosphopeptide 5 in Figures 23 and 38).

The demonstration that CK II and PKA in combination led to additional stimulation of the topoisomerase IIα activity compared to either kinase alone supports the hypothesis that the activation is sequential. In other words, whilst PKA could not reactivate the dephosphorylated topoisomerase IIα protein, it could activate the protein in the presence of CK II. To confirm this model of sequential activation, the activity of topoisomerase IIα protein will have to be measured following the ordered addition of each kinase following complete dephosphorylation of the protein.

Although several studies have implicated CK II in the regulation of activity of topoisomerase II proteins from lower eukaryotes, no role for PKA has been previously identified. Indeed, it was reported that PKA failed to modify topoisomerase II protein from Drosophila (Sayhoun *et al.*, 1986). This suggests that there are significant differences between lower eukaryotes (in which only one topoisomerase II is expressed) and human cells in the pathways for post-translational modification of topoisomerase II proteins. This means that non-mammalian species, such as yeast or *Drosophila*, may not be good models for a study of all aspects of the role of topoisomerase II phosphorylation in human cells.

It is possible that some of the phosphorylation sites for CK II, which are crucial for overall enzyme activity, are conserved between yeast and human enzymes (and perhaps in both α and β isozymes), whereas the human isozymes have evolved additional phosphorylation sites for other kinases due to the more specialized functions of each isozyme in these higher eukaryotes.

The observation that the β isozyme of human topoisomerase II is also a phosphoprotein *in vivo* suggests that there are indeed likely to be

some aspects of regulation by phosphorylation that are conserved in all eukaryotic type II topoisomerases. Although *in vitro* phosphorylation was only carried out on the stable 150kDa fragment of topoisomerase IIβ immunoprecipitated from human cells, it was clearly a substrate for both CK II and the catalytic subunit of PKA. It was not a substrate for PKC β1. It will be vital to identify whether the 30kDa region missing from this degraded form of topoisomerase IIβ comes from the N- or C-terminus of the protein. Both of these regions contain good potential phosphorylation sites and hence kinases which do not phosphorylate the 150kDa fragment may still phosphorylate the whole protein. Mapping of the epitopes of the topoisomerase IIβ-specific monoclonal antibodies may allow the identification of which terminus is cleaved off.

Likewise, it will be difficult to carry out measurements of the effect on activity of phosphorylation of this degraded form of topoisomerase IIβ as allosteric conformational changes resulting from phosphorylation may be altered in the 150kDa fragment compared to in the whole enzyme. It will be important to find a cell source or isolation conditions which allow the full-length human topoisomerase IIβ protein to be purified, perhaps by immunoaffinity chromatography. Chung *et al.* (1989) demonstrated the isolation of a 180kDa protein from U937 cells suggesting that this was full-length topoisomerase IIβ protein. However, a 145kDa cross-reactive polypeptide was also recognised by one of two antibodies they had raised to topoisomerase IIβ-specific peptides. It is possible then that from this cell line as well, topoisomerase IIβ was isolated as a partially degraded product.

Previous data from other groups suggest that topoisomerase IIβ protein in human cells seems to be far less responsive to changes in the cell cycle position and proliferation state than topoisomerase IIα. Hence, it is particularly interesting to speculate as to the role of phosphorylation of this isozyme. Perhaps CK II phosphorylation is essential for basal activity of topoisomerase IIβ, as it seems may be necessary for topoisomerase IIα. Phosphorylation may also play a role in the specific localization of the human topoisomerase IIβ protein to the nucleolus. The identification of which kinases phosphorylate topoisomerase IIβ *in vivo* by phosphopeptide mapping may begin to answer these questions.

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Residue Numbers	Sequence
24-33	AKKRL**S**VERI
102-112	MSCIRV**T**IDPE
446-455	AGGRN**S**TECT
1156-1165	TLKRK**S**PSDL
1218-1225	VIPRI**T**ID
1294-1305	PDSESDRS**S**DES
1311-1318	PRE**T**EPRR
1461-1470	NRRKRKP**S**TS

Table 6 Putative PKA sites in the human topoisomerase IIα protein. All sequences match the motifs RXS/T, RRXS/T or RKXS/T. The one letter amino acid code is used, and the residue numbers refer to the position in the human topoisomerase IIα protein sequence (see Figure 3, chapter 1). The human protein is 1530 residues in length. Potential serine or threonine acceptor sites are in bold-faced type.

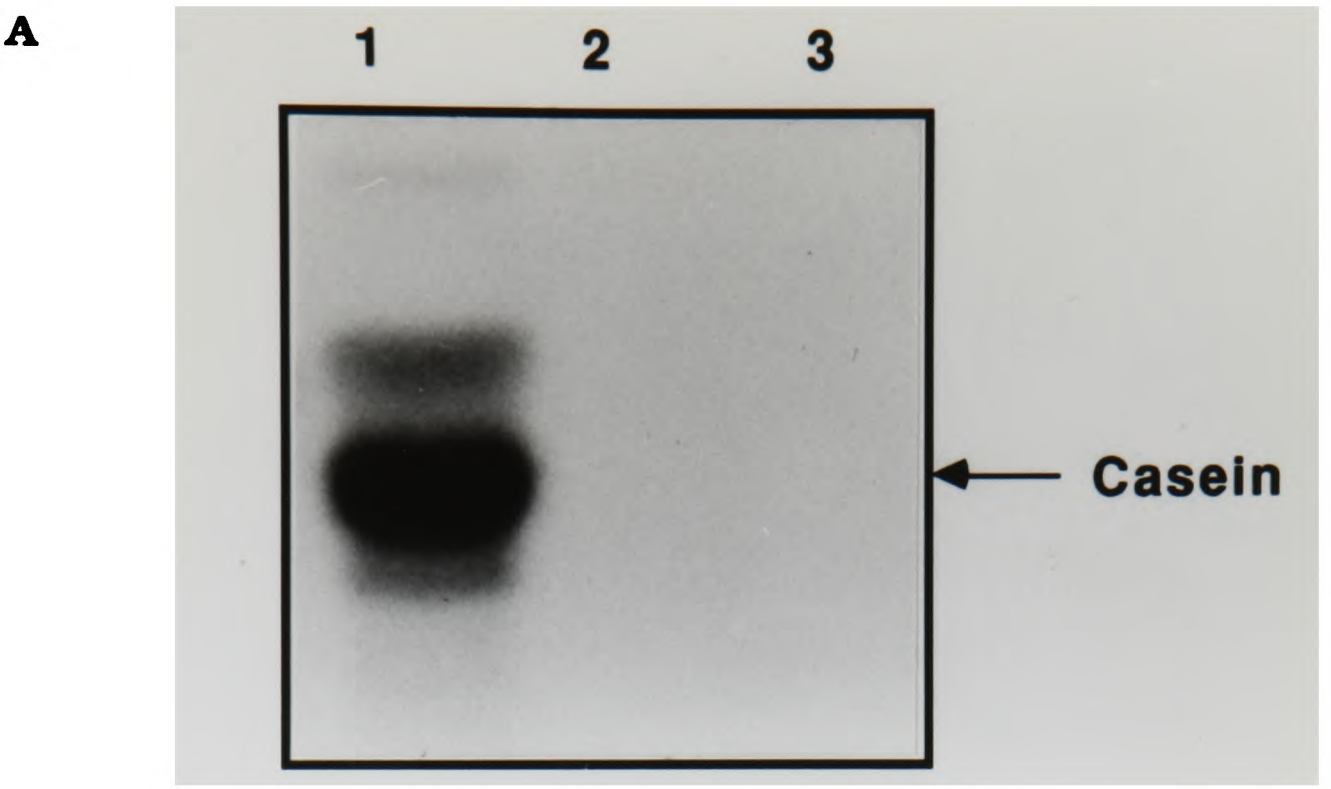


Figure 36 Demonstration of activity of PKA and PKG and the inhibitory effect of PKi on PKA phosphorylation. (A) Casein was incubated with [γ^{32}P]-ATP alone (lane 3) or in the presence of the catalytic subunit of PKA, with (lane 2) and without (lane 1) the inhibitory peptide, PKi. (B) A heptapeptide substrate was incubated with [γ^{32}P]-ATP alone (lane 1), with the catalytic subunit of PKA (lane 2), or with PKG (lane 3). PKG was also incubated alone with [γ^{32}P]-ATP (lane 4). All samples were incubated at 30°C for 10 minutes and then separated on either 15% (panel A) or 22% (panel B) SDS-PAGE. Gels were dried and autoradiographed.

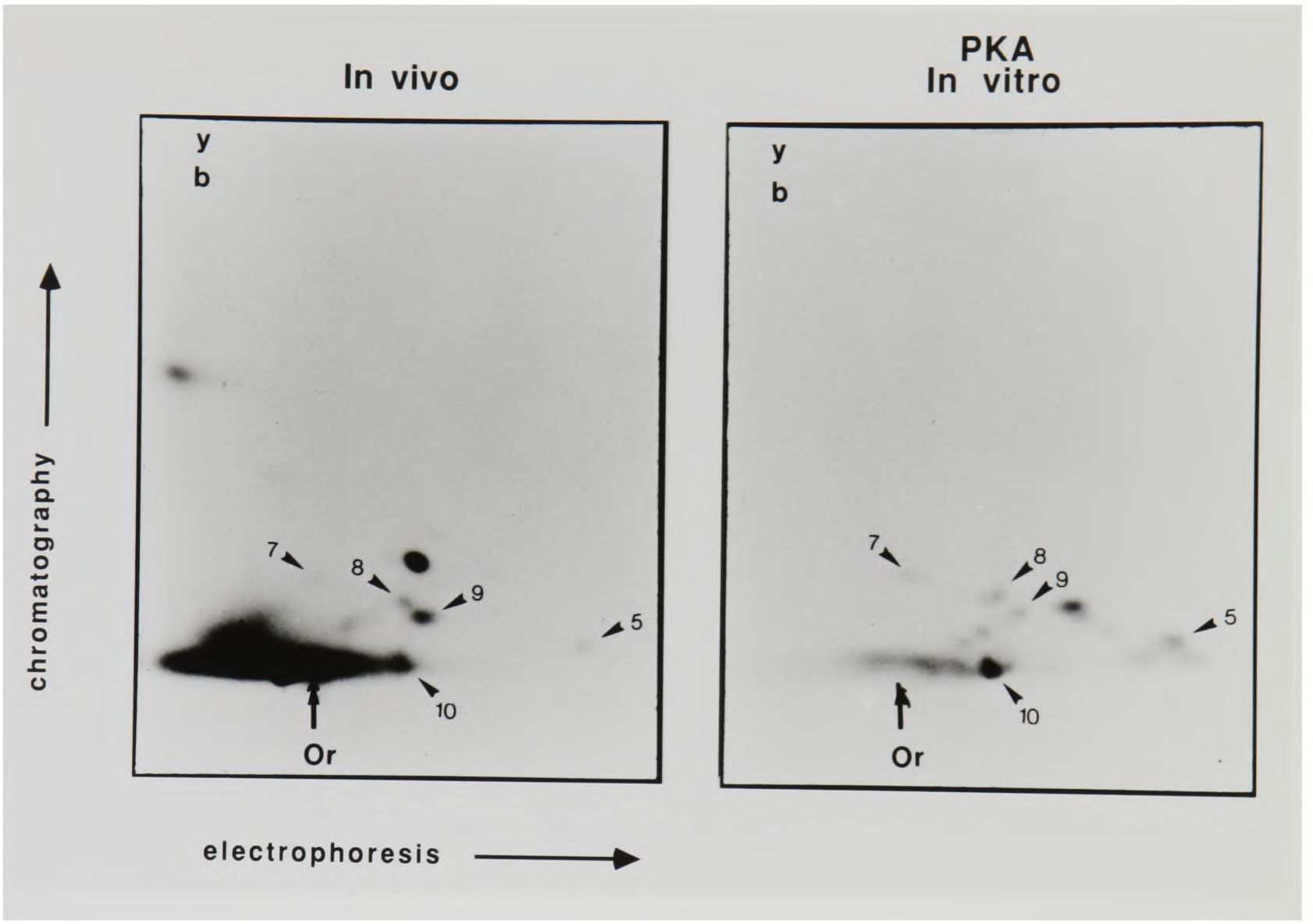


Figure 38 Two-dimensional tryptic phosphopeptide mapping of topoisomerase IIα protein phosphorylated *in vivo* or by PKA *in vitro*. Peptides were separated by high voltage electrophoresis followed by chromatography. Phosphopeptides were visualized by autoradiography. The positions of the origin (Or) and the two chromatographic markers, DNP-lysine (y) and xylene cyanol (b), are indicated. The numbered arrows indicate phosphopeptides present in the *in vivo* labelled sample which co-migrated with peptides phosphorylated *in vitro* by PKA. (See comparison with CK II phosphopeptide map for Nos. 1-4 and 6).

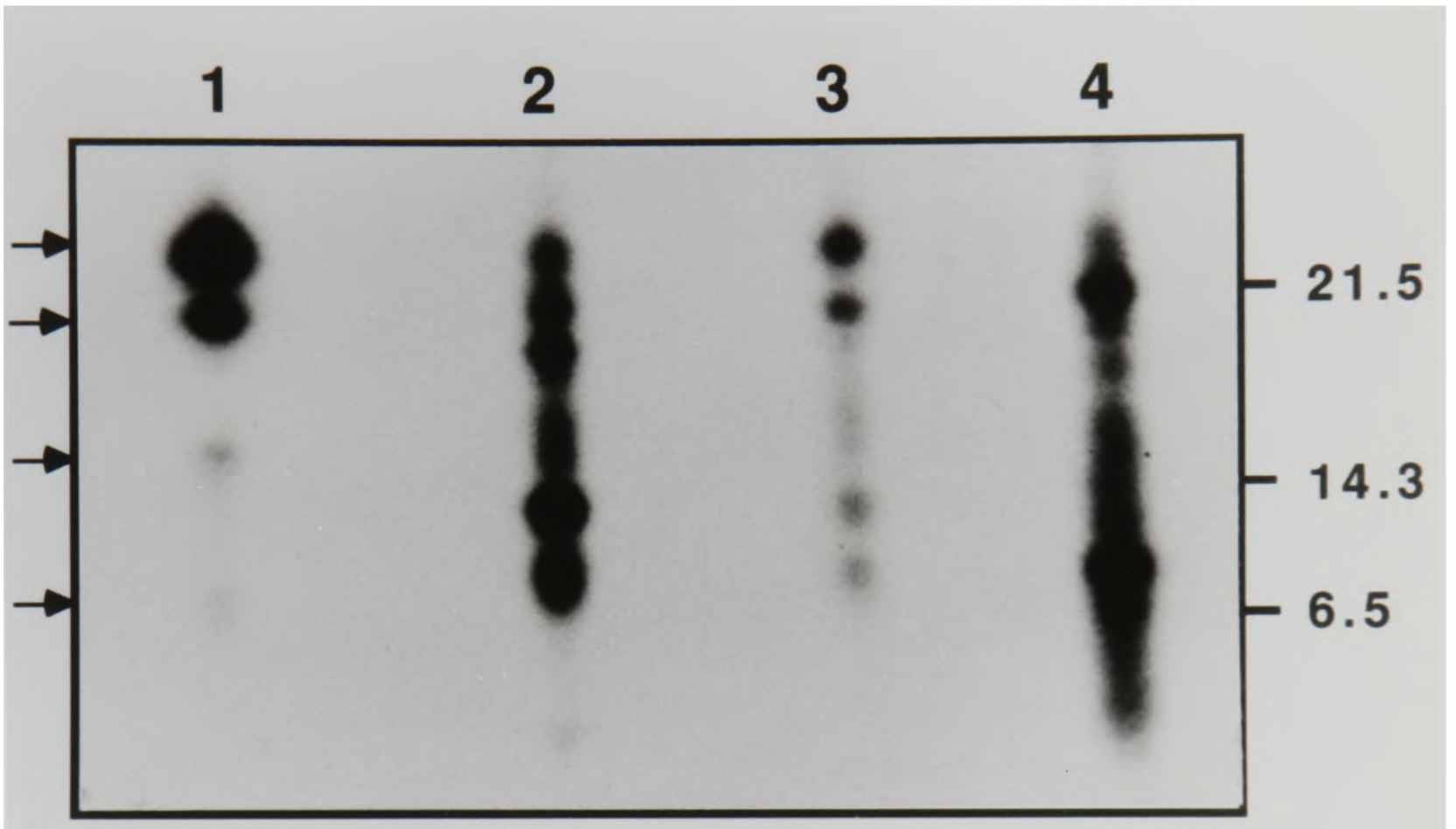


Figure 39 One-dimensional V8 phosphopeptide maps of topoisomerase IIα protein phosphorylated *in vivo* and by PKA *in vitro*. Topoisomerase IIα protein was labelled *in vivo* with ^{32}P-orthophosphate (lane 1), or phosphorylated *in vitro* with [γ^{32}P]-ATP in the presence of purified CK II (lane 2), the co-purifying kinase (lane 3), or purifed PKA catalytic subunit (lane 4). Samples were processed for one-dimensional phosphopeptide mapping as described in section 2.12. The position of molecular weight standards (kDa) are shown on the right and the four phosphopeptides present in the *in vivo* sample (lane 1) are indicated with arrows.

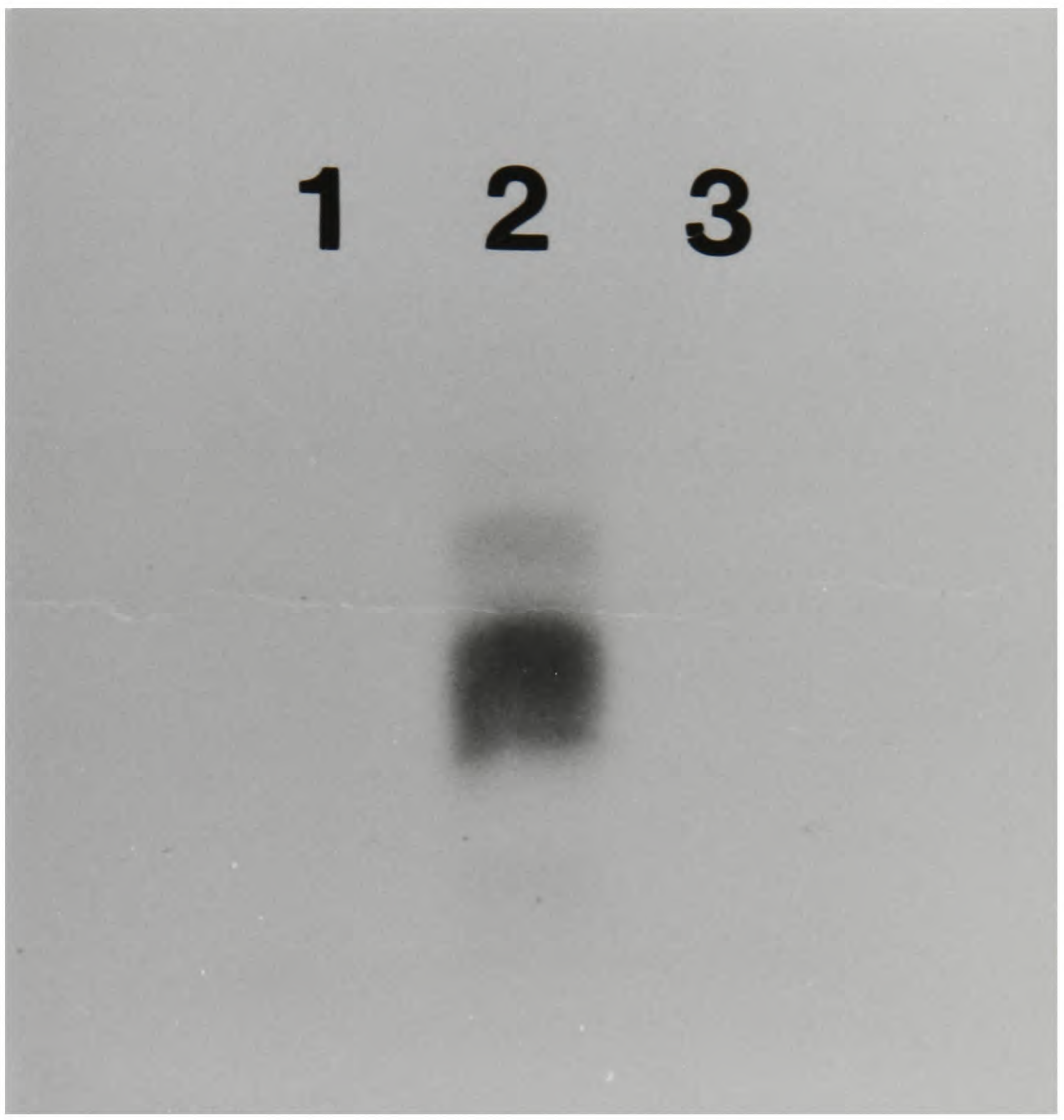


Figure 40 Phosphorylation of a peptide based on a region within the C-terminal domain of human topoisomerase IIα protein by PKA. The synthetic peptide was incubated with [γ^{32}P]-ATP in the absence (lane 1) or presence (lanes 2 and 3) of the PKA catalytic subunit. 5μM of the inhibitor PKi was included in the reaction shown in lane 3. After incubation at 30°C for 10 minutes, samples were electrophoresed on a 22% SDS-polyacrylamide gel and the gel fixed and autoradiographed.

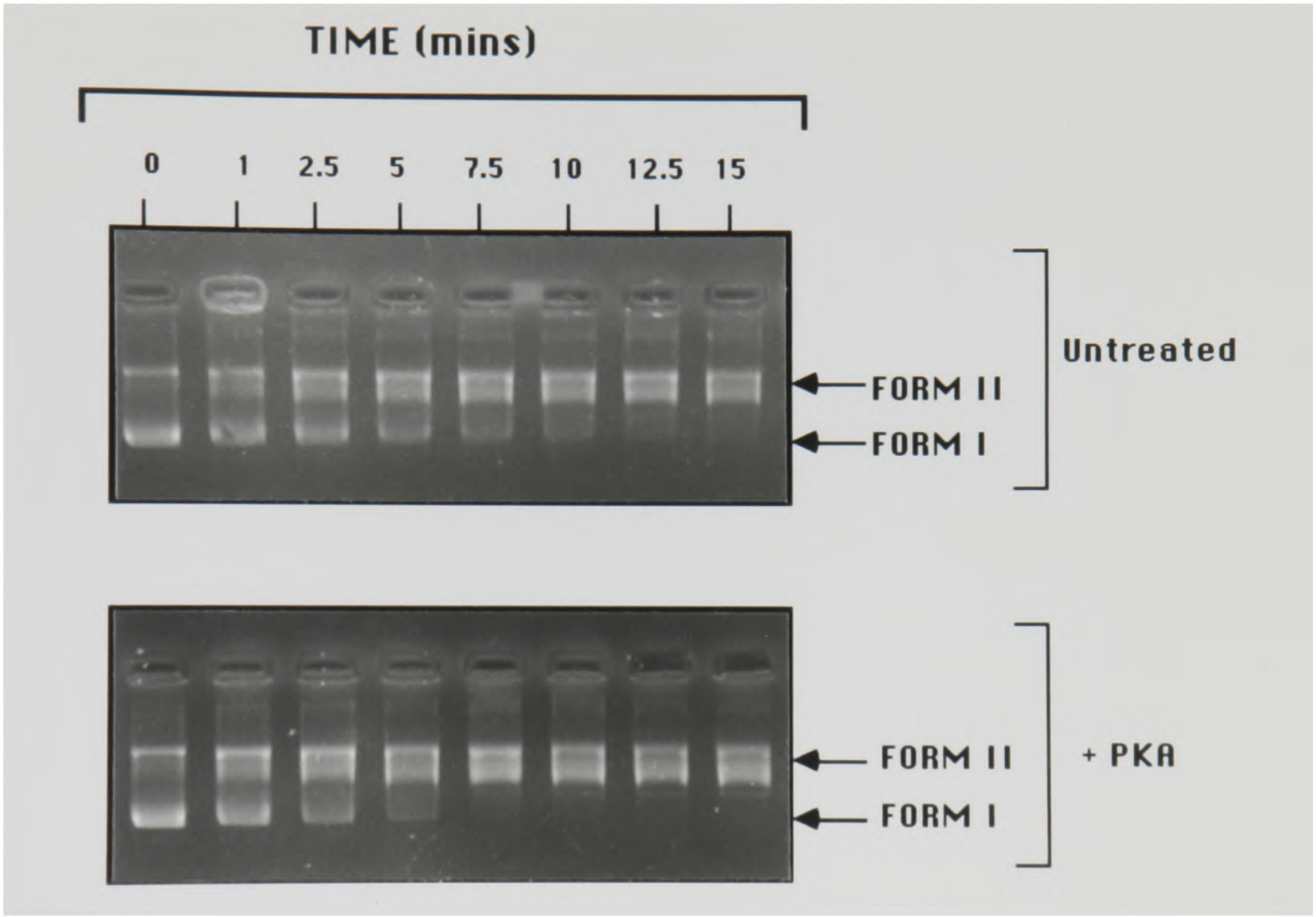


Figure 41 Relaxation activity of untreated and PKA phosphorylated human topoisomerase IIα protein. The relaxation activity was measured as the rate of relaxation of a supercoiled plasmid substrate from Form I (supercoiled) DNA to Form II (fully relaxed) DNA with time. Before carrying out the relaxation reaction the topoisomerase IIα protein was incubated at 30°C for 10 minutes in the absence (top panel) or presence (bottom panel) of the catalytic subunit of PKA. Samples were taken and stopped at time T=0, 1, 2.5, 5, 7.5, 12.5 and 15 minutes and electrophoresed on a 1% agarose gel. The gel was stained with ethidium bromide and photographed under UV-illumination.

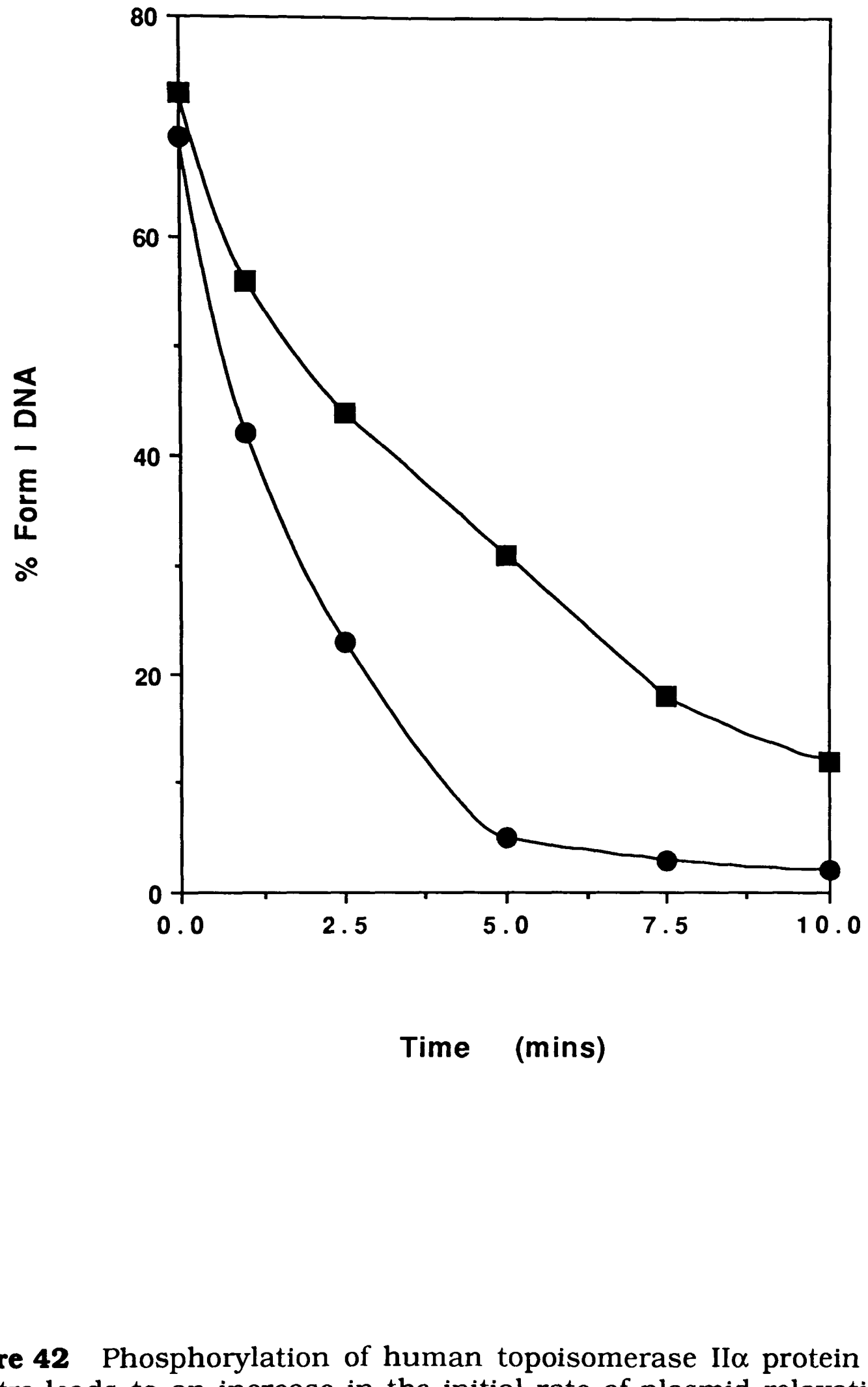


Figure 42 Phosphorylation of human topoisomerase IIα protein by PKA *in vitro* leads to an increase in the initial rate of plasmid relaxation. The photograph shown in Figure 41 was scanned by densitometry and the % of Form I (supercoiled) DNA plotted against time. Activity is shown in the absence (■) or presence (●) of the catalytic subunit of PKA.

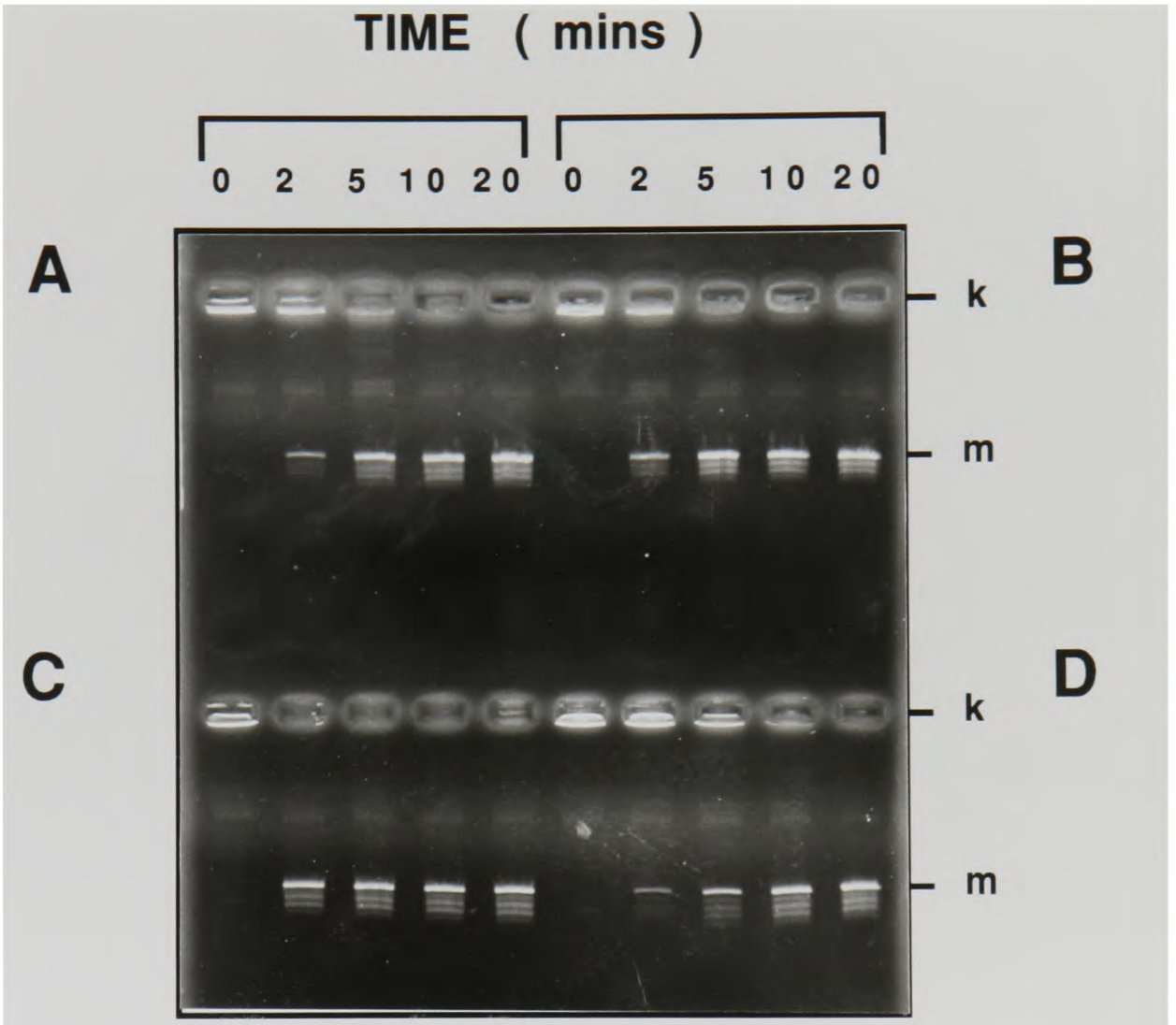


Figure 43 Changes in the decatenation activity of human topoisomerase IIα protein following phosphorylation by PKA or CK II. The decatenation activity was measured as the rate of release of minicircles (m) from kinetoplast DNA (k) with time. Before carrying out the relaxation reaction the topoisomerase IIα protein was incubated at 30°C for 10 minutes in the absence of kinase (A), or the presence of CK II (C) or the catalytic subunit of PKA (B and D). The inhibitory peptide for PKA, PKi, was included in both C and D. Samples were taken and stopped at time T=0, 2, 5, 10, and 20 minutes, and electrophoresed on a 1% agarose gel. The gel was stained with ethidium bromide and photographed under UV-illumination.

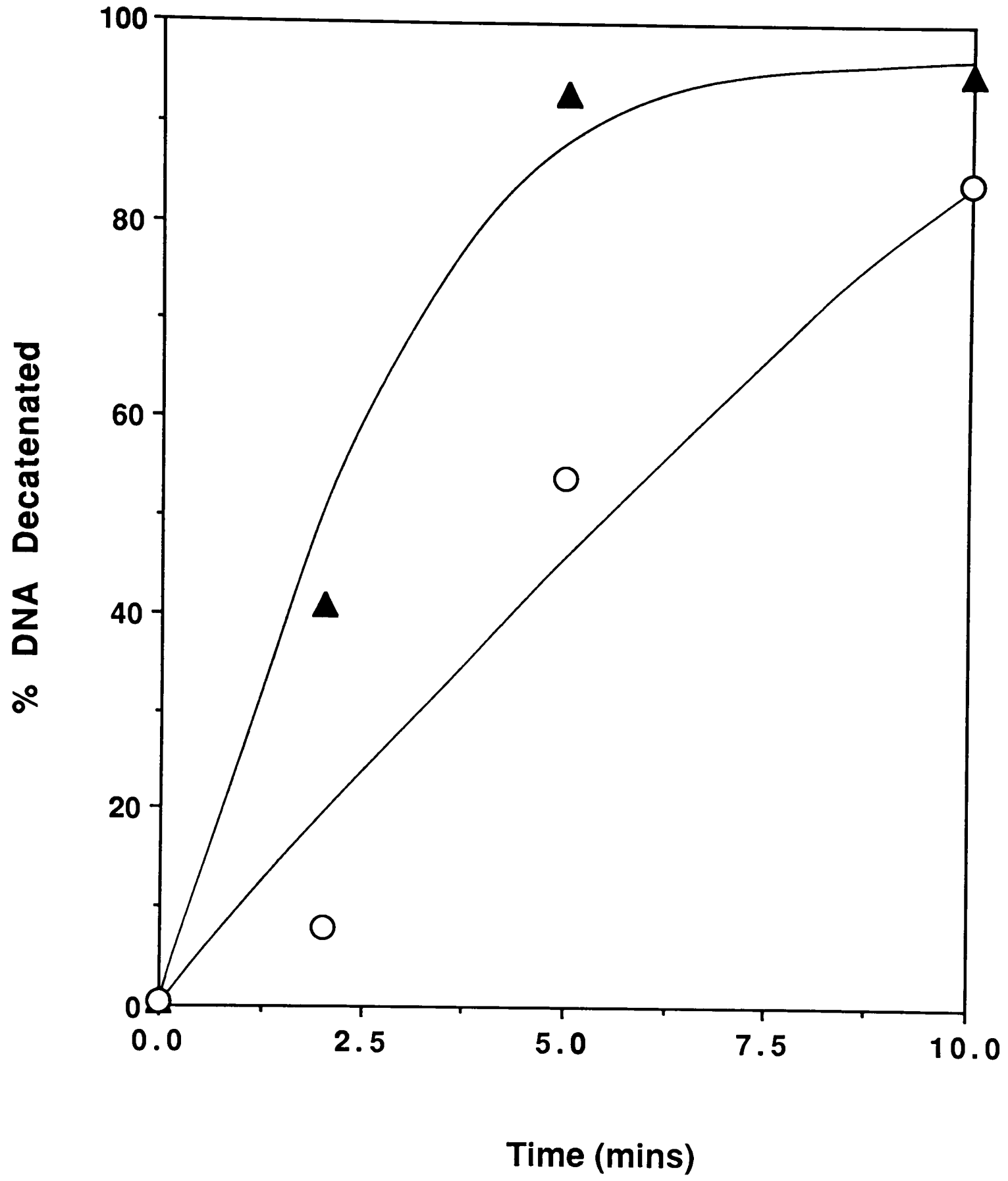


Figure 44 Phosphorylation by PKA enhances the initial rate of decatenation of human topoisomerase IIα protein by approximately 2-fold. The photograph shown in Figure 43 was scanned by densitometry and the % of DNA decatenated plotted against time. Activity is shown for topoisomerase IIα protein following phosphorylation by PKA in the absence (▲) or presence (○) of the inhibitory peptide, PKi.

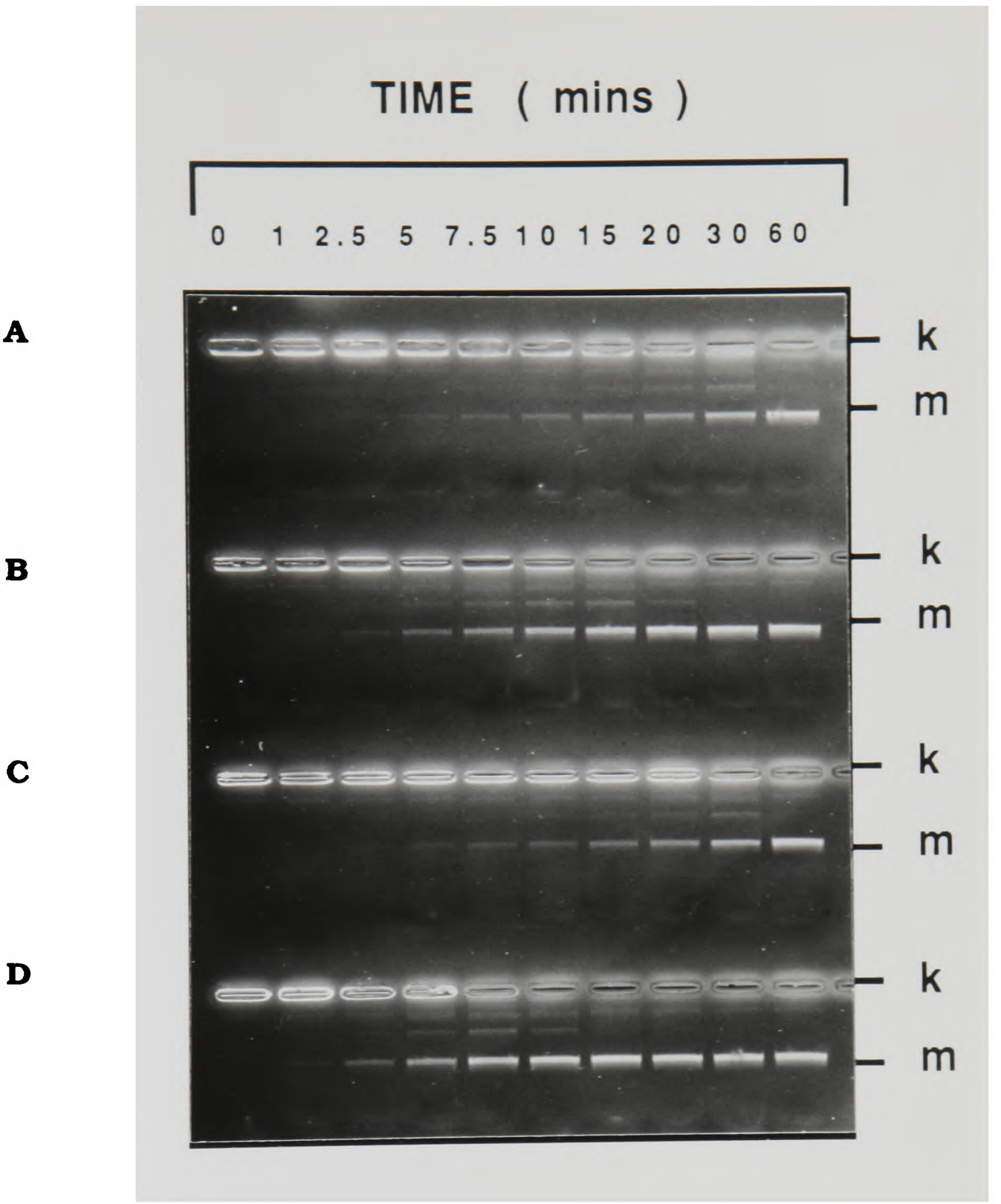


Figure 45 Changes in the decatenation activity of human topoisomerase IIα protein following phosphorylation by PKA and CK II separately and in combination. The decatenation activity was measured as the rate of release of minicircles (m) from kinetoplast DNA (k) with time. Before carrying out the relaxation reaction the topoisomerase IIα protein was incubated at 30°C for 10 minutes in the absence of kinase (A), or the presence of CK II (B), the catalytic subunit of PKA (C), or CK II and PKA together (D). Samples were taken and stopped at time T=0, 1, 2.5, 5, 7.5, 10, 15, 20, 30 and 60 minutes, and electrophoresed on a 1% agarose gel. The gel was stained with ethidium bromide and photographed under UV-illumination.

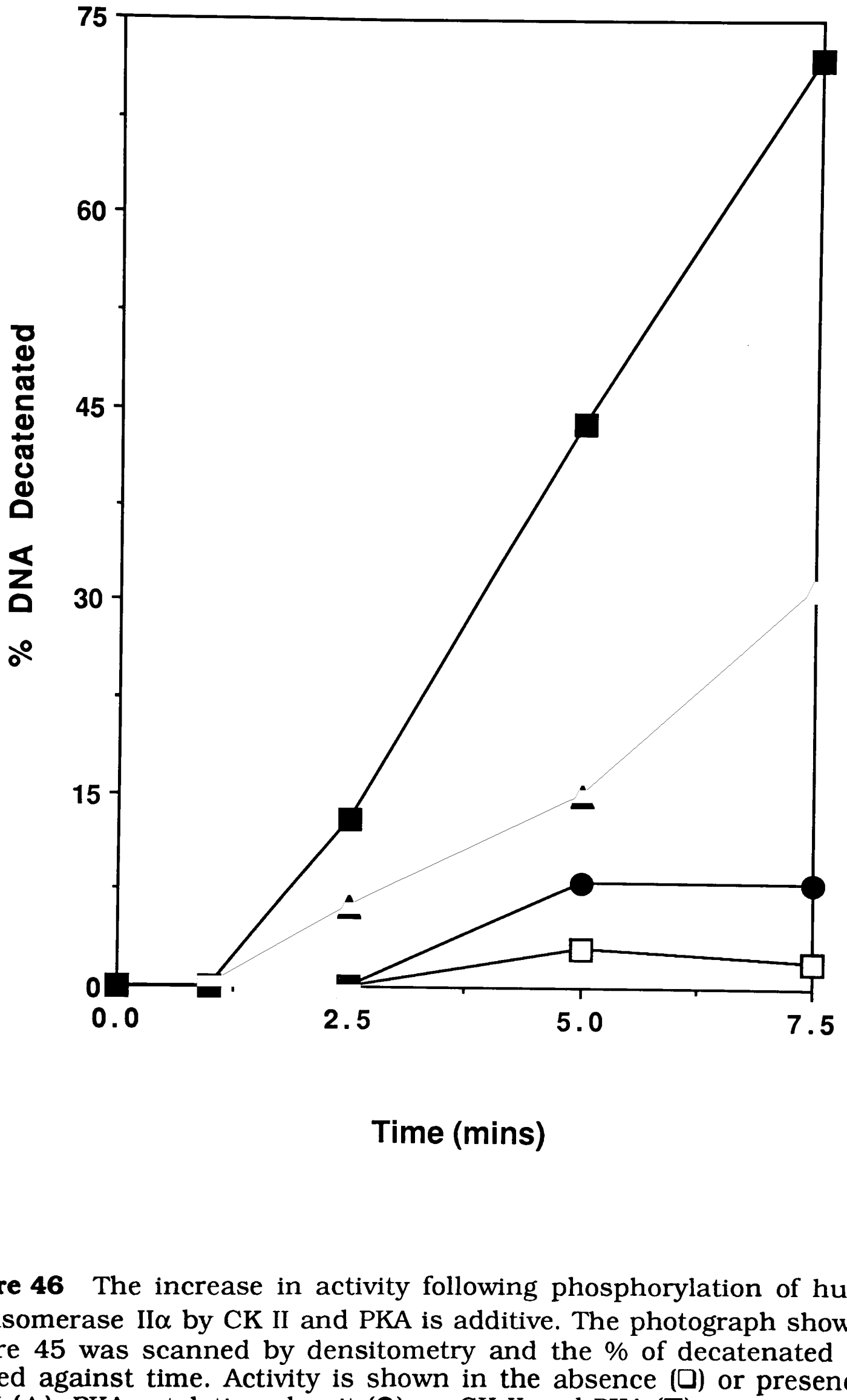


Figure 46 The increase in activity following phosphorylation of human topoisomerase IIα by CK II and PKA is additive. The photograph shown in Figure 45 was scanned by densitometry and the % of decatenated DNA plotted against time. Activity is shown in the absence (❑) or presence of CK II (▲), PKA catalytic subunit (●), or CK II and PKA (■).

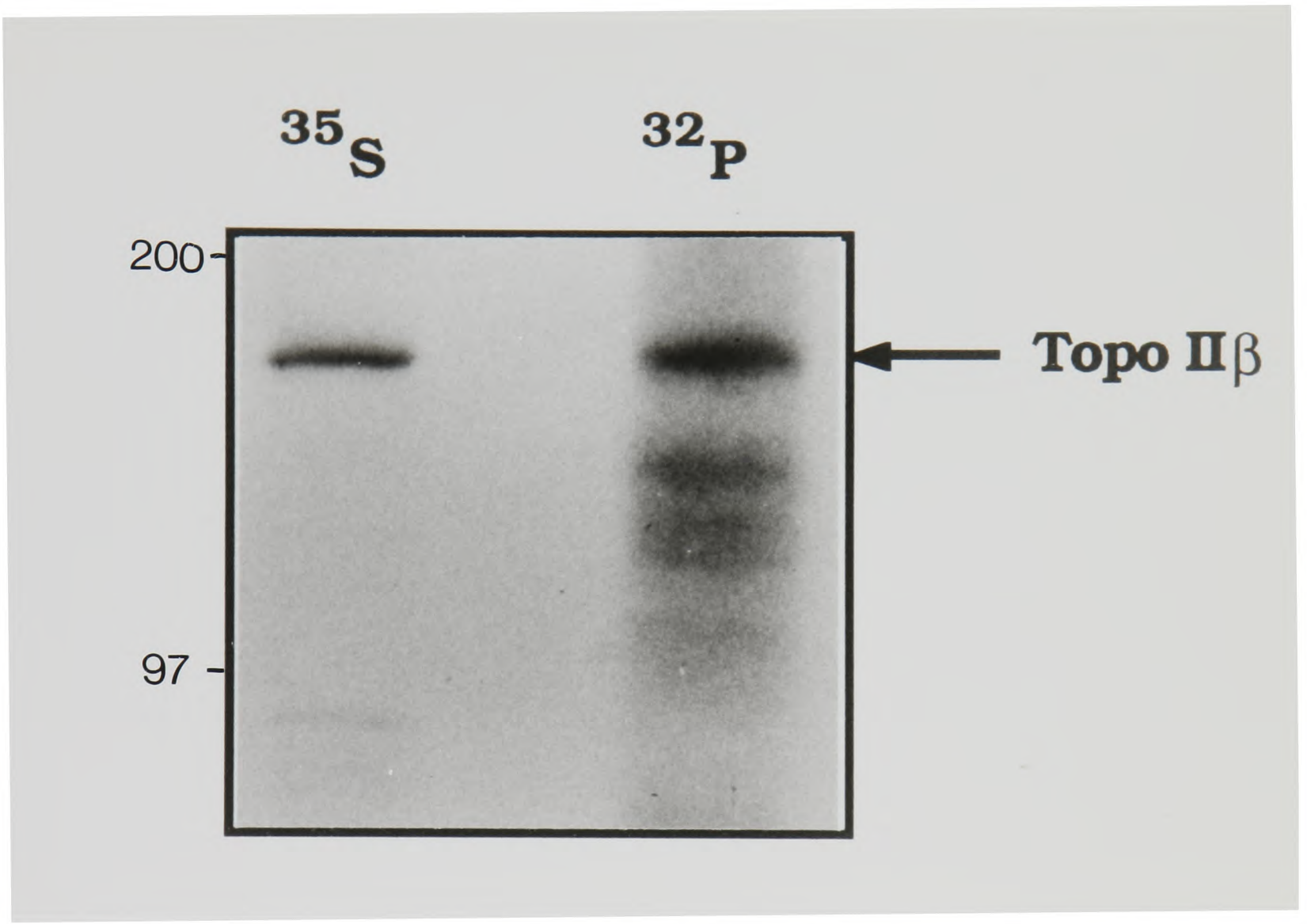


Figure 47 Topoisomerase IIβ is a phosphoprotein *in vivo*. The β isozyme of topoisomerase II was immunoprecipitated from nuclear extracts of HeLa cells metabolically labelled with either ^{35}S-methionine (left lane) or ^{32}P-orthophosphate (right lane). The position of the topoisomerase IIβ protein is indicated. Molecular weight standards (kDa) are shown on the left.

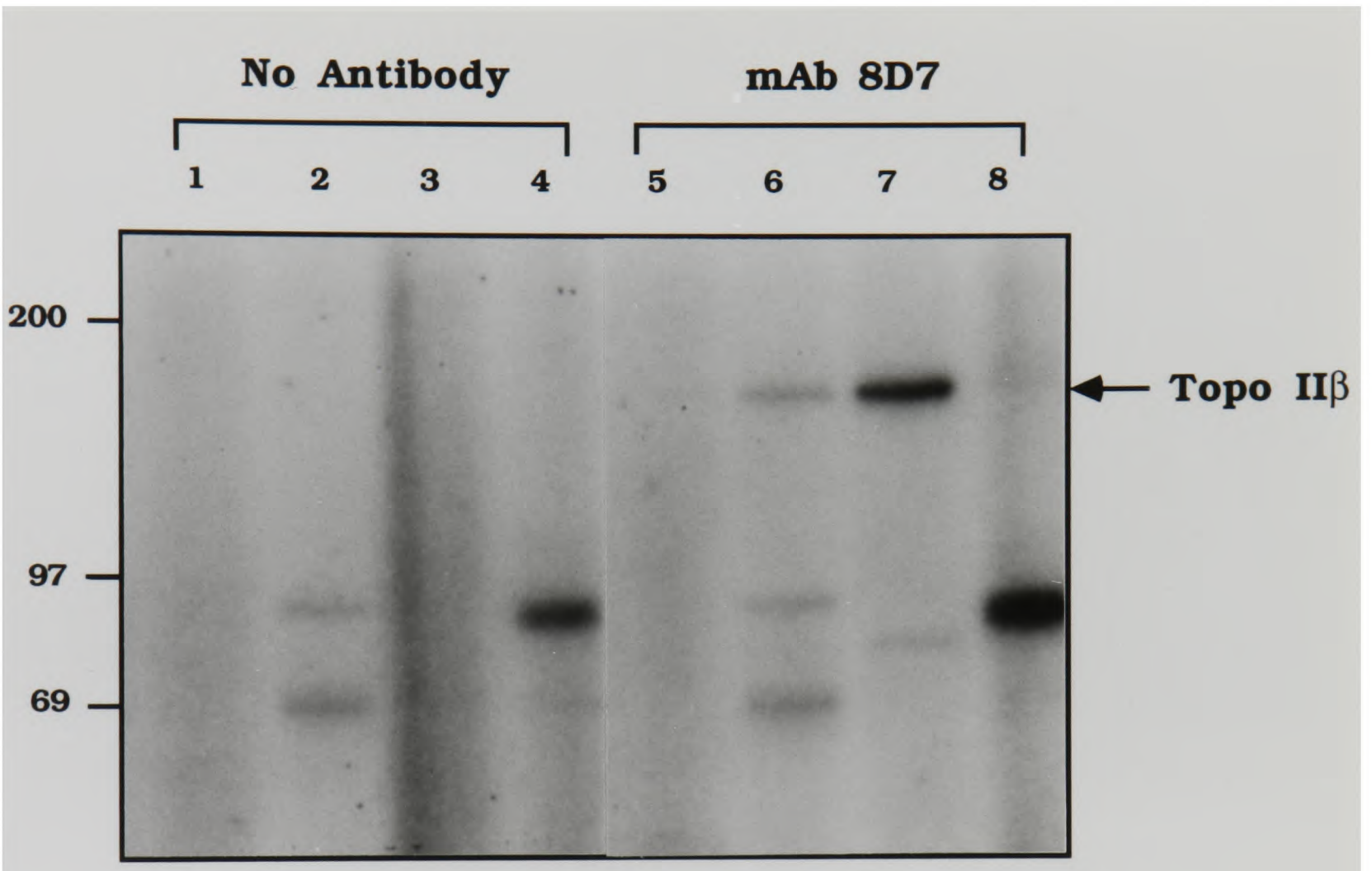


Figure 48 The 150kDa fragment of human topoisomerase IIβ is phosphorylated by CK II and PKA. Immunoprecipitates were prepared from HeLa cells either with no antibody (lanes 1-4) or the monoclonal antibody, 8D7 (lanes 5-8). The immunoprecipitates were incubated in the presence of [γ^{32}P]-ATP with no kinase (lanes 1 and 5), CK II (lanes 2 and 6), the catalytic subunit of PKA (lanes 3 and 7), and PKC-β1 (lanes 4 and 8). Following incubation at 30°C for 10 minutes, samples were separated by 7.5% SDS-PAGE and the gel dried and autoradiographed. The position of molecular weight standards (kDa) is shown on the left and the 150kDa fragment of human topoisomerase IIβ on the right.

Chapter 7 PHOSPHORYLATION OF HUMAN TOPOISOMERASE IIα PROTEIN BY PROTEIN KINASE C AND BY $p34^{cdc2}$ KINASE.

7.1 Introduction

The work described in chapters 5 and 6 of this thesis indicate that human topoisomerase IIα protein is phosphorylated *in vivo* by at least two protein kinases, casein kinase II and protein kinase A. These two kinases appear to account for most, but not all, the phosphopeptides seen in the two-dimensional map of *in vivo* phosphorylated protein, suggesting that topoisomerase IIα may be a physiological substrate for additional protein kinases. These kinases may play a more selective role in phosphorylating topoisomerase IIα protein, for example in response to a specific extracellular signal or at a particular point in the cell cycle.

Studies on phosphorylation of topoisomerase II from lower eukaryotes have identified at least three other kinases which can use this protein as a substrate *in vitro*. These are protein kinase C, calmodulin-dependent protein kinase II and $p34^{cdc2}$ kinase (Sahyoun *et al.*, 1986; Cardenas *et al.*, 1992). Moreover, protein kinase C was shown to phosphorylate topoisomerase II *in vivo* in the sponge, *Geodia cydonium* (Rottmann *et al.*, 1987) and, of these three kinases, only protein kinase C has been shown to affect the topoisomerase II activity *in vitro*, in this case leading to a modest activation (Rottmann *et al.*, 1987).

Two independent studies in vertebrates have demonstrated cell cycle dependent phosphorylation of topoisomerase II protein (Heck *et al.*, 1989; Saijo *et al.*, 1992). Both of these reports showed that maximal phosphorylation of topoisomerase II occurred at mitosis, although the kinase responsible was not identified. A good candidate would be the cell-cycle regulated kinase, $p34^{cdc2}$, which is involved in coordinating cell-cycle events and is maximally active at mitosis (Nurse, 1990). This kinase has so far only been shown to phosphorylate topoisomerase II *in vitro* in yeast and there was no evidence that this modification occurred *in vivo* (Cardenas *et al.*, 1992).

7.1.1 Protein Kinase C and Topoisomerase II.

Protein kinase C (PKC) is perhaps the most studied of the cellular kinases involved in signal transduction and tumourigenesis. It acts as an

internal target for a variety of growth factors, hormones, neurotransmitters and drugs. PKC is activated by diacylglycerol released following the receptor-mediated hydrolysis of inositol phospholipids by phospholipase C. Stimulation of PKC, in turn, elevates directly or indirectly the activity of nuclear proto-oncogenes such as *c-fos*, *c-myc* and *c-jun*. PKC is also implicated in the long-term stimulation of growth and differentiation. Moreover, PKC is a direct target of the phorbol esters, a potent class of tumour promoters. It is proposed that PKC itself can be oncogenic as, in appropriate cell types, overexpression leads to transformation. For general reviews on PKC activation and function see Nishizuka (1986 and 1988) and Blumberg (1991).

PKC has two functional domains, an N-terminal regulatory domain containing a pseudosubstrate site and a C-terminal kinase domain. PKC is activated by the displacement of the inhibitory pseudosubstrate region from the catalytic site. This is thought to occur *in vivo* as well as *in vitro*, either by proteolysis which cleaves off the regulatory domain or by the allosteric changes brought about by interaction with Ca^{2+}, anionic phospholipid and the inositol phospholipid breakdown product, *sn*-1,2-diacylglycerol. Phorbol esters mimic the action of diacylglycerol and are thought to lower the requirements for Ca^{2+} and phospholipid, thereby activating the enzyme under conditions of limiting cofactor.

Recently PKC has been shown to represent a large family of related isoforms, numbering at least 10 members at the last count. These appear to fall into two classes, those that are Ca^{2+}-dependent, including PKC-α, β1, β2 and γ, and those that are Ca^{2+}-independent, including PKC-δ, ε, λ, ζ, θ and η. Some members of the family exhibit distinct patterns of tissue expression and intracellular localization; for example, in the rat brain PKC-γ is most highly expressed in the cerebellum and hippocampus, whereas PKC-β is highest in the cerebral cortex (Shearman *et al.*, 1987; Saito *et al.*, 1988). Specific isozyme localization in different cell types or tissues can act as a determinant of isozyme function, by regulating accessibility to substrate. Also, as the different isoforms appear to be subtly different in their mode of activation, they could have distinct functions in the processing and modulation of different extracellular signals.

There is also pharmacological evidence for functional PKC heterogeneity. Not all phorbol esters are necessarily tumour promoting. Indeed, some appear to be antipromoting (Szallasi and Blumberg, 1991) and some appear to only act as second-stage promoters following

treatment with a first-stage promoter like PMA (Furstenburger *et al.*, 1981). Another class of PKC agonists, apart from the phorbol esters, are the bryostatins, macrocyclic lactones isolated from marine bryozoans and initially studied on the basis of their antitumour activity against P388 leukemic cells (Pettit *et al.*, 1970). *In vitro*, the bryostatins bind to and activate PKC. In cultured cells though, bryostatins only activate a subset of the responses induced by phorbol esters and paradoxically they block those responses that they do not induce (Gschwendt *et al.*, 1988). It is not known whether this reflects the action on different PKC isoforms.

Apart from the topoisomerases in DNA-containing organelles such as mitochondria, chloroplasts and kinetochores, topoisomerases are generally regarded as being restricted to the nucleus. Hence, the same arguments concerning the cellular localization of CK II and PKA also apply to PKC. If PKC is to directly phosphorylate human topoisomerase IIα protein, the two proteins must come into contact in some cellular compartment. There is still some debate as to whether topoisomerase II does in fact spend some time after synthesis in the cytoplasm and the immunostaining results described in chapter 3 also suggest that human topoisomerase IIα might be found in quite significant amounts in the cytoplasm.

There is considerable evidence that PKC becomes translocated to the nucleus under certain circumstances. Phorbol ester treatment has previously been shown to cause a shift of PKC from the cytosol to the plasma membrane fraction of cells (Nishizuka, 1986). Some reports also showed a shift from the cytosol to the nuclear membrane region (Halsey *et al.*, 1987; Kiss *et al.*, 1988) and Leach *et al.* (1989) showed specifically that PKC-α became localized to the nuclear envelope on PMA treatment of NIH 3T3 cells. Bryostatin-1, but not phorbol dibutyrate, was shown to cause selective translocation of PKC-β2 to the nucleus of HL-60 cells (Hocevar and Fields, 1991). Insulin-like growth factor-1 (IGF-1) was shown to cause an increase in nuclear PKC as a result of the hydrolysis of inositol phospholipids from the nuclear membrane itself, in contrast to bombesin which initiated the hydrolysis of plasma membrane inositol phospholipids leading to the translocation of PKC from the cytosol to the nuclear envelope (Divecha *et al.*, 1991).

Of particular interest to this study is the possible interaction between the PKC and the cAMP/PKA signal transduction pathways proposed by Nishizuka (1986). There are many examples of intracellular target proteins that are phosphorylated by both PKC and PKA. Moreover,

the consensus phosphorylation sites of these two kinases are quite similar and it has been shown that the same residues in a protein can be phosphorylated by both kinases, e.g. in myelin basic protein (Kishimoto *et al.*, 1985). Thus, it is possible for two separate pathways to target one protein, perhaps even through the same residues. However, more important than this, is the ability of these two major, but separate, signal transduction pathways to exert some sort of control over each other. This may be in the form of "bidirectional control", where the two pathways counteract each other by some negative feedback mechanism, or "monodirectional control", where the two pathways stimulate each other by a positive feedback mechanism. Examples of each type of interaction are given in Nishizuka (1986).

Having already demonstated *in vivo* phosphorylation of topoisomerase IIα by PKA (chapter 6), it is possible that the PKC signal transduction pathway could regulate topoisomerase IIα phosphorylation, not only through direct phosphorylation by PKC but also through modulation of the PKA pathway.

There are a few reports of the effect of PKC agonists or antagonists on human topoisomerase II expression or activity, although these do not distinguish between the two topoisomerase II isozymes. Constantinou *et al.* (1989) showed that PMA-induced differentiation of HL-60 leukemia cells was accompanied by a rapid reduction in topoisomerase II activity and an increase in topoisomerase II phosphorylation. All these effects could be blocked by the PKC inhibitor, H-7, suggesting a direct action of PKC on topoisomerase II. As a cautionary note, H-7 is also a potent inhibitor of cyclic nucleotide-dependent protein kinases including PKA, and thus these results could also be interpreted in terms of PKC modulation of the PKA pathway.

Zwelling *et al.* (1990) showed that within 24 hours of PMA-induced differentiation of HL-60 cells there was a 10-fold decline in etoposide-induced topoisomerase II-mediated DNA cleavage, but only a 3-fold decline in m-AMSA-induced DNA cleavage. There was also a decline in topoisomerase II gene expression. However, these reductions were not seen in a mutant HL-60 cell line that would not terminally differentiate. The same group went on to show that the PKC inhibitor, staurosporine, also caused an equivalent reduction in etoposide- and m-AMSA-induced DNA cleavage in a dose- and time-dependent manner (Zwelling *et al.*, 1991b). They concluded that these apparently conflicting results meant that a direct phosphorylation of topoisomerase II by PKC was unlikely to

explain the change in topoisomerase II expression. Likewise, the minimal effects of bryostatin-1 on drug-induced, topoisomerase II-mediated DNA cleavage and topoisomerase II gene expression in HL-60 cells, led to the conclusion that the phorbol ester effects are related to more downstream events on topoisomerase II than direct PKC phosphorylation (Zwelling *et al.*, 1991a).

7.1.2 $p34^{cdc2}$ Kinase and Topoisomerase II.

The cell cycle-regulated kinase, $p34^{cdc2}$, was first identified by a genetic approach in the fission yeast, *Schizosaccharomyces pombe*. Mutations in the *cdc2* gene led to cells becoming arrested at two different points in the cell-cycle, at START (in late G_1) and at G_2/M, the point of entry to mitosis (Beach *et al.*, 1982). Further work showed that the product of the *cdc2* gene was indeed the pivotal regulator for progression of *S. pombe* cells through these two major checkpoints of the eukaryotic cell cycle (reviewed in Nurse, 1990).

Other genes important for progression through the cell cycle were also cloned by complementation of mutants defective in cell cycle control (Nurse, 1990). Those gene products which appeared to control mitotic progression were shown to act in a regulatory network, either in a positive or negative fashion, to control the activation of the $p34^{cdc2}$ kinase. Many of these gene products have been shown to encode protein kinases or phosphatases, demonstrating that the cell cycle is regulated, at least in part, by phosphorylation/dephosphorylation cascades.

The human homologue of the *S. pombe cdc2* gene was cloned by complementation using a fission yeast $cdc2^{ts}$ mutant strain and a human cDNA expression library (Lee and Nurse, 1987). The ability of the human CDC2 gene to completely substitute for the yeast gene, pointed to the high degree of conservation of eukaryotic cell cycle control mechanisms. Indeed, regulation of the *S. pombe* cell cycle has proved to be a good model for regulation of the cell cycle of higher eukaryotes (Norbury and Nurse, 1989).

The steps leading to specific activation of $p34^{cdc2}$ kinase at mitosis are now well understood (reviewed in Nurse, 1990 and Draetta, 1990). Two things are crucial: firstly, the association with a specific member of the cyclin family, a group of proteins which are synthesized in a cell cycle-dependent manner; secondly, the dephosphorylation of a tyrosine residue in the ATP-binding site of the $p34^{cdc2}$ kinase by the *cdc25* gene

product. In higher eukaryotes, a threonine in the ATP-binding site must also be dephosphorylated for activation (Norbury *et al.*, 1991). Interestingly, this dephosphorylation is carried out by the same phosphatase as dephosphorylates the tyrosine residue; in other words, this gene product appears to be both a threonine and a tyrosine phosphatase (Millar *et al.*, 1991). There is also evidence that in *S. pombe* both phosphorylations are effected by the dual action threonine/tyrosine kinase, *wee1* (Featherstone and Russell, 1991).

Hence, phosphorylation plays an absolutely critical role in mitotic control in the eukaryotic cell cycle. Firstly, the regulatory network coordinating mitotic activation involves phosphorylation cascades, coordinated by a series of kinases and phosphatases. Secondly, the crucial switch to activate $p34^{cdc2}$ kinase appears to involve the specific dephosphorylation of residues in the *cdc2* gene product. Thirdly, it is the phosphorylation of a wide range of substrates by $p34^{cdc2}$ kinase, and perhaps other downstream kinases, which actually leads to the cellular reorganisation seen at mitosis.

Whilst many cytoplasmic and nuclear proteins have been put forward as potential substrates of $p34^{cdc2}$ kinase based on *in vitro* phosphorylation results, very few have been clearly defined as substrates *in vivo* (reviewed in Moreno and Nurse, 1990). Those that do appear to be physiological substrates include histone H1 (Langan *et al.*, 1989) and the nuclear lamin B protein (Peter *et al.*, 1990a). Phosphorylation of these substrates appears to lead to chromosome condensation and nuclear lamina disassembly, respectively. The nucleolar antigens, NO38 and nucleolin, are also phosphorylated *in vivo* by $p34^{cdc2}$ at mitosis and this may account for disassembly of the nucleolus and inhibition of nucleolar activities during mitosis (Peter *et al.*, 1990b). There is also good evidence for the phosphorylation of $pp60^{c\text{-}src}$ at mitosis (Shenoy *et al.*, 1989; Morgan *et al.*, 1989). Activation of the tyrosine kinase of $pp60^{c\text{-}src}$ by $p34^{cdc2}$ phosphorylation might be associated with the cytoskeletal reorganization that occurs at mitosis.

The evidence for these proteins being *in vivo* substrates is convincing because they are appropriate cellular targets at mitosis and the phosphorylation ellicits an appropriate biological response. With several of the other proposed substrates this is not so clear. These proposed substrates include RNA polymerase II, protein synthesis elongation factors and SV40 large T antigen (Moreno and Nurse, 1990). It is possible that $p34^{cdc2}$ kinase phosphorylates a wide range of DNA-

binding proteins simply in order to remove them from DNA by electrostatic repulsion and thus allow complete condensation of the chromosomes. If this were the case, an active biological role in mitosis for phosphorylation of these substrates may not be evident.

Topoisomerase II plays an essential role in mitosis (discussed in detail in section 1.6.5). Yeast *top2*^{ts} mutants incubated at the restrictive temperature can neither complete chromosome condensation nor segregate their chromosomes (Uemura *et al.*, 1987a). In mammalian cells, topoisomerase II poisons inhibit entry into mitosis, block chromosome condensation and segregation, and cause a variety of chromosomal lesions at mitosis (Downes *et al.*, 1991; Sumner, 1992). Furthermore, a stable cell-free mitotic extract has been derived from *Xenopus* eggs that contains activities necessary for nuclear envelope breakdown and chromosome condensation (Newport and Spann, 1987). These authors showed that chromosome condensation specifically required topoisomerase II protein and was an independent and separable biochemical process from nuclear envelope vesicularization and lamina disassembly.

In vertebrate species, topoisomerase II expression has been shown to be cell cycle-dependent with the level peaking in late G_2/M (discussed in section 1.9). Furthermore, the level of phosphorylation of the topoisomerase II protein appears to be cell cycle-dependent, also with maximal phosphorylation at G_2/M (Heck *et al.*, 1989). The increase in the level of phosphorylation appears to be independent of the increase in protein level (Saijo *et al.*, 1992). Hence, based on both functional and biochemical observations topoisomerase II would seem to be a good candidate substrate for the cell cycle-regulated p34^{cdc2} kinase.

7.1.3 Strategy for Studying the Potential Phosphorylation of Human Topoisomerase IIα Protein by PKC and p34^{cdc2} Kinase.

Once human topoisomerase IIα protein had been purified it could be added to individually purified PKC isozymes or purified p34^{cdc2} kinase in *in vitro* phosphorylation assays. However, studies on p34^{cdc2} kinase were started before this became available so alternative strategies were adopted at the outset. This initially involved further characterization of a mutant CHO cell line, ADR-1, generated in this laboratory which had an altered cell cycle distribution as well as an enhanced level of topoisomerase II expression. It was hypothesized that if topoisomerase

IIα was a substrate for $p34^{cdc2}$ kinase then the elevated topoisomerase II expression in ADR-1 cells might be due to an altered $p34^{cdc2}$ kinase activity manifested by the abnormal cell cycle distribution. Phosphorylation by $p34^{cdc2}$ kinase, for instance, might be important for increasing the stability of topoisomerase IIα protein, leading to the increase in protein level normally seen at mitosis.

Peptides were also designed based on potential $p34^{cdc2}$ kinase consensus target sites within the human topoisomerase IIα sequence. Phosphorylation of these peptides by $p34^{cdc2}$ kinase would demonstrate that isolated sequences in the topoisomerase IIα protein could act as acceptor sites for $p34^{cdc2}$ phosphorylation. Some previous studies have also used a peptide approach to try and identify phosphorylation sites within target proteins; for instance, the phosphorylation of peptides from within the tumor suppressor protein, RB, by $p34^{cdc2}$ kinase (Taya *et al.*, 1989). These results though would only be very preliminary evidence and would not prove that the native protein can be phosphorylated by $p34^{cdc2}$ kinase. In the native protein, the individual peptide sequences may be hidden within the interior of the protein or might adopt completely different conformations which are not recognized by $p34^{cdc2}$ kinase.

If phosphorylation of the purified topoisomerase IIα protein was shown for one of the kinases tested, then evidence for *in vivo* phosphorylation would be sought by phosphopeptide mapping. Sites which get phosphorylated *in vitro* should also be phosphorylated *in vivo.* Moreover, phosphorylation should be modulated by agents that alter activity of the kinases *in vivo*; for example, PMA treatment in the case of PKC, or nocodazole treatment which arrests cells in mitosis, in the case of $p34^{cdc2}$ kinase. Finally, if changes in topoisomerase II activity could be demonstrated upon phosphorylation by these kinases *in vitro*, then changes in topoisomerase IIα activity *in vivo* would be sought under conditions which stimulate the kinase. For instance, in the case of PKC, survival curves could be measured with the topoisomerase II poison, VM-26, in the presence or absence of PMA or bryostatin.

7.2 Results

7.2.1 Human Topoisomerase IIα Protein is Phosphorylated by Protein Kinase C Isotypes α, β1, β2, γ and ε.

The Ca^{2+}-dependent PKC isotypes, α, β1, β2 and γ and the Ca^{2+}-independent PKC isotype, ε, were kindly provided by Dr. P. J. Parker (I.C.R.F., London). The α, β1, β2 and γ isotypes were purified from bovine brain and identified with specific polyclonal antisera (Marais and Parker, 1989). PKC-ε was purified following overexpression of the murine cDNA in insect cells using a baculovirus expression system (Schaap and Parker, 1990). To demonstrate activity of the PKC α, β1, β2 and γ isozymes, equal amounts of the kinase isozymes were incubated with lysine-rich histone type IIIS in the presence of [γ^{32}P]-ATP and separated on a 12% SDS-polyacrylamide gel (Figure 49; data not shown for PKC-β2). Reactions were then repeated with equal units of each PKC isozyme, based on their activity against histone type IIIS, using the purified human topoisomerase IIα protein as the substrate (Figure 49; data not shown for PKC-β2). This demonstrated that PKC α, β1, β2 and γ could all phosphorylate human topoisomerase IIα protein *in vitro* and with approximately equal efficiency .

The control activity of the PKC-ε isozyme, along with PKC-β1 and PKC-γ, was measured against a peptide [Ser]^{25}PKC(19-31) from GIBCO BRL, which is based on the pseudosubstrate site of PKC but where alanine at position-25 has been changed to serine so that phosphorylation can occur. All three PKC isozymes were shown to phosphorylate this peptide as detemined by spotting samples on phosphocellulose discs and Cerenkov counting following incubation in the presence of [γ^{32}P]-ATP. PKC-ε was then shown to be able to phosphorylate the purified human topoisomerase IIα protein in the absence, but not the presence, of phosphatidylserine and TPA (Figure 50). Phosphorylation of topoisomerase IIα by PKC-ε could also be inhibited by a peptide representing the normal pseudosubstrate site, PKC(19-36) from GIBCO BRL (Figure 50).

7.2.2 Activation of Human Topoisomerase IIα by Protein Kinase C Phosphorylation.

The specific activity of the purified human topoisomerase IIα protein following either no treatment or phosphorylation by PKC-α, β1 or

γ, was measured in a plasmid relaxation assay (Figure 51). Phosphorylation by each PKC isozyme led to a generally increased rate of plasmid relaxation compared to untreated topoisomerase IIα as measured by loss of Form I (fully supercoiled DNA). However, there also appeared to be a more specific change in the nature of reaction intermediates produced by the PKC phosphorylation of topooisomerase IIα protein.

Plasmid relaxation catalysed by the untreated topoisomerase IIα enzyme led to a slow shift from fully supercoiled to fully relaxed DNA but with an apparently constant number of products of intermediate linking number (Figure 51). Plasmid relaxation by PKC-phosphorylated topoisomerase IIα led to a rapid loss of the fully supercoiled DNA but without an initial increase in fully relaxed DNA; instead there is stepwise move of intermediates from high to low linking number (Figure 51). Assuming that the DNA substrate is in excess, this perhaps suggests a shift from a more processive reaction mechanism to a more distributive reaction mechanism (discussed in section 7.3.1).

Inactivation of the PKC isozymes by heating to 80°C for 15 minutes completely blocked any change in the reactionmechanism of topoisomerase IIα enzyme (Figure 51). Densitometry of the photographs of these gels showed that phosphorylation by each isozyme led to a greater than 10-fold increase in the initial rate of reduction of Form I DNA compared to the untreated enzyme (Figure 52). All three isozymes had approximately the same effect. Hence under these conditions, these three Ca^{2+}-dependent isozymes appear to phosphorylate and activate human topoisomerase IIα protein *in vitro* to a similar extent. The effect on activity of topoisomerase IIα by phosphorylation with PKC-β2 or PKC-ε was not measured.

7.2.3 Stimulation of the Phosphorylation of Human Topoisomerase IIα Protein *In Vivo* with PMA.

Having demonstrated that each of the PKC isozymes tested was able to phosphorylate the purified topoisomerase IIα protein *in vitro*, we looked to see whether the level of topoisomerase IIα phosphorylation *in vivo* could be altered by short-term exposure of cultured cells to phorbol ester or bryostatin. HeLa cells were labelled *in vivo* with ^{32}P-orthophosphate for 16 hours to allow a steady-state level of topoisomerase IIα phosphorylation to be reached. Cells were then exposed for 1 hour to 10nM PMA in 0.01% DMSO, 100nM bryostatin in

0.01% DMSO or to 0.01% DMSO alone. The labelled topoisomerase IIα was then immunoprecipitated, electrophoresed on a 7.5% SDS-polyacrylamide gel and the gel dried and autoradiographed (Figure 53). No significant difference in the overall level of topoisomerase IIα protein phosphorylation was detected.

It was possible either that the HeLa cells were relatively unresponsive to these agents, perhaps having down-regulated the level of intracellular PKC, or that a change in phosphorylation due to PKC was masked by the phosphorylation due to other kinases, particularly CK II and PKA. To test the first possibility, the induction of *c-fos* protein synthesis was assayed by western blotting following incubation with PMA or bryostatin under the same conditions as described above. Phorbol esters have been shown to cause a rapid, transient induction of *c-fos* in many cell types (reviewed in Curran, 1988). Figure 54 shows that a 1 hour treatment of HeLa cells with either of these PKC agonists leads to a dramatic induction of the *c-fos* protein demonstrating the integrity of the PKC pathway in this cell type.

Phosphopeptide mapping would allow a change in the phosphorylation of one particular phosphopeptide to be seen in a background of other phosphorylations. Thus one-dimensional phosphopeptide maps were produced of topoisomerase IIα protein phosphorylated *in vivo*, either in the absence or presence of PMA as described above, and of purified topoisomerase IIα protein phosphorylated by PKC-β2 *in vitro*. Scanning the autoradiograph shown in Figure 55 by densitometry showed that the phosphorylation of the 20kDa phosphopeptide (labelled 'b' in Figure 55) is increased by two-fold on PMA stimulation with respect to phosphopeptide 'a'. This 20kDa phosphopeptide also co-migrates with the peptide phosphorylated most efficiently by PKC-β2 *in vitro*. It has already been shown that this phosphopeptide is probably derived from the very C-terminal region of the topoisomerase IIα protein (see section 5.2.7); this region is also phosphorylated *in vitro* by CK II and PKA.

7.2.4 VM26 Cytotoxicity is Moderately Reduced by Short-term PMA or Bryostatin Treatment.

Stimulation of PKC by phorbol ester leading to a change in topoisomerase IIα phosphorylation *in vivo* may therefore lead to a change in topoisomerase IIα activity *in vivo*. This may be reflected in an altered

sensitivity to topoisomerase II poisons. Thus, HeLa cells were incubated for 60 minutes with a range of VM-26 concentrations, in the absence or presence of 10nM PMA or 100nM bryostatin. Cells were then washed and left to grow in a colony forming assay; the colonies were then fixed, stained and counted, and the mean number of counts from four independent experiments plotted against VM-26 concentration (Figure 56). Both PMA and bryostatin appeared to give a modest degree of protection against the cytotoxicity of VM-26 in a 60 minute co-incubation experiment. The D_{25} concentration (dose to give 25% survival) of VM-26 in the presence of each agent was increased to approximately 0.5μg/ml compared to 0.3μg/ml in the absence of PMA or bryostatin. However, this effect is minimal and the experiment would need to be repeated many more times to prove any statistical significance.

7.2.5 Cell Cycle Distribution of the Topoisomerase II-Drug Sensitive CHO Cell Line ADR-1 .

A Chinese hamster ovary cell line, ADR-1, was isolated from wild-type cells, CHO-K1, by chemical mutagenesis and screening for hypersensitivity to a range of drugs which inhibit topoisomerase II (Robson *et al.*, 1987). This mutant cell line was shown to have an elevated level of topoisomerase II protein which correlated with an elevated number of DNA strand breaks upon exposure to the topoisomerase II poison, m-AMSA (Davies *et al.*, 1988). ADR-1 was also shown to have an altered cell cycle distribution compared to its parental line, CHO-K1, with a much higher proportion of cells in G_1 and much lower proportion in G_2/M (Davies *et al.*, 1988). The primary defect in this cell line has still to be determined and it is not clear whether the elevated level of topoisomerase II expression is a cause or effect of an abnormal cell cycle distribution.

The abnormal cell cycle distribution of ADR-1 cells was confirmed by flow cytometry on exponentially growing CHO-K1 and ADR-1 cells. Figure 57 shows a typical FACS scan for both cell types and confirms that the mutant ADR-1 cells do indeed have an altered distribution, with a greater percentage of cells in G_1 phase, about the same in S-phase and a smaller percentage in G_2/M phase.

To assess whether there was also a difference in the proportion of cells undergoing mitosis, each cell type was grown on glass coverslips, fixed whilst in exponential phase and stained with a fluorescent DNA

intercalating agent. Mitotic figures were counted as a proportion of total numbers of cells. From a mean of 5 independent experiments the proportion of cells with mitotic figures was 3.3% for the wild-type CHO-K1 cells and 2.9% for the mutant ADR-1 cells. Thus there was not a significant difference in the proportion of cells in mitosis.

The doubling time for ADR-1 cells has been shown to be 15 hours, compared to 13 hours for the CHO-K1 cells (Robson *et al.*, 1987). Hence the actual time spent in each phase of the cell cycle can be calculated from the cell cycle distribution (figures taken from Davies *et al.*, 1988), the mitotic counts and the doubling time (Table 7). Interestingly, this shows that the real time taken to complete S-phase and M-phase is the same for both mutant and wild-type cell line. In contrast though, the mutant ADR-1 spends a far longer time in G_1 and only half as long in G_2 compared to CHO-K1.

7.2.6 Altered Histone H1 Kinase Activity in ADR-1 Cells.

Activation of the human $p34^{cdc2}$ kinase through interaction with a B-type cyclin and dephosphorylation on threonine and tyrosine causes cells to enter mitosis. It is possible that the rapid passage of ADR-1 cells through G_2 phase and into mitosis is due to a premature activation of the cell cycle-regulated $p34^{cdc2}$ kinase in ADR-1 cells. The histone H1 kinase activity of the mutant ADR-1 cell line was compared to that of the parental line under a variety of conditions by immunoprecipitating $p34^{cdc2}$ kinase from HeLa cell extracts and assaying its activity against an excess of histone H1. In an exponential population of cells there was up to a 2-fold increase in histone H1 kinase activity in the mutant ADR-1 cell line compared to CHO-K1, despite having only half as many cells in the G_2/M phase of the cell cycle (Figure 58).

As a difference was sometimes very difficult to detect, a mitotically enriched population of each cell type was compared; this should increase the proportion of cells with an active $p34^{cdc2}$ kinase. Mitotic arrest can be induced in eukaryotic cells with tubulin inhibitors such as nocodazole which block the formation of mitotic spindles. Duplicate samples of $p34^{cdc2}$ kinase were immunoprecipitated from both cell lines incubated for 6 hours, either in the absence or presence of 1μg/ml nocodazole. The histone H1 kinase activity of both cell lines was increased by about 5-fold by incubation in nocodazole (Figure 59). Furthermore, it could be seen that both the untreated and nocodazole-arrested ADR-1 cells had an

approximately 2- to 3-fold greater level of histone H1 kinase activity than the equivalent CHO-K1 cells (Figure 59).

Mitotic figures were also counted from each cell type treated with 1μg/ml nocodazole for 6 hours, to check that the mutant cell line did not respond differently to this mitotic block. For each cell type the proportion of cells in mitosis increased from approximately 3% to 38% and, as 6 hours is approximately 40% of the doubling time for each cell type, this suggested the block was effective in both cell types.

To further confirm this increased level of $p34^{cdc2}$ kinase activity in ADR-1 cells, both cell types were synchronized by serum-starvation and then refeeding. The $p34^{cdc2}$ kinase activity was then measured as cells synchronously progressed through G_2 into mitosis and then back into G_1 (Figure 59). It can be seen as expected that synchronized cells in the G_2/M phase of the cell cycle have a much greater histone H1 kinase activity than an asynchronous population. The mutant ADR-1 cells reached peak histone H1 kinase activity 16 hours after release from the serum block and by 20 hours this activity had dropped by approximately 5-fold, suggesting passage of cells back into G_1. The synchronized CHO-K1 cells also reached peak histone H1 kinase activity at 16 hours, but with a slightly lower overall histone H1 kinase activity; the peak was more drawn out though, so that at 20 hours the activity had still not started to drop. It is possible that this was the result of a poor synchronization of the CHO-K1 cell population, but it could also be due to the greater time CHO-K1 cells spend traversing G2/M compared to ADR-1 cells.

7.2.7 Phosphorylation by $p34^{cdc2}$ Kinase of Peptides from the Human Topoisomerase IIα Protein Sequence.

Before purified human topoisomerase IIα protein was available, the protein sequence was scanned for $p34^{cdc2}$ kinase consensus target sites. Unfortunately, the primary sequence consensus is weak, consisting at best of S/TPK or S/TPXK/R. Other basic residues and particularly lysine, either immediately before or after this consensus, appear to strengthen it, but several sites shown to be phosphorylated at least *in vitro* by $p34^{cdc2}$ kinase only contain the core consensus (Moreno and Nurse, 1990). Presumably the secondary and tertiary protein structures around the potential phosphorylation site play an important determining role for this kinase.

Nevertheless, nine peptides were synthesized based on sequences in the human topoisomerase IIα protein which contained the best matches to the consensus. These are shown in Table 1 (chapter 3). Peptide T2K5 was of particular interest as it contained a potential $p34^{cdc2}$ phosphorylation site only three residues away from the topoisomerase II active site tyrosine which forms the covalent interaction with the broken DNA strand in the DNA breakage-rejoining reaction. Peptide T2K8 contained both the T2K3 and T2K6 potential target sites on the same peptide as these sequences are contiguous in the protein. A peptide, H1K, based on one of the general $p34^{cdc2}$ kinase phosphorylation sites from histone H1 was also synthesized to use as a control peptide.

Each of these peptides, together with histone H1 protein, was tested for its ability to act as a substrate for $p34^{cdc2}$ kinase immunoprecipitated from HeLa cell extracts using an antiserum raised to a peptide based on the C-terminus of human $p34^{cdc2}$ kinase. The samples were run out on a 22% SDS-polyacrylamide gel, dried and autoradiographed (Figure 60; data is not shown for T2K9 as this was tested separately from the others and showed no phosphorylation). Both the histone H1 protein and the H1K peptide were efficiently phosphorylated by the immunoprecipitated $p34^{cdc2}$ kinase. Of the topoisomerase II peptides, T2K3, T2K4 and T2K8 (which contains the T2K3 site) were phosphorylated as efficiently as the H1K peptide. However long exposure of the autoradiograph showed that T2K1, T2K2 and T2K7 were also phosphorylated but with a much lower efficiency.

7.2.8 Phosphorylation of Human Topoisomerase IIα Protein by Recombinant, but not Immunoprecipitated, $p34^{cdc2}$.

Once the human topoisomerase IIα protein had been purified it was tested as a substrate for the immunoprecipitated $p34^{cdc2}$ kinase. Phosphorylation of the control substrate histone H1 by immunoprecipitates prepared with the anti-$p34^{cdc2}$ immune antiserum, but not by immunoprecipitates prepared with pre-immune serum, was used to demonstrate specific kinase activity (Figure 61). A low level of histone H1 phosphorylation by the pre-immune precipitates suggests that a minor kinase activity is precipitated in a non-specific manner by this technique.

Whilst phosphorylation of human topoisomerase IIα protein by immune precipitates of $p34^{cdc2}$ kinase was seen, it was never significantly greater than with pre-immune precipitates and certainly not in the same order of phosphorylation seen using histone H1 as a substrate (Figure 61). Hence, this suggested that the form of $p34^{cdc2}$ immunoprecipitated by this antiserum did not efficiently phosphorylate human topoisomerase IIα protein, if at all.

We then tested whether purified recombinant chicken $p34^{cdc2}$ kinase could phosphorylate the human topoisomerase IIα protein; again histone H1 was used as a control substrate (Figure 62). This time there was stronger phosphorylation of the human topoisomerase IIα protein in the presence of $p34^{cdc2}$ kinase than in its absence (Figure 62). This was reproducible and the increase in phosphorylation was in the order of 1.5- to 5-fold over background phosphorylation. Thus, purified human topoisomerase IIα protein appeared to be a better substrate *in vitro* for recombinant $p34^{cdc2}$ kinase than for $p34^{cdc2}$ kinase immunoprecipitated with the antibody raised to the C-terminal peptide of human $p34^{cdc2}$ kinase.

7.2.9 One-dimensional Phosphopeptide Analysis of Topoisomerase IIα Protein Phosphorylated by $p34^{cdc2}$ Kinase.

A one-dimensional partial V8 protease cleavage map was generated of human topoisomerase IIα protein phosphorylated *in vitro* with the recombinant $p34^{cdc2}$ kinase in the same way as described for both CK II and PKA. Maps were generated of *in vivo* labelled topoisomerase IIα protein and CK II *in vitro* phosphorylated topoisomerase IIα protein at the same time for comparison (Figure 63). The pattern of phosphopeptides generated by $p34^{cdc2}$ phosphorylation differed markedly from that generated by CK II phosphorylation, suggesting phosphorylation of different regions of the protein.

The major 23kDa fragment phosphorylated *in vivo* (fragment 'a') co-migrated with the major site of phosphorylation by $p34^{cdc2}$, whereas fragment 'b', shown to come from the C-terminus of the protein (chapter 5), was not apparently a phosphorylation site for $p34^{cdc2}$ *in vitro*.

7.3 Discussion

We have demonstrated that human topoisomerase IIα protein can be phosphorylated *in vitro* by five different isozymes of protein kinase C and by recombinant chicken $p34^{cdc2}$ kinase. The *S. cerevisiae* topoisomerase II protein was also shown to be a substrate *in vitro* for PKC and $p34^{cdc2}$ kinase (Cardenas *et al.*, 1992). Hence, human topoisomerase IIα and yeast topoisomerase II show similar abilities to act as substrates for multiple kinases *in vitro*. The yeast topoisomerase II, unlike human topoisomerase IIα, was not shown to be a substrate for PKA (see chapter 6).

7.3.1 Topoisomerase II and Protein Kinase C.

The PKC isozymes can be divided into two groups according to their dependence on Ca^{2+} and lipids; often members of one group have similar substrate specificity which differ from that of members of the other group (Blumberg, 1991). However, in this case human topoisomerase IIα was phosphorylated by both Ca^{2+}-dependent and Ca^{2+}-independent PKC isozymes. The four Ca^{2+}-dependent PKC isozymes tested, α, β1, β2 and γ, all phosphorylated topoisomerase IIα to a similar extent. Three of these also each caused an approximate 10-fold increase in the plasmid relaxation activity of topoisomerase IIα *in vitro* as measured by rate of loss of Form I DNA. Whilst PKC-β2 and the Ca^{2+}-independent isozyme PKC-ε phosphorylated topoisomerase II, the effect on activity was not measured due to time constraints.

An apparent change in the reaction intermediates catalysed by PKC-phosphorylated topoisomerase IIα protein was also noted. Following phosphorylation there was a rapid loss of fully supercoiled DNA but without any immediate build-up of fully relaxed DNA. Instead, there was a gradual shift down the gel with a stepwise decrease in the level of supercoiling of the entire plasmid population. In the absence of PKC phosphorylation the build-up of fully relaxed DNA coincided with the loss of fully supercoiled DNA, with a constant number of DNA molecules of intermediate superhelicity. This suggests that PKC phosphorylation might alter the "on-off" rate of DNA-topoisomerase II binding in favour of the off-rate. This would lead to the enzyme shifting from a generally processive reaction mechanism to a more distributive one. In other words, instead of remaining associated with a single plasmid DNA

molecule until fully relaxed, each topoisomerase IIα molecule completes only one catalytic cycle before falling off and then reassociating with the most supercoiled molecule available. This preferential binding to supercoiled DNA (Zechiedrich and Osheroff, 1990) means that in general the PKC-phosphorylated topoisomerase IIα isozymes relax all the most fully supercoiled DNA before moving on to the next most supercoiled DNA molecules. This apparent change in mechanism clearly needs further investigation to confirm the hypothesis discussed.

The marked change in topoisomerase II activity by those PKC isozymes tested suggests that this phosphorylation is not an *in vitro* artefact, as it would be surprising to find such activation of an enzyme resulting from a fortuitous non-physiological phosphorylation event. Moreover, PKC has been suggested to phosphorylate topoisomerase II *in vivo* in lower eukaryotic species (Rottmann *et al.*, 1987).

We looked for evidence of *in vivo* phosphorylation of human topoisomerase IIα by PKC using phosphopeptide maps of topoisomerase II phosphorylated *in vivo* in either untreated cells or cells treated with phorbol ester. One dimensional separation of phosphopeptides generated by partial V8 protease cleavage suggested that PMA induced a 2-fold increase in the phosphorylation of a peptide *in vivo* which is also phosphorylated by PKC-β2 *in vitro*. These data suggest that short-term exposure to phorbol ester can indeed cause a change in the phosphorylation of the C-terminal domain of topoisomerase IIα protein *in vivo*. This also agrees with the data of Constantinou *et al.* (1989) who observed a general increase in the level of topoisomerase II phosphorylation in HL-60 cells treated with phorbol ester.

Based on the differential effect of the two PKC activators, phorbol ester and bryostatin, Matthes *et al.* (1990) concluded that phosphorylation of topoisomerase II in HIV-1 infected human H9 cells was mediated by the α and γ isoforms of PKC. Blocking this phosphorylation resulted in an inhibition of HIV-1 production. These authors highlighted the possibility of inhibiting PKC phosphorylation of topoisomerase II as a potential therapeutic approach in HIV treatment.

An altered expression of the PKC isozymes has been implicated in resistance to topoisomerase II poisons. Comparing Adriamycin-resistant HL-60 cells to wild-type, Aquino *et al.* (1990) showed that the resistant cells contained an equivalent level of PKC-α and a lower level of PKC-β, whilst PKC-γ was only detected in the resistant cells. However, it is by no means clear that altered phosphorylation of topoisomerase II is the cause

of resistance to Adriamycin, as Adriamycin is an inhibitor of PKC as well as topoisomerase II and PKC expression has been directly implicated in resistance to Adriamycin (O'Brian *et al.*, 1989). PKC has also been shown to phosphorylate the Multidrug Resistance membrane transporter protein (Chambers *et al.*, 1990), possibly leading to increased drug efflux capacity.

It is still not clear whether PKC phosphorylation causes short-term changes in topoisomerase II activity in the nucleus. To complicate issues, the activation of PKC itself appears to be more complex than first thought. The initial activation of PKC seems to result from hydrolysis of inositol phospholipids by phospholipase C leading to the production of diacylglycerol and the opening of Ca^{2+} channels. However, it is becoming clear that agonist-induced hydrolysis of other membrane phospholipids, particularly choline phospholipids, by phospholipase D and phospholipase A_2 may enhance and prolong the activation of PKC (Nishizuka, 1992). Hence, even long-term changes in the level of topoisomerase IIα phosphorylation in response to PKC agonists may be through direct phosphorylation via sustained PKC activation.

In conclusion, the results presented here suggest that human topoisomerase IIα protein has the potential to be phosphorylated and activated by a variety of PKC isozymes. Furthermore, it appears that direct phosphorylation of topoisomerase IIα by PKC *in vivo* may be an important part of the PKC signalling pathway, perhaps as part of the response leading to differentiation (Zwelling *et al.*, 1990). It remains to be proven by two-dimensional tryptic phosphopeptide mapping that the sites phosphorylated *in vitro* by PKC are the same sites that get phosphorylated *in vivo* in response to PKC agonists. This would exclude the possibility that this phosphorylation might be mediated through intermediary kinases such as PKA.

7.3.2 Topoisomerase II and $p34^{cdc2}$ Kinase.

Confirmation that human topoisomerase IIα protein is a physiological substrate for the cell cycle-regulated $p34^{cdc2}$ kinase has proved very difficult to determine despite using a variety of approaches. In an indirect approach, the level of $p34^{cdc2}$ histone H1 kinase activity was measured in a CHO mutant cell line with an increased sensitivity to topoisomerase II poisons and an altered cell cycle distribution. The primary defect in this cell line had not been characterized and it was

hypothesized that the increased drug sensitivity might be the result of abnormal phosphorylation of topoisomerase II by $p34^{cdc2}$ correlating with an abnormal cell cycle distribution.

The CHO mutant cell line, ADR-1, did appear to have an elevated level of histone H1 kinase activity as measured in an asynchronous population and a mitotically-enriched population. However, these results were not always reproducible and sometimes there was no detecable difference between CHO-K1 and ADR-1; CHO-K1 never had a greater level of histone H1 kinase activity than ADR-1. It was unlikely that the differences observed reflected purely the different cell cycle distributions of these two cell lines, as ADR-1, with only half as many cells in the G_2/M population, might have been expected to have a reduced histone H1 kinase level.

In cells synchronized by serum starvation, there was only a minor elevation of peak histone H1 kinase activity in ADR-1 cells compared to CHO-K1 cells. Furthermore, in accordance with the suggestion that these cells have a reduced G_2 period, the ADR-1 cells appeared to re-enter G_1 phase more rapidly than CHO-K1 cells as indicated by their earlier loss of histone H1 kinase activity; though this could also be explained by better synchronization in this mutant cell line.

These results do not demonstrate that the abnormality in ADR-1 cells is necessarily in a component of the cell cycle control machinery such as $p34^{cdc2}$ kinase. They do suggest though that the abnormal distribution of the ADR-1 cell cycle in some way leads to an increased proportion of asynchronous cells with active $p34^{cdc2}$ kinase, despite having less cells in the G_2/M portion of the cell cycle. This may explain why ADR-1 cells pass more rapidly into mitosis leading to a shorter G_2 period. The cells then need to spend longer in the G_1 phase to allow growth-regulated events to "catch up" with cell cycle-regulated events.

The increased level of topoisomerase II protein and concomitant increased sensitivity to topoisomerase II poisons in the ADR-1 cell lines may or may not be related to these cell cycle changes. The overall level of phosphorylation of the topoisomerase II protein in vivo in ADR-1 cells compared to CHO-K1 cells has not been measured as antibodies which immunoprecipitate topoisomerase II from hamster cells are not presently available. The tight association of topoisomerase II expression with the cell cycle and its essential role in mitosis point to this alteration as being more than a mere coincidence. Perhaps a more rapid passage through

G_2/M might require a greater topoisomerase II activity at this point in the cell cycle stimulating the cell to synthesize more protein.

Nine peptides were synthesized based on potential $p34^{cdc2}$ kinase consensus motifs within the topoisomerase IIα sequence. However, some of these sequences fitted only the absolute core consensus, i.e. (Ser/Thr)-Pro in a basic environment, and indeed only two sites appeared to be good substrates for immunoprecipitated human $p34^{cdc2}$ kinase. The T2K3 phosphorylation site is Serine-1360 in the human topoisomerase IIα sequence. It lies in the C-terminal regulatory domain and the Ser-Pro motif is conserved in the topoisomerase IIβ isozyme according to some, but not other alignments. The motif does not seem to be conserved in the *Drosophila* or yeast topoisomerase II enzymes, but alignments are very difficult and perhaps not very meaningful in this poorly conserved C-terminal region. The T2K4 phosphorylation site is Threonine-601 in the human topoisomerase IIα sequence. It lies in a region homologous to the GyrB subunit of *E. coli* DNA gyrase but C-terminal to the ATPase domain itself. This motif is clearly not conserved in the topoisomerase IIβ isozyme nor in any of the other known type II topoisomerase sequences.

Results from the phosphorylation of peptides should not be overinterpreted. It is possible that those sequences phosphorylated in a short peptide are not the ones phosphorylated in the holoenzyme, and vice-versa. Kinases such as $p34^{cdc2}$, which have weak primary consensus target sequences, may well be much more strongly influenced by secondary and tertiary structures not necessarily present in the peptide.

Purified human topoisomerase IIα protein was tested as a substrate for both immunoprecipitated human $p34^{cdc2}$ and recombinant chicken $p34^{cdc2}$ in kinase assays. The results again were not unequivocal. Using immunoprecipitated $p34^{cdc2}$ the difference between using pre-immune serum and anti-$p34^{cdc2}$ serum was minimal, with a small increase in phosphorylation using the anti-$p34^{cdc2}$ serum on some, but not all, occasions. Using recombinant $p34^{cdc2}$ kinase, there did at least seem to be a reproducible phosphorylation of human topoisomerase IIα protein compared to background. One-dimensional mapping of topoisomerase IIα following incubation with the recombinant $p34^{cdc2}$ kinase showed a pattern clearly distinct from any other kinase tested, supporting the suggestion that this phosphorylation is indeed attributable to the $p34^{cdc2}$ kinase.

This difficulty in proving phosphorylation of human topoisomerase IIα protein by $p34^{cdc2}$ might have several causes. The first one that must

be considered is that topoisomerase IIα is not a true physiological substrate of $p34^{cdc2}$ and hence *in vitro* it acts only as a very poor substrate. Secondly, efficient phosphorylation might require other post-translational modifications (including phosphorylation) of either kinase or substrate which are not present in this artificial highly purified system; hence, the phosphorylation that does occur is weak. Thirdly, if phosphorylation by $p34^{cdc2}$ kinase occured at only one site on the protein, it might be very difficult to measure in a background of other phosphorylations. For instance, phosphopeptide mapping suggests that CK II phosphorylates topoisomerase IIα at multiple sites (chapter 5). Moreover, some preparations of topoisomerase IIα also appear to contain some co-purifying CK II (chapter 4). So, although heparin is included in these assays to block any CK II phosphorylation, minor (though potentially very important) phosphorylations may still be difficult to measure. Fourthly, it has recently been shown that there are, in fact, a whole family of cdc2-related proteins which may play co-ordinated, but distinct, roles in the control of the higher eukaryotic cell cycle (Meyerson *et al.*, 1992). It is possible that human topoisomerase IIα is a good substrate for one member of this family which is not necessarily the same one as those tested. In other words, if the correct family member was used, in association with its natural cyclin partner, then a much stronger phosphorylation may be seen.

The regulation of topoisomerase IIα activity at mitosis would appear to be critical for proper cell division. The overall protein level and degree of phosphorylation of topoisomerase IIα both appear to gradually increase and peak in mitosis, presumably because of the requirement for a high degree of decatenation activity needed to achieve chromosome condensation and segregation. Yet it still remains unclear how the extremely tight temporal regulation of chromosome condensation and segregation at mitosis is achieved.

It is possible that the untangling of replicated chromosomes by decatenation occurs as the chromosomes are replicated. In this case a separate "chromatid separase" would regulate the initiation of segregation (Sumner, 1992) even though inhibition of topoisomerase II activity would only be revealed at this point. Microinjection of $p34^{cdc2}$ kinase into mammalian cells leads to premature chromosome condensation despite the cells not passing further into cell division (Lamb *et al.*, 1990), showing that initiation of condensation is triggered by $p34^{cdc2}$ phosphorylation. However, it is possible that chromosome condensation

is triggered not by topoisomerase II phosphorylation, but perhaps by the phosphorylation of histone H1 by $p34^{cdc2}$ (Langan *et al.*, 1989). Hence, whilst it is clear that topoisomerase II activity is very important in allowing mitotic integrity, it remains to be proven whether phosphorylation of topoisomerase IIα protein plays a role in the timing of mitotic events.

Cell Line	Doubling Time	Percentage of Population in each Cell Cycle Stage		Time for Cell Cycle Stage[a]	
CHO-K1	13 hours	G_1	37%	G_1	4.8 hours
		S	46%	S	6.0 hours
		G_2/M	17%	G_2	1.8 hours
		Mitotic Count	3.3%	M	0.4 hours
ADR-1	15 hours	G_1	51%	G_1	7.7 hours
		S	40%	S	6.0 hours
		G_2/M	9%	G_2	0.9 hours
		Mitotic Count	2.9%	M	0.4 hours

Table 7 Cell cycle distribution of CHO-K1 and ADR-1 cells. The doubling times are taken from Robson *et al.* (1987) and the percentages in G_1, S and G_2/M from Davies *et al.* (1988).[a] The real time spent in each part of the cell cycle is calculated from the doubling time and the percentage of cells in each stage of the cycle (the percentage in G_2 is calculated from G_2/M minus the mitotic count).

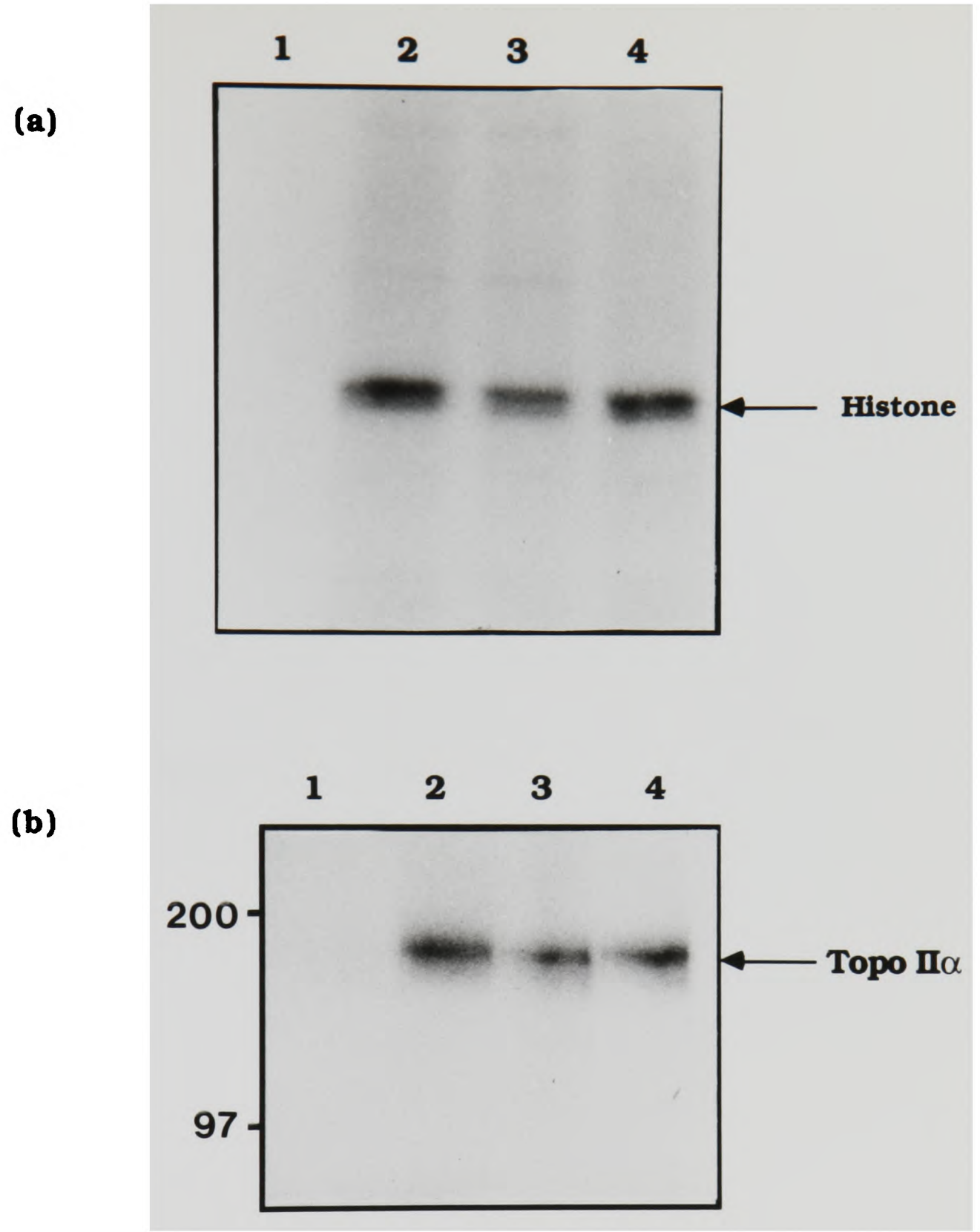


Figure 49 Human topoisomerase IIα protein is a substrate *in vitro* for PKC-α, β1 and γ isozymes. (a) The three purified mammalian PKC isozymes were shown to be active by their ability to phosphorylate the lysine-rich fraction of histone type III-S. (b) Each isozyme was then shown to phosphorylate the purified preparation of human topoisomerase IIα. In both panels the reaction was carried out with substrate alone (lane 1), or substrate with PKC-α (lane 2), PKC-β1 (lane 3), or PKC-γ (lane 4). Molecular weight markers are shown on the left in panel (b).

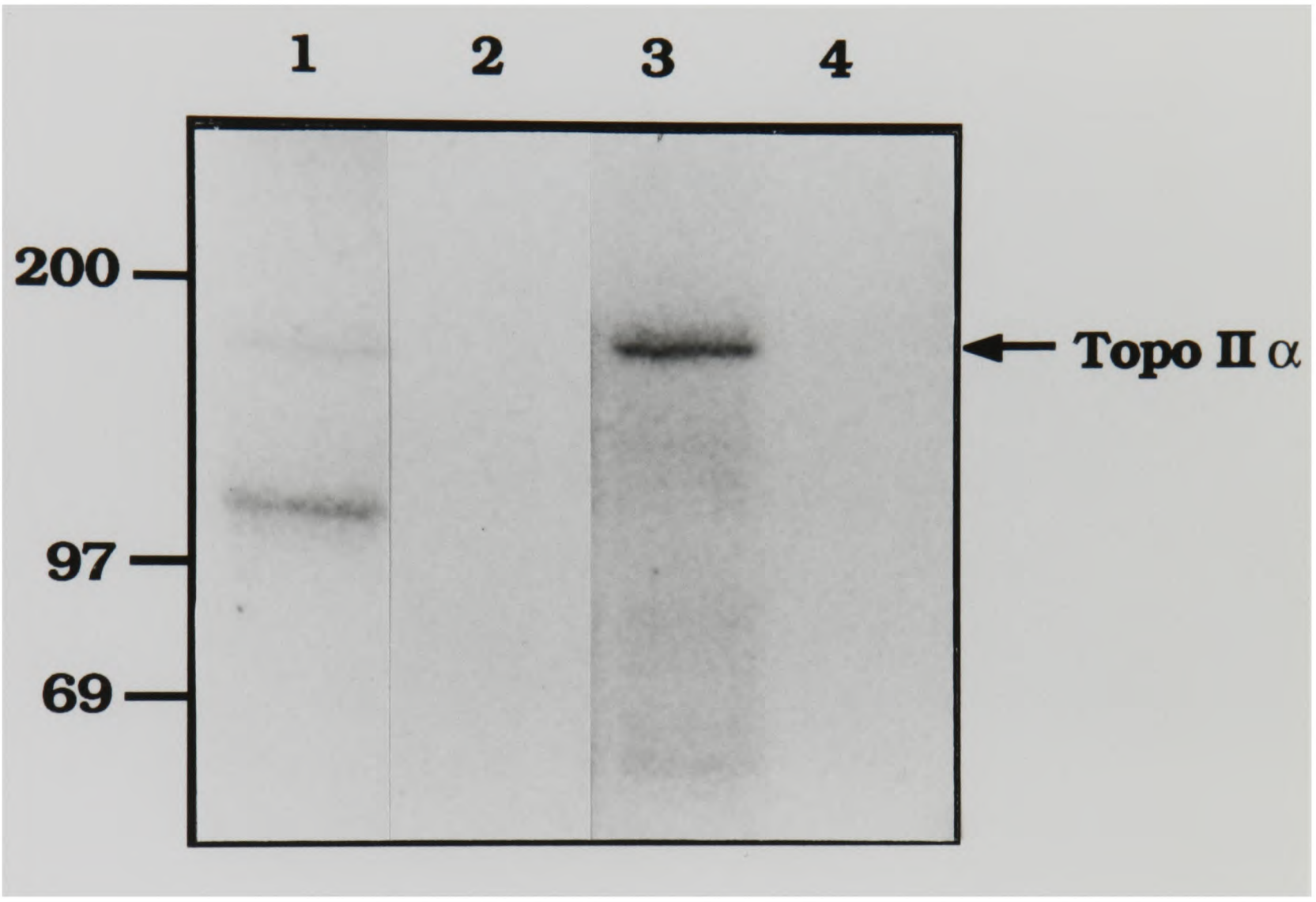


Figure 50 Human topoisomerase IIα protein is a substrate *in vitro* for PKC-ε isozyme. Purified human topoisomerase IIα protein was incubated in the presence of [γ^{32}P]-ATP alone (lane 1) or with purified PKC-ε in the presence (lane 2) or absence (lanes 3 and 4) of phosphatidylserine and TPA; a competitive peptide substrate was also added to the reaction in lane 4. Molecular weight markers (kDa) are shown on the left, and the position of the phosphorylated topoisomerase IIα protein on the right.

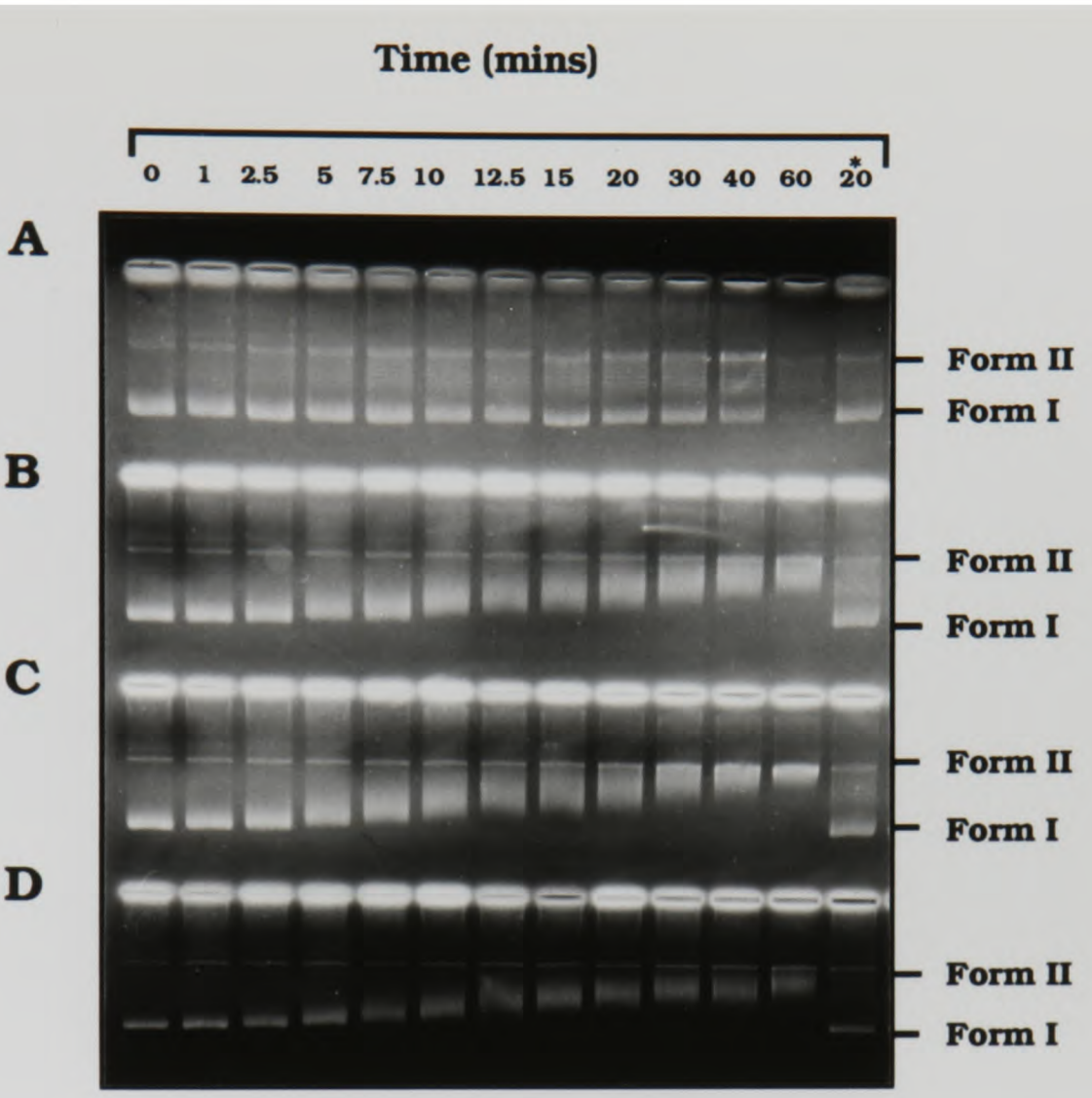


Figure 51 Relaxation activity of untreated and PKC phosphorylated human topoisomerase IIα protein. The relaxation activity was measured as the rate of relaxation of a supercoiled plasmid substrate from Form I (supercoiled) to Form II (relaxed) DNA with time. Before carrying out the relaxation reaction the topoisomerase IIα was incubated at 30°C for 10 minutes in the absence (panel A) or presence of PKC-α (panel B), PKC-β1 (panel C), or PKC-γ (panel D). Samples were taken and stopped at time T= 0, 1, 2.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40 and 60 minutes and run on a 1% agarose gel. Similar reactions were carried out but with PKC isozymes which had been previously heated to 80°C for 15 minutes. A single sample from these reactions was stopped after 20 minutes and run in the last lane of each panel (labelled *). The gel was stained with ethidium bromide and photographed under UV-illumination.

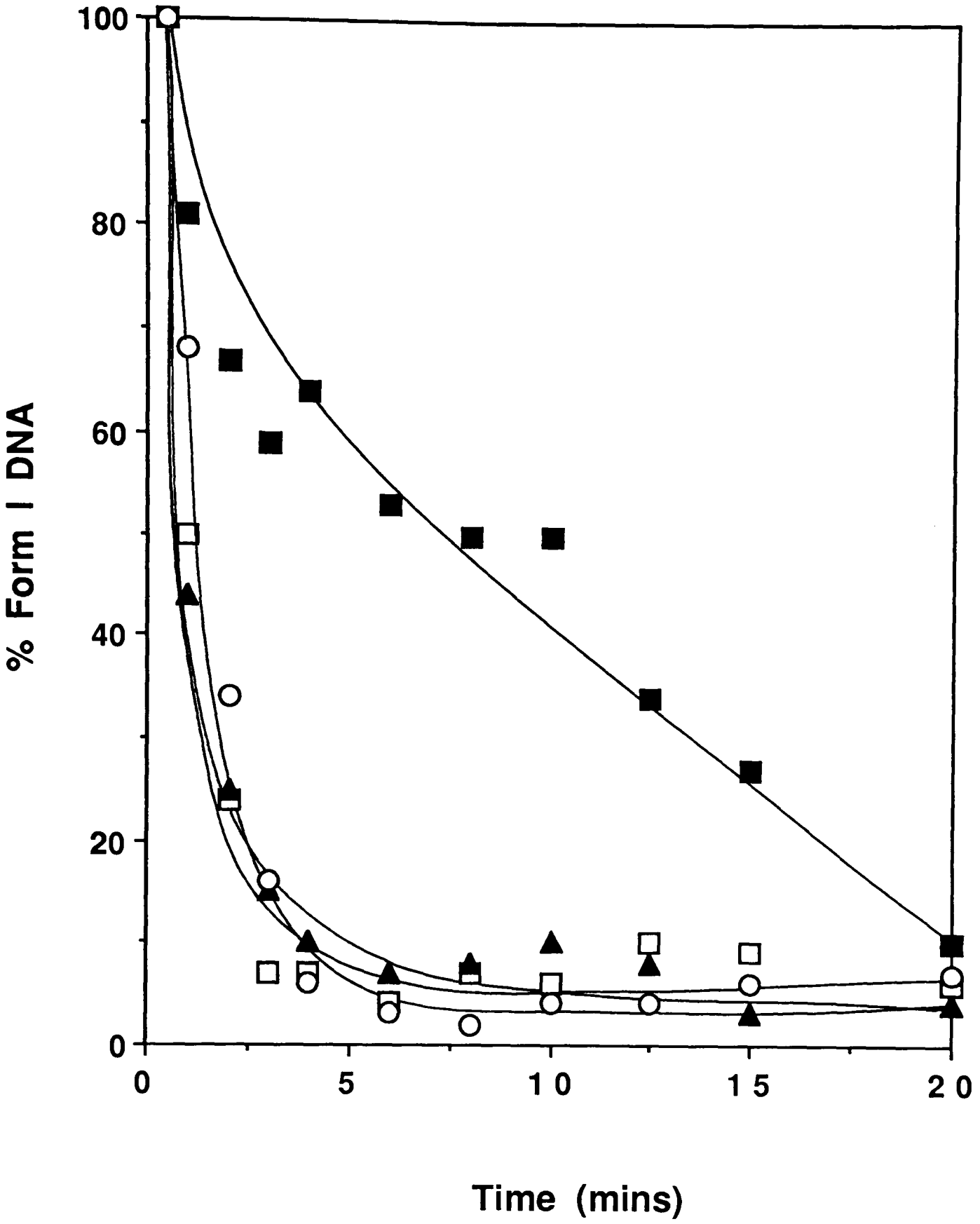


Figure 52 Phosphorylation of human topoisomerase IIα by PKC-α, β1 or γ *in vitro* leads to an increase in the specific relaxation activity of the enzyme. A reaction similar to that shown in Figure 51 was carried and the autoradiograph scanned by densitometry and the % of Form I (supercoiled) DNA plotted against time. Activity is shown in the absence (■) or presence of PKC-α (○), PKC-β1 (□), or PKC-γ (▲).

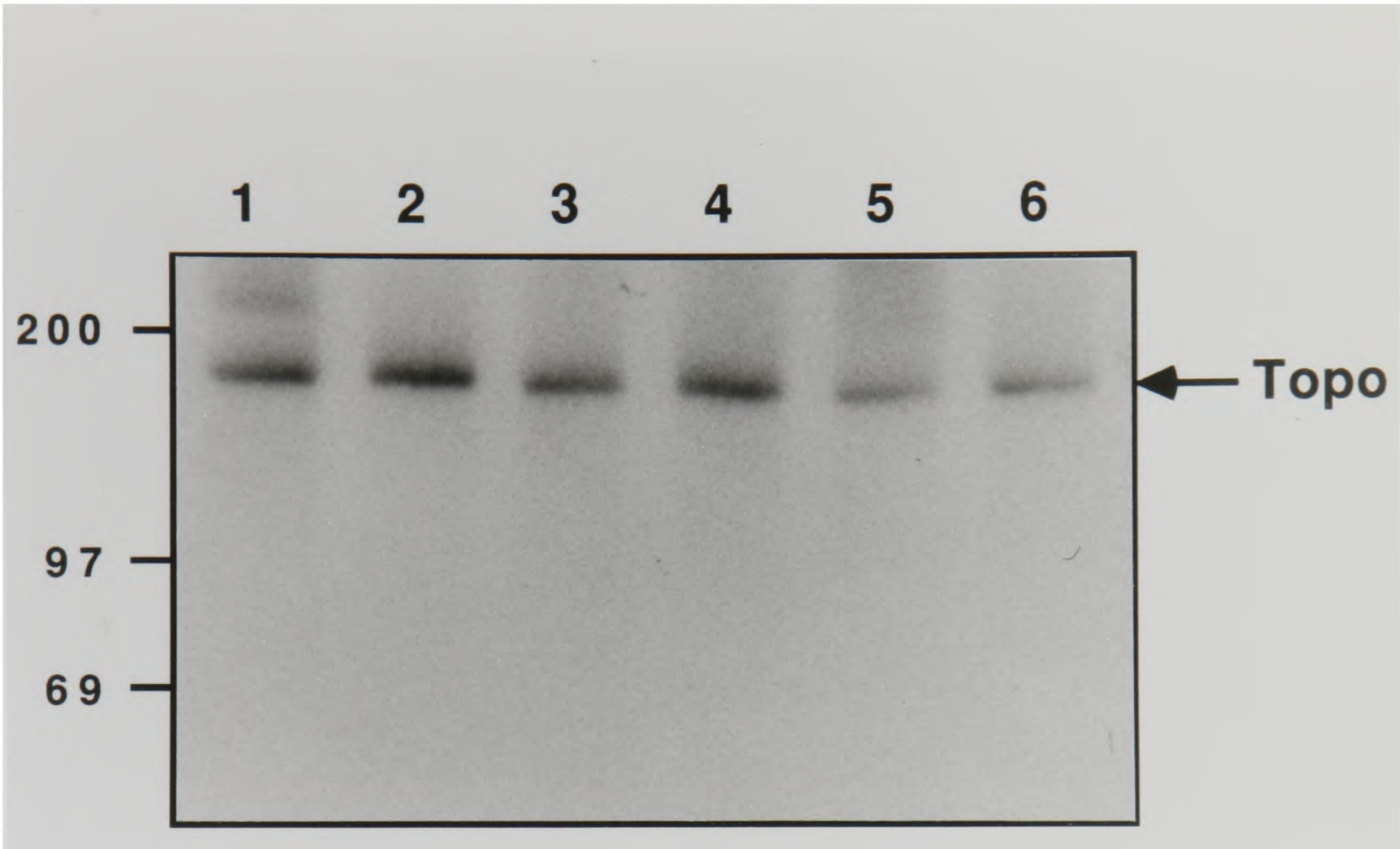


Figure 53 Short-term treatment with PMA or bryostatin does not cause significant changes in the overall level of human topoisomerase IIα phosphorylation *in vivo*. HeLa cells were treated with 0.01% DMSO only (lanes 1 and 2), with 10nM PMA in 0.01% DMSO (lanes 3 and 4) or with 100nM bryostatin-1 in 0.01% DMSO (lanes 5 and 6) for 1 hour. Nuclear extracts were then prepared and human topoisomerase IIα immunoprecipitated using the CRB polyclonal antibody. The samples were electrophoresed on a 7.5% SDS-polyacrylamide gel and the gel was dried and autoradiographed. Molecular weight standards are shown on the left and the position of human topoisomerase IIα protein indicated on the right.

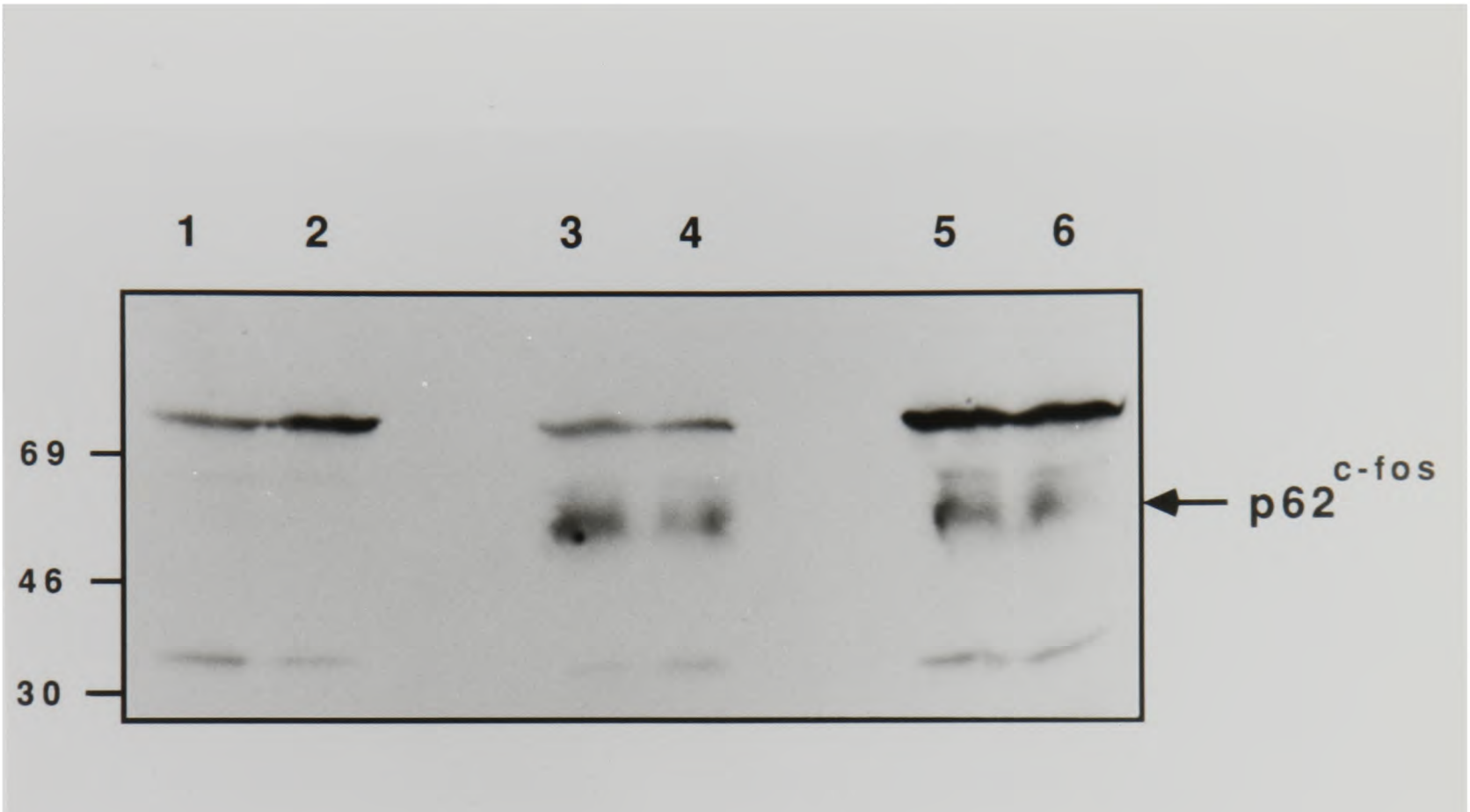


Figure 54 Induction of *c-fos* protein by PMA demonstrates integrity of PKC pathway. HeLa cells were treated with 0.01% DMSO only (lanes 1 and 2), with 10nM PMA in 0.01% DMSO (lanes 3 and 4) or with 100nM bryostatin-1 in 0.01% DMSO (lanes 5 and 6) for 1 hour. Cells were then lysed directly into SDS/sample buffer and electrophoresed on a 10% SDS-polyacrylamide gel. The gel was electroblotted onto membrane and probed with a polyclonal antibody to *c-fos*. Molecular weight standards are shown on the left and the postion of the $p62^{c\text{-}fos}$ protein on the right.

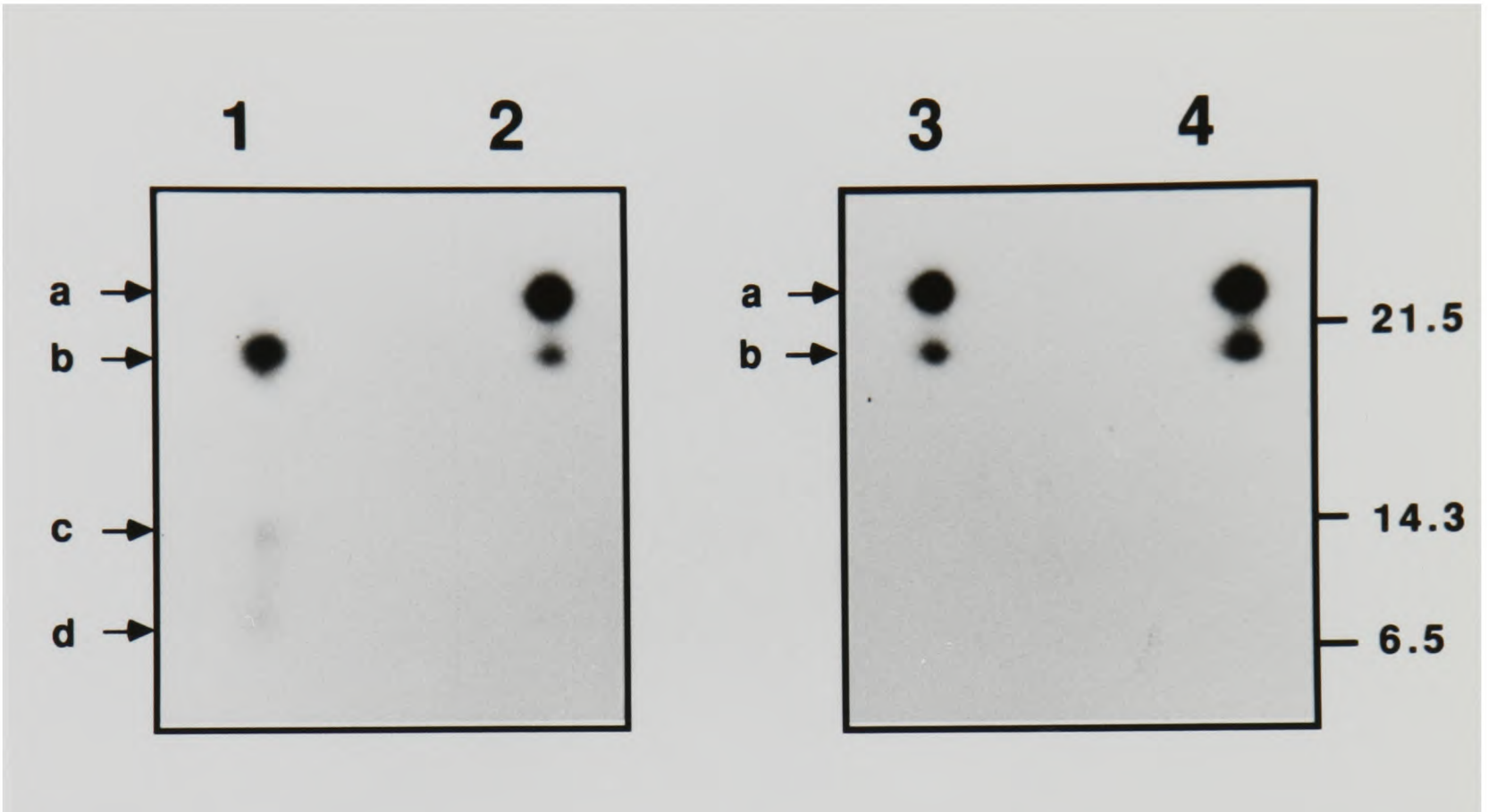


Figure 55 One dimensional phosphopeptide mapping shows that PMA treatment *in vivo* increases the level of phosphorylation of topoisomerase IIα. Topoisomerase IIα protein was labelled *in vivo* with ^{32}P-orthophosphate following no treatment (lanes 2 and 3) or 60 minutes incubation with 10nM PMA (lane 4), or phosphorylated *in vitro* with purified PKC-β2 (lane 1). Phosphopeptides were identified using the one-dimensional electrophoresis method of Cleveland *et al.* (1977). The positions of molecular weight standards are shown on the right and the positions of the 4 major phosphopeptides present in the *in vivo* labelled sample (designated 'a-d') are indicated by arrows on the left.

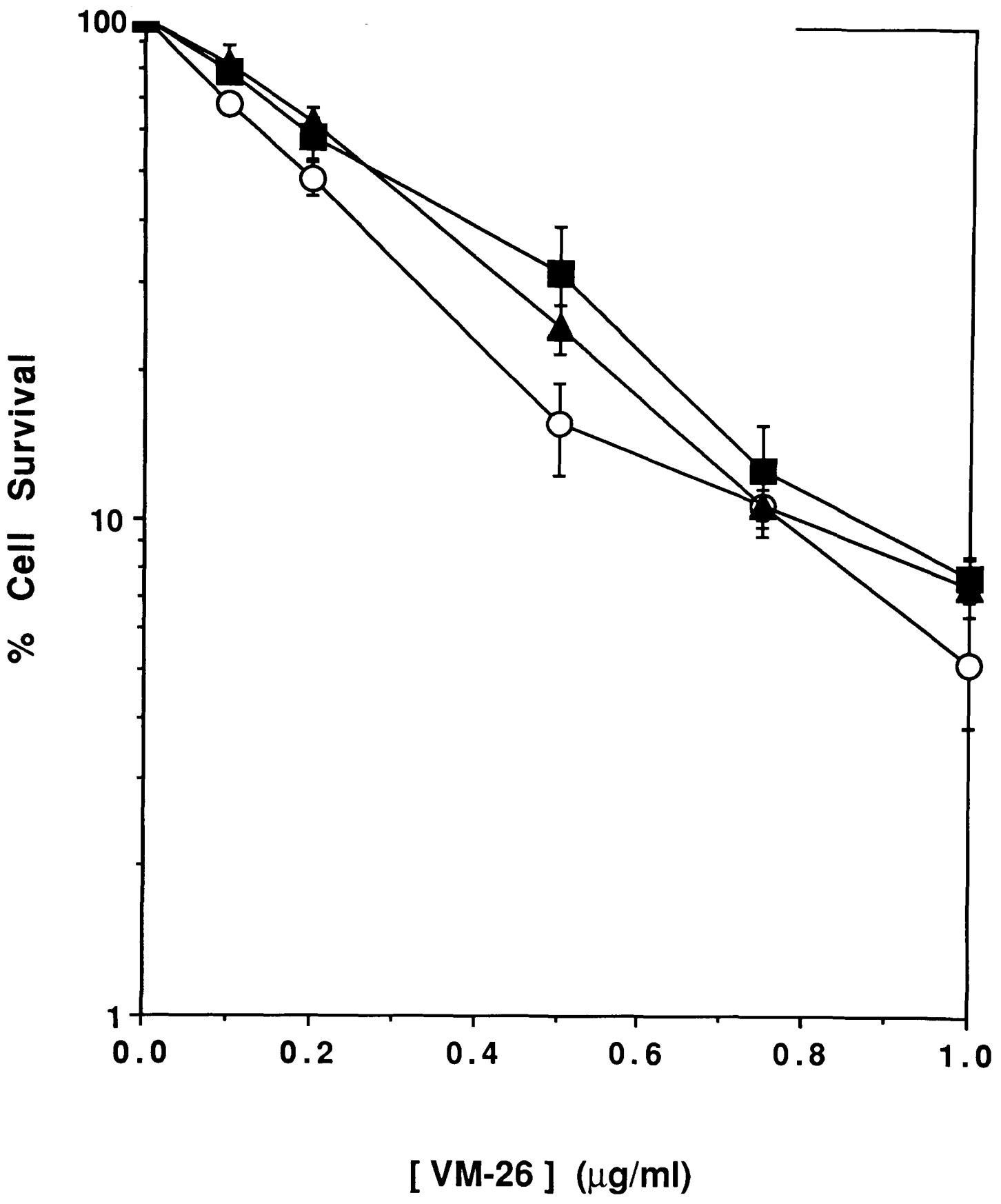


Figure 56 PMA and bryostatin do not significantly alter the degree of cytotoxicity of VM26 to HeLa cells. Clonogenic assays were carried out with HeLa cells following a 60 minute treatment with a range of VM-26 concentrations alone (○), or with 10nM PMA (▲), or with 100nM bryostatin (■). After the 1 hour treatment the drugs were washed off and the cells allowed to grow up into colonies over 10-14 days. Colonies were then counted and plotted against VM-26 concentration. Results show the mean and standard error from four independent experiments.

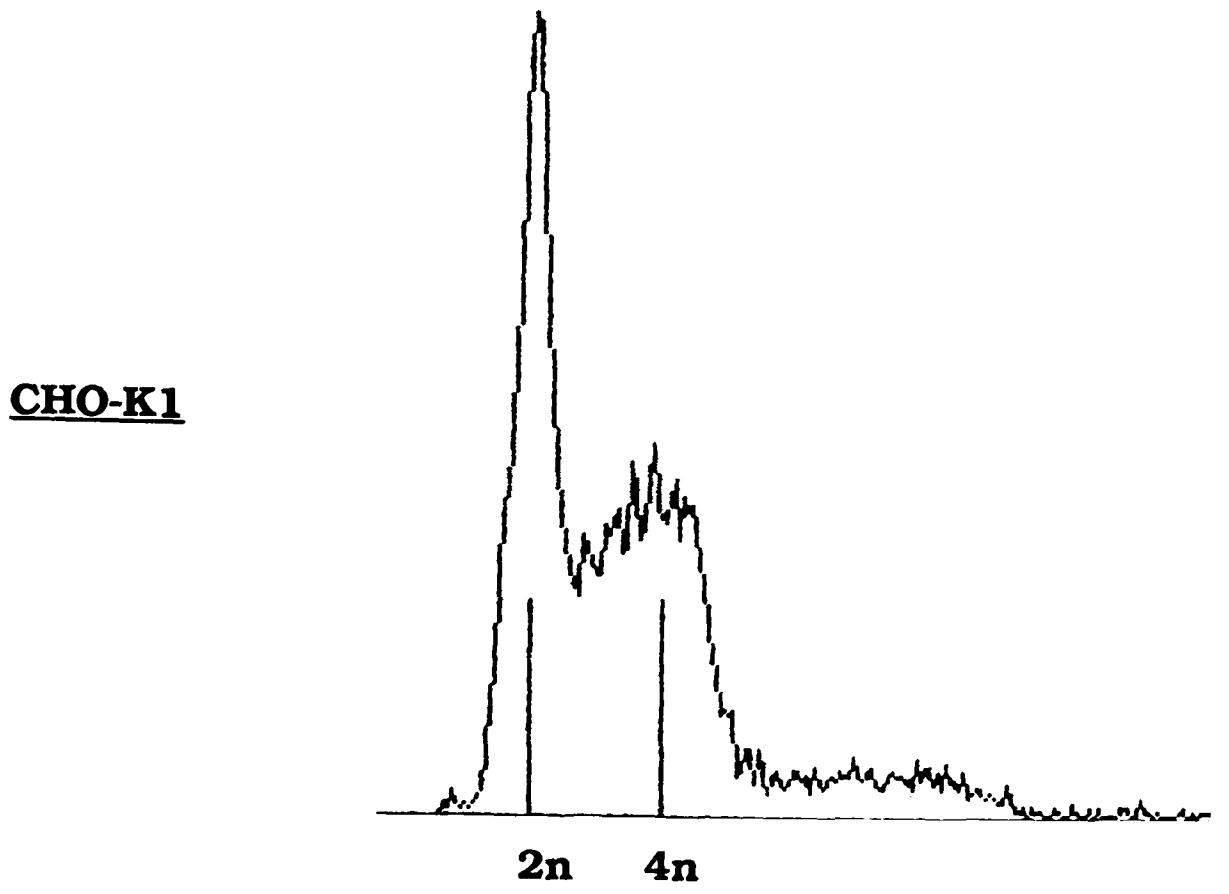


Figure 57 Exponentially growing ADR-1 cells have an altered cell cycle distribution compared to CHO-K1 cells. FACS analysis was carried out on exponentially growing CHO-K1 (top panel) and ADR-1 (bottom panel) cells. The proportion of cells with unreplicated diploid DNA content (2n) compared to replicated diploid DNA content (4n) is shown.

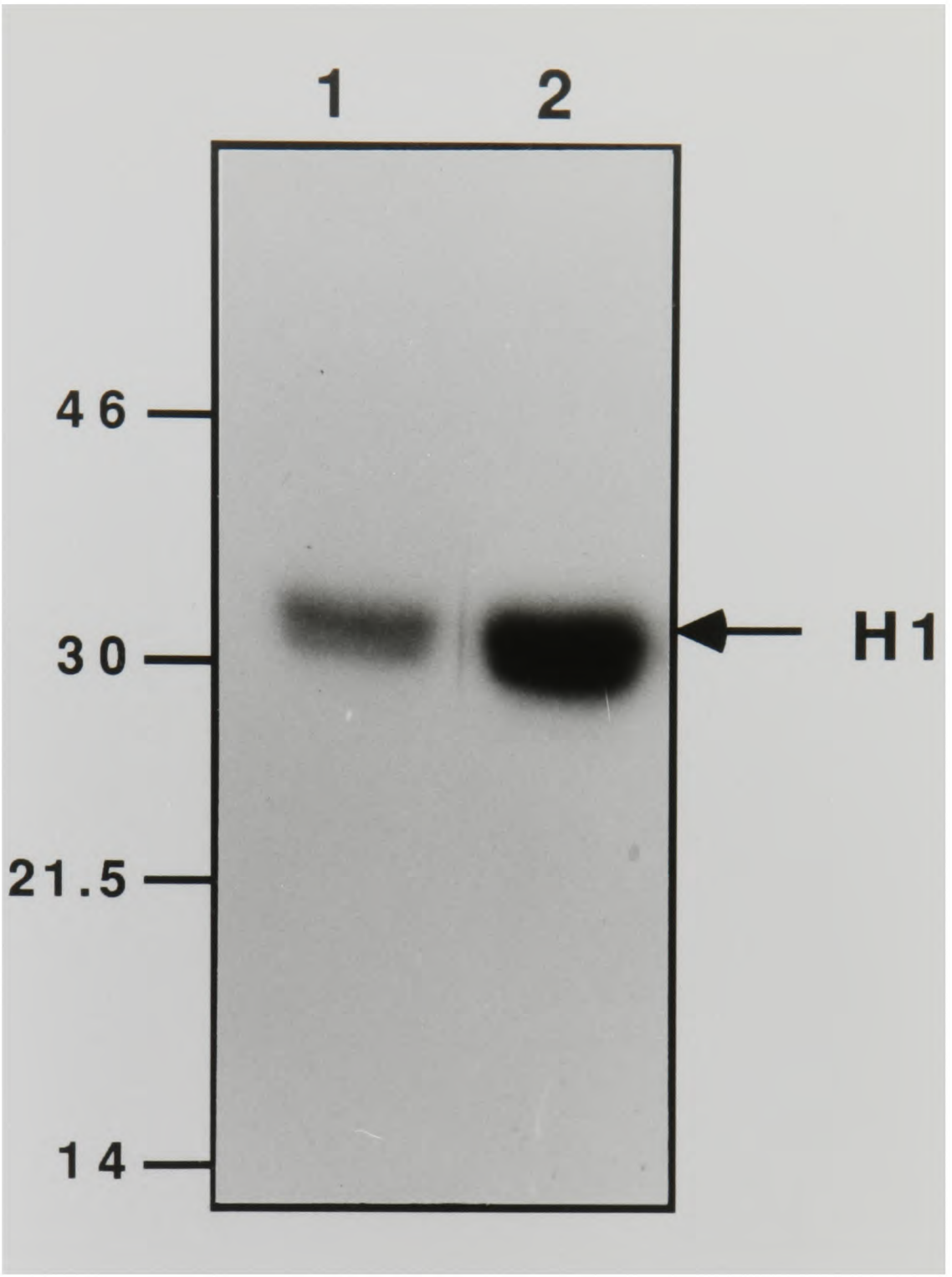


Figure 58 Histone H1 kinase activity of exponentially growing ADR-1 cells compared to CHO-K1 cells. $p34^{cdc2}$ kinase was immunoprecipitated from either CHO-K1 cells (lane 1) or ADR-1 cells (lane 2) in log phase and added to histone H1 in the presence of [γ^{32}P]-ATP. Kinase reactions were carried out for 10 minutes at 30°C and the samples then separated by SDS-PAGE. The position of the phosphorylated histone H1 and the molecular weight standards (kDa) are indicated.

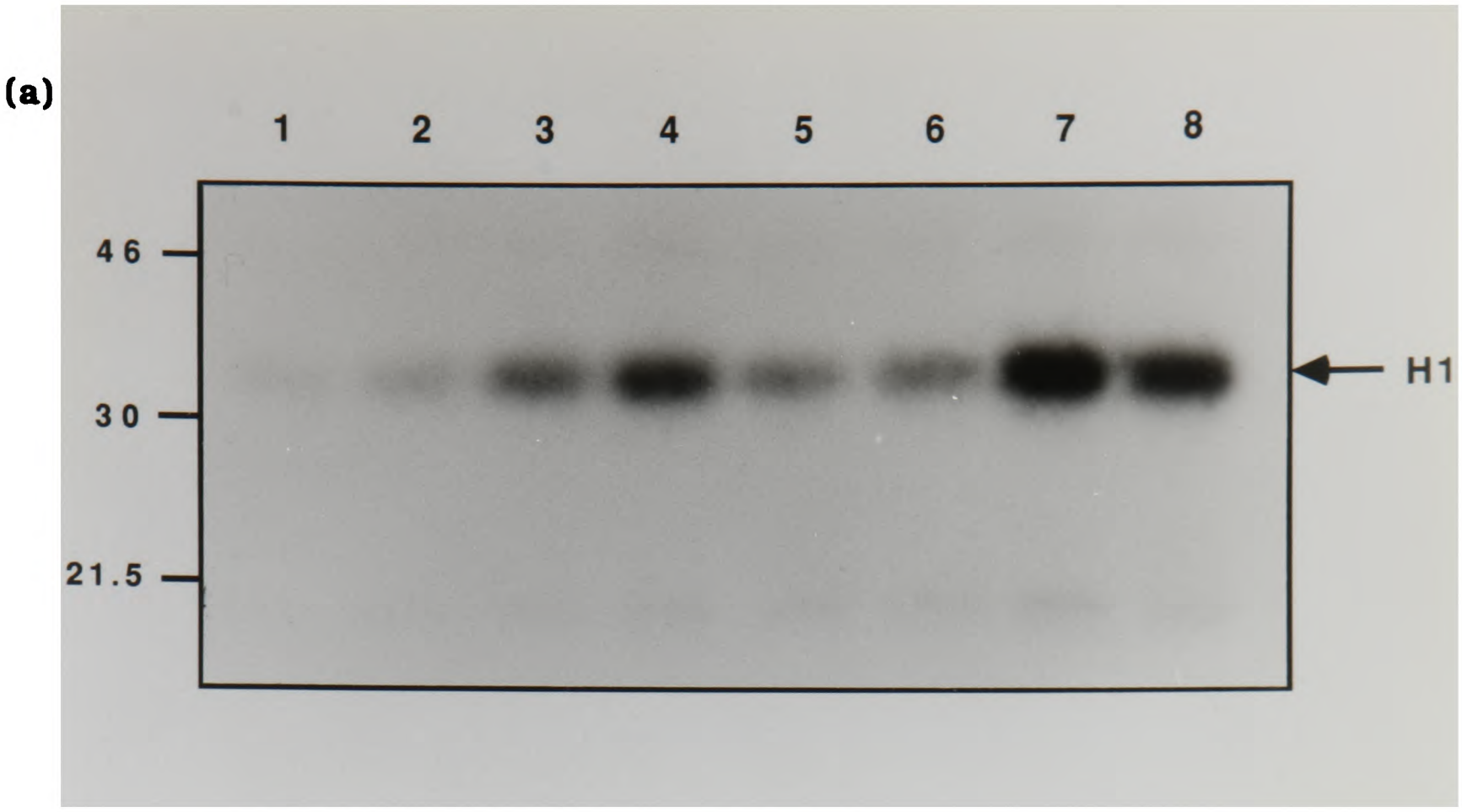


Figure 59 Histone H1 kinase activity of mitotically-arrested and synchronized ADR-1 and CHO-K1 cells. In the top panel (a) $p34^{cdc2}$ kinase was immunoprecipitated from either CHO-K1 cells (lanes 1-4) or ADR-1 cells (lanes 5-8) which had either been untreated (lane 1,2 and 5,6) or treated with 1μg/ml nocodazole for 6 hours prior to harvesting (lanes 3,4 and 7,8). In the bottom panel (b) CHO-K1 and ADR-1 cells (as indicated) were either untreated (U) or synchronized by serum starvation and refeeding; the times of harvesting after refeeding are indicated in hours. Each immunoprecipitate was then added to histone H1 in the presence of [γ^{32}P]-ATP. Kinase reactions were carried out for 10 minutes at 30°C and the samples then separated by SDS-PAGE. The position of the phosphorylated histone H1 and the molecular weight standards (kDa) are indicated.

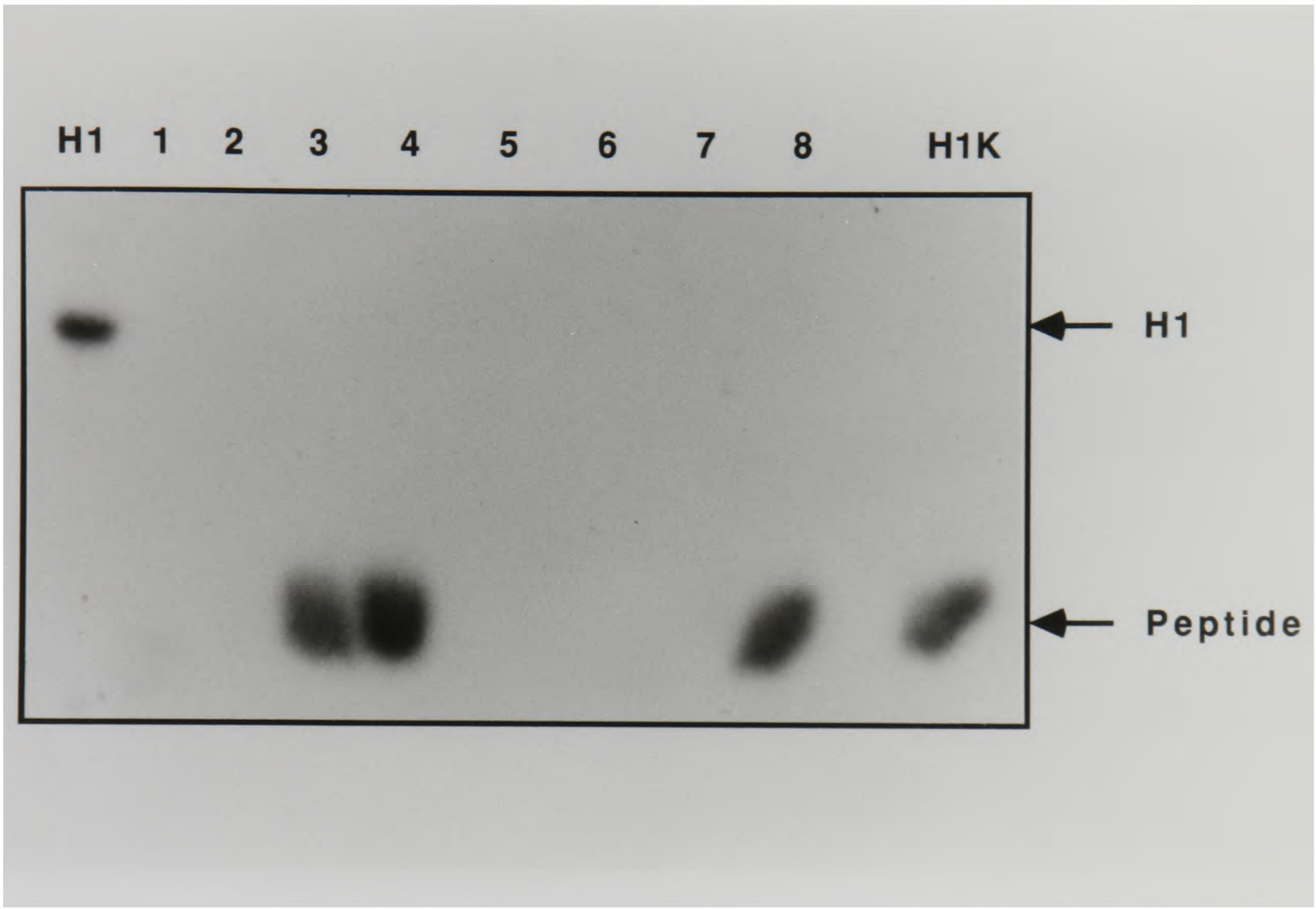


Figure 60 Phosphorylation of human topoisomerase IIα-specific peptides by immunoprecipitated human $p34^{cdc2}$ kinase. Human $p34^{cdc2}$ kinase was immunprecipitated from HeLa cells and added to histone H1 (H1), peptides T2K1 to T2K8 (labelled 1 to 8 respectively), or the histone H1-specific peptide (H1K), in the presence of [γ^{32}P]-ATP. Following phosphorylation the samples were separated by electrophoresis on a 22% SDS-polyacrylamide gel which was then autoradiographed. The position of the phosphorylated histone H1 protein and peptides is shown on the right.

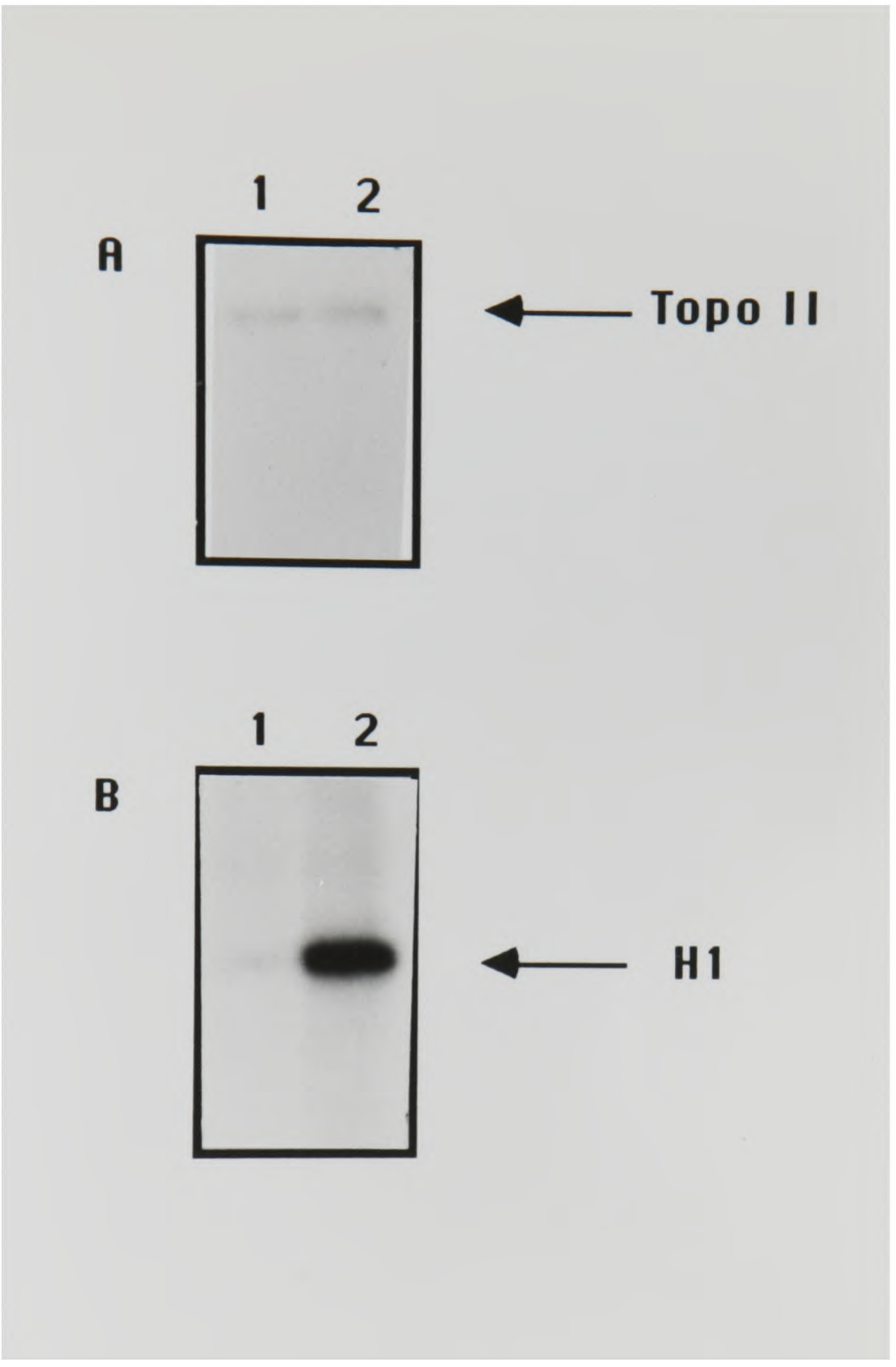


Figure 61 Immunoprecipitated human p34^{cdc2} kinase does not appear to phosphorylate human topoisomerase IIα protein *in vitro*. Purified human topoisomerase IIα protein (top panel) or histone H1 (bottom panel) were incubated with [γ^{32}P]-ATP in the presence of immunoprecipitates from HeLa cells prepared with pre-immune serum (lane 1) or anti-p34^{cdc2} serum (lane 2). After incubation at 30°C for 10 minutes the reactions were stopped and subjected to SDS-PAGE. Dried gels were then autoradiographed. The position of phosphorylated topoisomerase IIα and histone H1 are shown on the right.

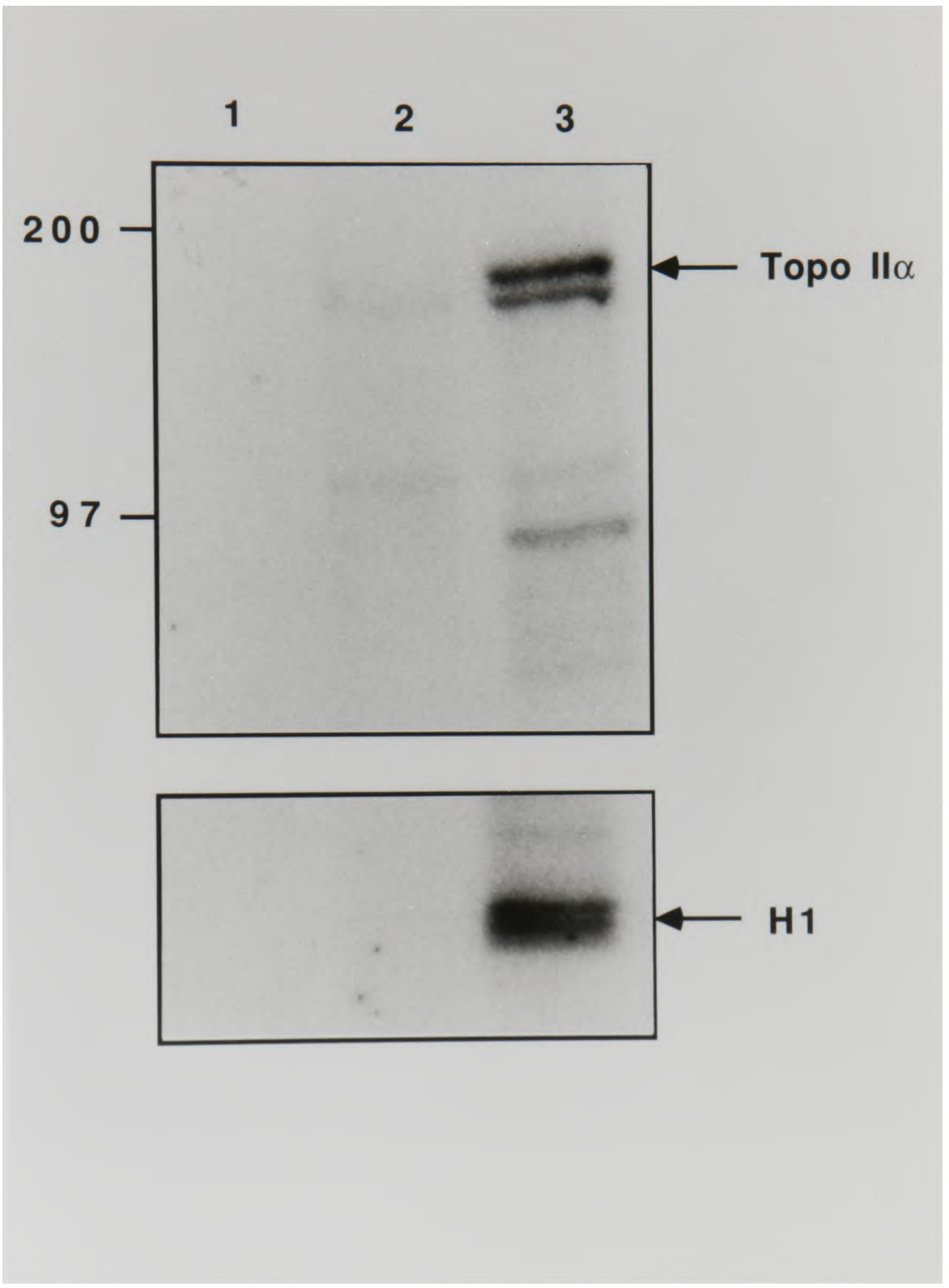


Figure 62 Recombinant chicken $p34^{cdc2}$ kinase phosphorylates human topoisomerase IIα protein kinase *in vitro*. Purified human topoisomerase IIα protein (top panel) or histone H1 (bottom panel) were incubated with [γ^{32}P]-ATP in the absence (lane 2) or presence (lane 3) of recombinant $p34^{cdc2}$ kinase. The kinase was also incubated alone with [γ^{32}P]-ATP (lane 1). After incubation at 30°C for 10 minutes the reactions were stopped and subjected to SDS-PAGE. Dried gels were then autoradiographed. The position of molecular weight standards for the topoisomerase IIα gel are shown on the left, and the position of phosphorylated topoisomerase IIα and histone H1 shown on the right.

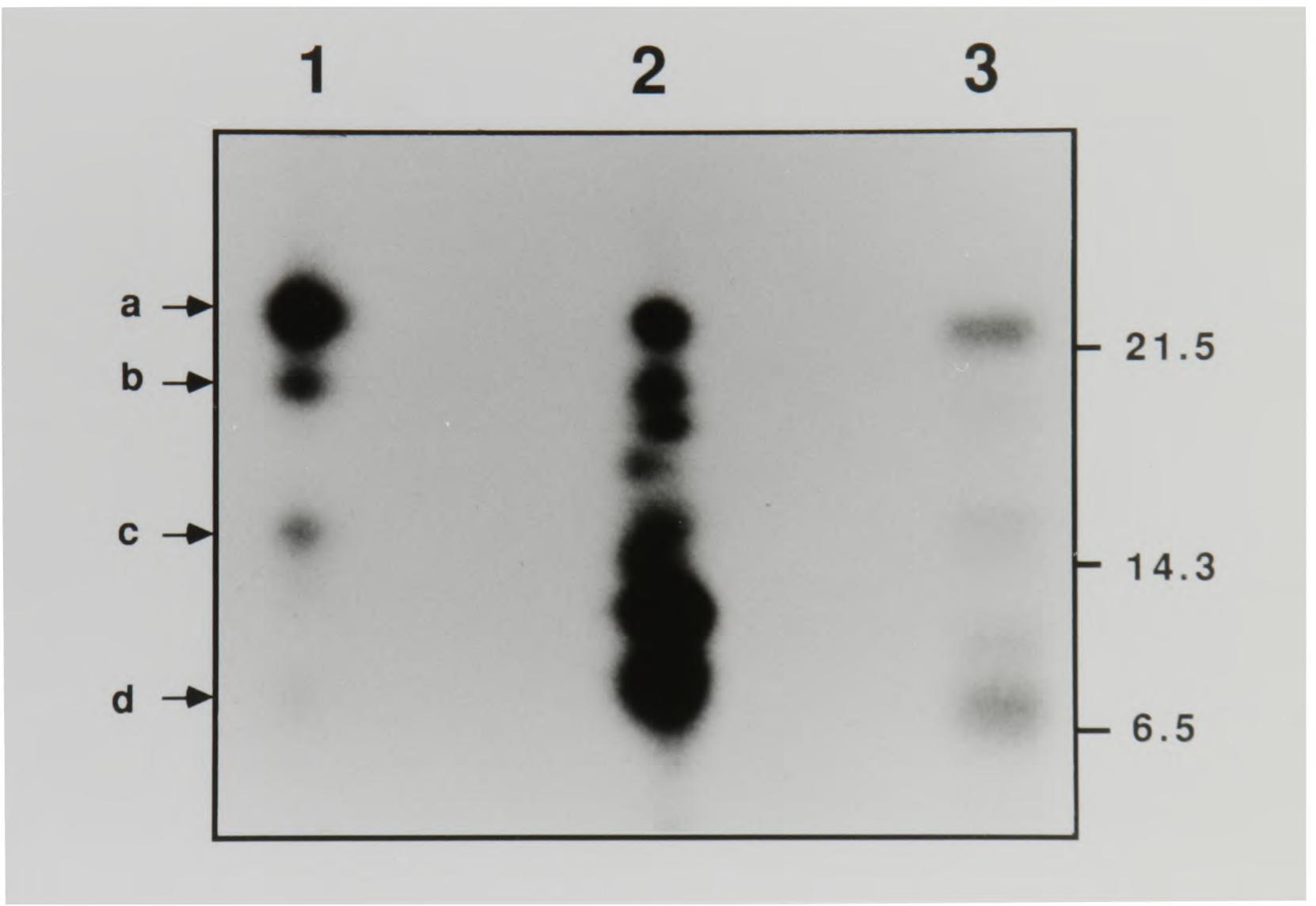


Figure 63 One-dimensional phosphopeptide map of topoisomerase IIα protein phosphorylated by $p34^{cdc2}$ kinase *in vitro*. Topoisomerase IIα was labelled *in vivo* with ^{32}P-orthophosphate (lane 1), or phosphorylated in vitro with [γ^{32}P]-ATP in the presence of CK II (lane 2) or $p34^{cdc2}$ kinase (lane 3). Samples were processed for one-dimensional phosphopeptide mapping as described in section 2.12. The position of molecular weight standards are shown on the right (kDa). The four peptides present in the in vivo labelled sample (lane 1) have been indicated with arrows on the left (a, b, c, d).

Chapter 8 **GENERAL DISCUSSION**

8.1 Does Regulation of Human Topoisomerase IIα Protein occur through Multisite Hierarchical Phosphorylation ?

This study represents the first investigation into the regulation of the two different isozymes of human DNA topoisomerase II protein by phosphorylation. The work demonstrates that the α and β isozymes are phosphoproteins and suggests that both are phosphorylated by multiple kinases *in vivo*. Furthermore, in all cases looked at, phosphorylation of the human topoisomerase IIα protein stimulated its enzymatic activity *in vitro* and dephosphorylation by a general phosphatase reduced the activity to a very low level.

Whilst at least four protein kinases can phosphorylate human topoisomerase IIα protein *in vitro*, some phosphorylation events appear to be more critical than others. For instance, phosphopeptide maps showed that phosphorylation by CK II and PKA appear to account for most of the phosphopeptides produced *in vivo* under non-stimulated conditions. Furthermore, *in vitro* activity could only be restored to dephosphorylated topoisomerase IIα by CK II phosphorylation and not by PKA phosphorylation.

Based on these results, we have built up a speculative model of how topoisomerase IIα protein might be regulated by multisite phosphorylation. It suggests that phosphorylation of topoisomerase IIα protein by CK II may be essential for a basal level of activity. However, it is almost certain that CK II phosphorylates topoisomerase IIα on several sites and it is not clear whether some of these are more critical than others.

The model proposes that following this framework phosphorylation by CK II, the activity of the topoisomerase IIα protein can be further modulated by phosphatases or other kinases through dephosphorylation or additional phosphorylation, respectively (Figure 64). These other kinases not only include PKA, but possibly also PKC and the cell cycle-regulated kinase $p34^{cdc2}$. Although phosphopeptide mapping suggests that in untreated, exponentially growing HeLa cells, most of the phosphorylation is due to either CK II or PKA, other kinases may have a significant effect on function under specific cellular conditions (see below).

Within the time constraints, only a limited number of kinases have been tested for their effect on modulating the activity of human topoisomerase II. It is possible that other kinases may also be involved in this regulation. Of particular interest may be both the calmodulin-dependent protein kinase, as this was shown to phosphorylate the *Drosophila* topoisomerase II (Sayhoun *et al.*, 1986), and the recently reported DNA-activated protein kinase (Lees-Miller and Anderson, 1991). This latter kinase is a nuclear serine/threonine protein kinase that, when activated by DNA, phosphorylates several DNA-binding substrates, including the tumour suppressor protein p53, SV40 large T-antigen, and several transcription factors, e.g. c-fos, Sp1, Oct-1 and SRF.

Phosphorylation of proteins is a reversible modification, and as the activity of topoisomerase II protein is dependent upon its phosphorylation status, it is clear that the action of protein phosphatases can be as crucial to its regulation as the action of protein kinases. We have concentrated on looking at which kinases are responsible for the phosphorylation of topoisomerase II, but equally an investigation should be carried out to look at which phosphatases are responsible for dephosphorylating topoisomerase II and under what circumstances they operate.

Initial studies showed that there are four major protein serine/threonine phosphatases, termed protein phosphatase 1, 2A, 2B and 2C, but, not surprisingly, it has recently been shown that each of these probably represents a family of proteins (reviewed in Cohen *et al.*, 1990, and Tonks, 1990). The catalytic subunits of these phosphatases are thought to form complexes with other regulatory proteins within the cell, and it is highly likely that these regulatory subunits are also subject to control by phosphorylation.

The idea of multisite protein phosphorylation is emerging as potentially important for the regulation of many key proteins in the cell, including metabolic enzymes, cytoskeletal proteins, membrane receptors and nuclear transcription factors (Roach, 1991). Some of these multisite phosphorylations involve independent recognition of multiple sites in the substrate by one or more protein kinases; however, some phosphorylations are interdependent, whereby one phosphorylation event influences another. In these 'hierarchical' phosphorylations, a primary phosphorylation affects the course of a secondary phosphorylation usually by a different kinase. This could occur at close range where a phosphorylated amino acid forms part of the recognition

sequence for a kinase, or at longer range, where the primary phosphorylation event affects the secondary event through allosteric changes in the protein.

An excellent example, and a potential model for topoisomerase II, is glycogen synthase. This metabolic enzyme is not a substrate for glycogen synthase kinase 3 (GSK-3) when totally dephosphorylated. Upon introduction of phosphate at Ser-656 (in rabbit glycogen synthase) by CK II, the protein becomes a substrate for GSK-3, which sequentially modifies four serine residues (Roach, 1990).

Another example of interdependent phosphorylation is that of DARPP-32 (dopamine- and cAMP-regulated phosphoprotein). This protein is an inhibitor of protein phosphatase-1, and is enriched in dopaminoceptive neurons possessing the D1 dopamine receptor. Phosphorylation of DARPP-32 by CK II at Ser-45 and/or Ser-102 facilitated phosphorylation of Thr-34 by PKA, with a 2.2-fold increase in the V_{max} and a 1.4-fold increase in the apparent K_m (Girault *et al.*, 1989). Phosphorylation of DARPP-32 by CK II *in vivo* may therefore modulate its phosphorylation in response to an increased level of cAMP.

We have not yet demonstrated whether the different phosphorylations of human topoisomerase IIα protein are interdependent. The model proposes only that CK II phosphorylation is essential for regulation of activity by other kinases, such as PKA or PKC. Current work is in progress in the laboratory to ascertain whether there is hierarchical phosphorylation of human topoisomerase IIα protein. In other words, is CK II phosphorylation required for topoisomerase IIα to become a substrate for PKA? As PKA recognizes serines and threonines in a positively charged target environment, one would not expect a phosphorylated residue to increase the likelihood of phosphorylation of a neighbouring residue by PKA. However, CK II phosphorylation may cause an allosteric conformational change in a domain of the protein which increases the rate of phosphorylation of a more distant site by PKA.

8.2 How does Topoisomerase II Phosphorylation affect its Catalytic Cycle ?

Another important aspect to investigate is how phosphorylation leads to an increase in the catalytic rate of the topoisomerase II reaction. Work on the *Drosophila* topoisomerase II enzyme suggested that CK II phosphorylation had no effect on any of the reaction steps which

preceded hydrolysis of the enzyme's high energy co-factor, ATP (Corbett *et al.*, 1992). This included the enzyme's binding to DNA and recognition of DNA topology, its prestrand passage DNA cleavage/religation equilibrium, its double-stranded DNA passage event, and its post-strand passage DNA cleavage/religation equilibrium. In contrast, the rate of ATP hydrolysis was stimulated by CK II phosphorylation to the same degree as the increase in overall catalytic rate. Hence the conclusion was that CK II phosphorylation stimulated the enzyme's ability to hydrolyse ATP and subsequently initiate a new round of catalysis.

Gel retardation assays showed that phosphorylation of *S. cerevisiae* topoisomerase II by CK II led to an increase in the association rate of topoisomerase II with DNA, although the binding affinity was unaltered (Alghisi *et al.*, 1992). This is consistent with the possible enhancement of protein-DNA interactions during chromosome condensation.

Another study has looked at the effect of phosphorylation on the discrete catalytic steps of *Drosophila* topoisomerase II in the presence of the topoisomerase II poisons, etoposide and m-AMSA (DeVore *et al.*, 1992). Although phosphorylation had little effect on the DNA cleavage/religation equilibrium in the absence of drugs, phosphorylation by CK II or PKC led to a decrease in the level of drug-induced DNA cleavage products. This correlated with an increased rate of DNA religation mediated by phosphorylated topoisomerase II. Hence, under these conditions, phosphorylation of topoisomerase II by CK II and PKC should lead to a reduced sensitivity to these drugs.

Clearly this work is at an early stage in these lower eukaryotes, and there are no reports of how phosphorylation modulates the human topoisomerase II catalytic cycle. It is possible that phosphorylation may affect different catalytic steps in the mechanism of the topoisomerase IIα isozyme from the topoisomerase IIβ isozyme, since the isozymes appear to operate differently in terms of the former having a more distributive, and the latter a more processive, mechanism (see sections 1.5 and 1.12)

The final step in understanding how phosphorylation controls topoisomerase II isozyme function, is to investigate how phosphorylation allows each isozyme to correctly carry out its biological role in the cell. In other words, what is the function of each independent phosphorylation event on the topoisomerase II isozyme. It is unlikely that phosphorylation events catalysed by different kinases all perform the same function or all have the same effect on topoisomerase II activity *in vivo*.

Based on the results presented here, two areas stand out where phosphorylation of topoisomerase II may be playing a very important biological role: one is in regulation of the cell cycle, and the other is in signal transduction. Unfortunately, a major problem at the moment is that no biological role has been clearly defined for one or other particular isozyme of topoisomerase II. This makes defining the biological role of phosphorylation of one particular isozyme that much more difficult. From studies of their different expression patterns, it has been proposed that the α isozyme is involved in the mitotic function(s) of topoisomerase II and in the nuclear scaffold, whereas the β isozyme may play a structural role in the nucleolus and be important for the transcription of the ribosomal genes. Definitive evidence for these suggestions is still awaited.

8.3 Does Topoisomerase II Phosphorylation have a Role in Cell Cycle Control ?

One of the best defined functions of yeast topoisomerase II is its role in mitosis. Lack of topoisomerase II decatenation activity leads to mitotic arrest, due to an inability to condense chromosomes, and a failure to segregate replicated daughter chromosomes at anaphase (Holm *et al.*, 1985; Uemura *et al.*, 1987a; Adachi *et al.*, 1991). The cell cycle-dependent expression of the human topoisomerase IIα protein, with peak levels at mitosis, is evidence that this isozyme is carrying out the essential decatenation functions in mitosis in human cells, which have been shown to be essential for cell division in yeast. Furthermore, the level of phosphorylation of yeast topoisomerase II and the human topoisomerase IIα isozyme, are both significantly elevated at mitosis.

One obvious question is which kinase is responsible for this cell cycle-dependent phosphorylation? We have presented evidence that purified human topoisomerase IIα protein can be phosphorylated *in vitro* by the cell cycle-regulated kinase, $p34^{cdc2}$. This kinase has also been shown to phosphorylate the yeast topoisomerase II protein *in vitro*, although there was no evidence that the sites phosphorylated *in vitro* by $p34^{cdc2}$ were the sites that increased in phosphorylation at mitosis (Cardenas *et al.*, 1992). Indeed in this species it appeared to be sites phosphorylated by CK II which became more phosphorylated at mitosis. As there is scant evidence that CK II is itself cell cycle-regulated, the situation is likely to be more complex. If confirmed, the report that CK II is phosphorylated by $p34^{cdc2}$ kinase (Mulner-Lorillon *et al.*, 1990), would

suggest one possible mechanism by which topoisomerase II becomes hyperphosphorylated at mitosis.

So what is the role of this phosphorylation at mitosis? The fact that mitotic chromosomes immunostain strongly for the presence of topoisomerase II protein (Earnshaw *et al.*, 1985; Gasser *et al.*, 1986), suggests that phosphorylation does not simply occur to dislodge the protein from the DNA, as has been suggested as the purpose of other mitotic phosphorylations (Moreno and Nurse, 1990). This leaves two broad possibilities: the first is that the high level of decatenation activity required at mitosis is achieved through having a high protein content, which is either stabilized or further activated through phosphorylation. The second is that the phosphorylation and dephosphorylation of topoisomerase II plays a more specific role in controlling the timing or sequence of mitotic events.

The possible effect of phosphorylation on the enzymatic mechanism of topoisomerase II has already been discussed earlier in this chapter. However, regulation of protein stability through phosphorylation may be as relevant *in vivo*, as modulation of specific activity. The sudden decrease in topoisomerase IIα content as cells exit mitosis in vertebrate cells appears to be the result of a sudden decrease in stability of the protein, such that it is rapidly degraded. Other examples of proteins which are rapidly degraded in late mitosis are the cyclins, which make up part of the maturation promotion factor (MPF) complex of cell cycle control, and the thymidine kinase protein. Degradation of cyclins is an essential step in cells being able to switch off MPF activity and exit mitosis.

An important question is whether the dephosphorylation of topoisomerase IIα at this point in the cell cycle is a cause or effect of its loss of stability. It is possible that *in vivo* the main function of phosphorylation changes is to control the stability of the topoisomerase IIα protein. Hence the increase in phosphorylation across S-phase and G_2 enables a build-up of protein to occur, which is suddenly degraded upon dephosphorylation in late mitosis. Clearly then, the question of regulation of topoisomerase IIα protein stability through phosphorylation is one that needs to be addressed. Cyclins appear to have a particular protein sequence motif, termed the "destruction box", which directs their ubiquitin-mediated degradation (Hunt, 1991). Although topoisomerase IIα does not contain a homologous sequence, it is possible that another functional motif will be identified which directs its degradation in an equally specific manner.

A recent study used the phosphatase 1 and 2A inhibitor, okadaic acid, to look at the general effect on mitosis of inhibiting these phosphatases in HeLa cells (Ghosh *et al.*, 1992). At concentrations which specifically inhibit only phosphatase 2A no effects were seen. However, at concentrations which also inhibit phosphatase 1, major defects appeared in the cells ability to complete mitotic events. Mitotic chromosomes became scattered, the mitotic spindle deranged and cells failed to enter anaphase i.e. the cells could not segregate or decondense their chromosomes. Removal of okadaic acid allowed chromosome decondensation and reformation of the nuclear envelope, although this usually led to multinucleate cells, due to the scattered chromosomes.

Genetic experiments in yeast have also shown that whilst phosphatase 2A is required for entry into mitosis, phosphatase 1 is required later in mitosis (Kinoshita *et al.*, 1990). It will be interesting to find out whether topoisomerase IIα is one of the proteins which need to be dephosphorylated in order to allow late mitotic events to occur. Initially, one would need to look *in vitro* whether phosphatase 1 can significantly dephosphorylate human topoisomerase IIα protein at the sites that get dephosphorylated *in vivo* on M to G_1 transition.

8.4 Do Signal Transduction Pathways modulate Topoisomerase II Phosphorylation ?

Apart from their crucial role in cell division and chromosome structure, the main function of DNA topoisomerases appears to be in the active processes of DNA metabolism, namely transcription, replication, recombination and repair. The exact role of topoisomerases in these processes is still not well understood and has already been discussed in section 1.6. Clearly though, not all these processes are proceeding at an equal rate in the cell all of the time. Growth stimuli which impinge upon a quiescent cell may stimulate a burst of transcriptional activity, a move into the replicative phase of the cell cycle, and an elevated repair requirement as a result of this increase in DNA metabolism. Hence, it seems reasonable that coordinate regulation may exist between the requirement for elevated topoisomerase II activity under these circumstances and the precise catalytic activity of topoisomerase II.

The activation of topoisomerase II must be an end-point of a signal transduction pathway initiated by the growth stimulus. Many signal transduction pathways use cascades of protein kinases to exert their

diverse cellular effects. Perhaps the best characterized protein kinases with respect to their roles in signal transduction are PKA and PKC (see sections 6.1 and 7.1.1 respectively), both of which have been shown here to phosphorylate human topoisomerase IIα isozyme *in vitro*. However, CK II has also been implicated in growth control (see section 5.1). Hence it is possible that the growth-stimulated regulation of topoisomerase IIα is carried out primarily by CK II, but that PKA and PKC may also regulate topoisomerase IIα activity, both through direct phosphorylation of topoisomerase IIα protein and through direct or indirect phosphorylation of CK II.

The use of kinase cascades allows the amplification and propagation of responses to external growth signals. It also allows complex regulation through an integrated network of protein kinases and phosphatases, leading to growth-related protein phosphorylation and dephosphorylation. Each individual phosphorylation state of the topoisomerase II protein is therefore a precise function of the overall state of these pathways at any one moment. Hence the apparent complex nature of topoisomerase II phosphorylation may simply be one way of allowing this universal player in DNA metabolism to integrate and respond to the myriad of instructions that are being directed at it via multiple signal transduction pathways.

8.5 Future Directions.

Previous work into the regulation of type II topoisomerases by phosphorylation had been carried out primarily in lower eukaryotes and particularly in *Drosophila* and S. *cerevisiae*. These studies had demonstrated that CK II and PKC phosphorylation could modulate the enzyme activity in these species, although the biological function of these phosphorylations is not known. However, higher eukaryotic species have been shown to possess two genetically distinct isozymes of topoisomerase II about which very little is known concerning their specific patterns of post-translational modification. This study has gone some way to answering these questions in human cells and may be of particular relevance to the development of topoisomerase II poisons for new chemotherapeutic approaches.

Ultimately, with an understanding of the regulation of human type II topoisomerases, the goal will be to modify topoisomerase II activity in conjunction with the administration of topoisomerase II poisons in the

hope of improving the clinical efficacy of these compounds. The data presented here on the effect of phosphorylation on topoisomerase II activity supports the reports in the literature that the cytotoxic effect of these compounds can be modulated by cellular factors such as protein kinases (Darkin-Rattray and Ralph, 1991; Sinha and Elliot, 1991).

At the moment, it is not clear which isozyme of human topoisomerase II is targetted by the various topoisomerase II poisons used in chemotherapy are targeting (discussed more in the Appendix). Until this is known, the effect of modulating topoisomerase II activity through phosphorylation cannot be predicted. A detailed study of the regulation of the α isozyme of human topoisomerase II has been presented here. Future work on topoisomerase II phosphorylation will need to address the same questions with the β isozyme. Some preliminary work on topoisomerase IIβ phosphorylation has also been reported here. Initially, similar approaches to those used here for topoisomerase IIα can be carried out for topoisomerase IIβ. These include the purification of human topoisomerase IIβ protein, perhaps through the use of an antibody-affinity column prepared with the β-specific monoclonal antibodies discussed in chapter 3, to measure *in vitro* activity changes upon phosphorylation, and the study of *in vivo* phosphorylation by immunoprecipitation and phosphopeptide mapping.

Once the basic regulation of both human topoisomerase II isozymes by phosphorylation has been worked out, more detailed studies can be carried out into the control and function of topoisomerase II phosphorylation. Mapping the specific sites of phosphorylation is currently underway in this laboratory using a variety of approaches. Direct sequencing of radiolabelled phosphopeptides from tryptic maps can be carried out in conjunction with manual Edman degradation to pinpoint the phosphorylated residue in the peptide. The C-terminal domain containing many of the potential sites of phosphorylation has been overexpressed in bacteria (C. Addison, unpublished results) and this can be used to identify phosphorylation sites using site-directed mutagenesis combined with two-dimensional mapping. One-dimensional mapping following more selective cleavage of the protein, such as with formic acid or cyanogen bromide, should also help localise the phosphorylation sites.

Most importantly, the cellular function of topoisomerase II phosphorylation must be identified. Initially, the functional effects both *in vitro* and *in vivo* of mutating the phosphorylation sites will be studied. These experiments could be done in a yeast $top2^{ts}$ mutant which,

according to unpublished data (J. Jenkins, this laboratory and O. Westergaard, University of Aarhus, personal communication), can be complemented at the restrictive temperature by both the human topoisomerase IIα and β isozymes. Only then will it become apparent what is the role of the different phosphorylation states of the topoisomerase II isozymes.

In conclusion, it is hoped that the work presented here, together with future experiments such as those discussed in this chapter, will reveal a clear picture of how the two isozymes of human topoisomerase II are regulated through phosphorylation with respect to their specific roles in DNA metabolism, mitotic processes and nuclear structure.

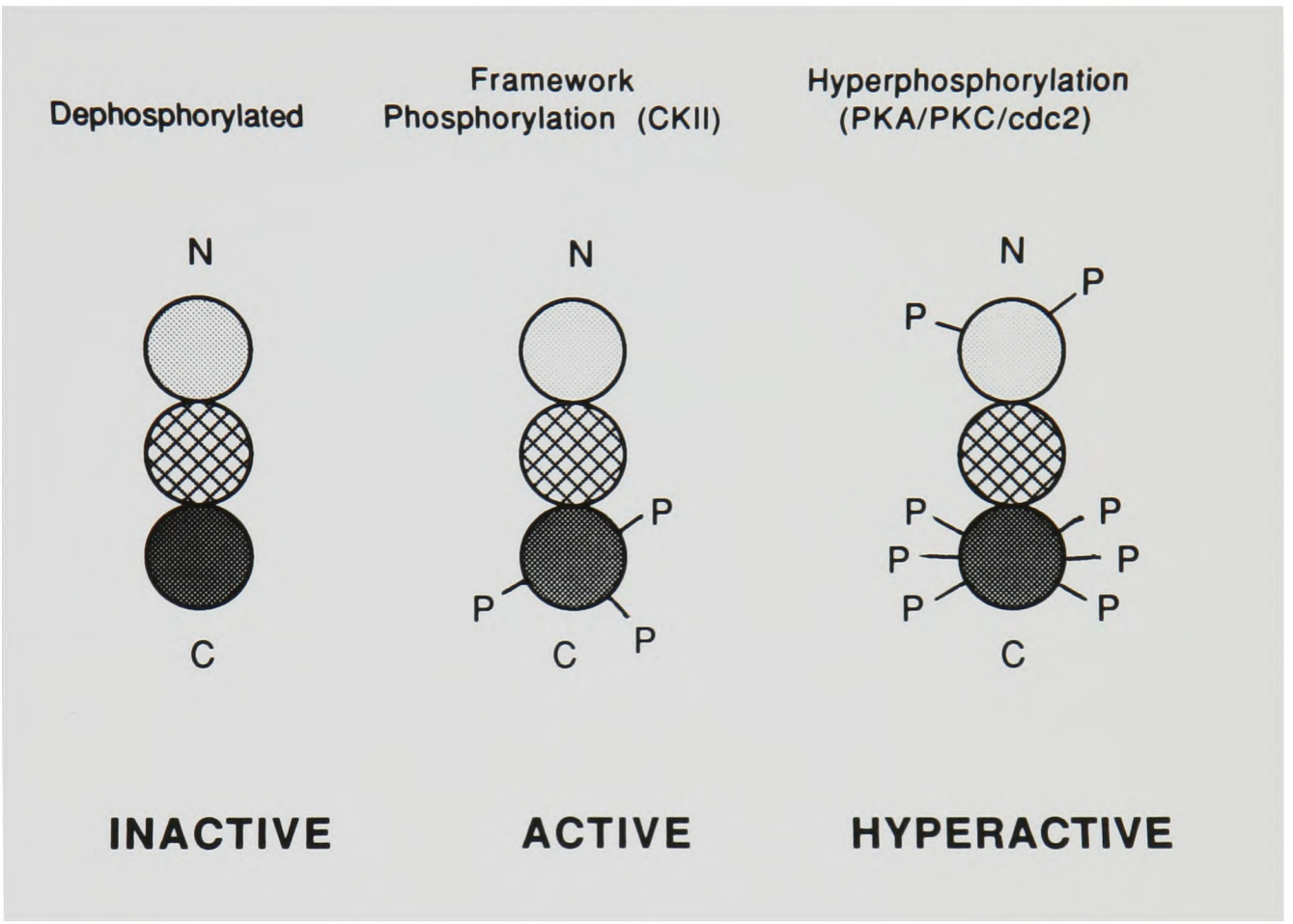


Figure 64 Hypothetical model of regulation of human topoisomerase IIα protein through multisite heirarchical phosphorylation. Completely dephosphorylated topoisomerase IIα protein is either completely or virtually inactive (left hand figure). CK II provides the framework phosphorylation necessary for activity (central figure), which can then be further stimulated through phosphorylation by other kinases (right hand figure). Phosphorylation sites for CK II appear to be primarily clustered in the C-terminal domain, although data from studies in yeast suggest phosphorylation may also occur at the extreme N-terminus (Shiozaki and Yanagida, 1991).

Appendix **Use of Purified Human Topoisomerase IIα Protein to Screen for Novel Topoisomerase II Poisons.**

Most of the topoisomerase II poisons commonly used both clinically and for experimental purposes do not exert their cytotoxic effects purely through topoisomerase II. For instance, anthracyclines such as doxorubicin (Adriamycin) and daunomycin are known to generate free radicals in cells which can lead to DNA damage and lipid peroxidation (Keizer *et al.*, 1990). Adriamycin also inhibits cytochrome c oxidase in mitochondria (Goormaghtigh *et al.*, 1982). Mitoxantrone inhibits polymerisation of microtubules (Ho *et al.*, 1991) and therefore could cause mitotic arrest independently of action on topoisomerases. Novobiocin and nalidixic acid, as well as having some effect on prokaryotic and eukaryotic topoisomerases, are primarily ATPase inhibitors (Gallagher *et al.*, 1986). Amsacrine (m-AMSA), another topoisomerase II inhibitor regarded as being highly specific, also inhibits aldehyde oxidase (Gormley *et al.*, 1983).

Furthermore, since the identification of two isozymes for topoisomerase II in human cells, there have only been a few reports demonstrating which isozyme each topoisomerase II poison specifically targets. Harker *et al.* (1991) demonstrated that mitoxantrone appeared to inhibit mainly topoisomerase IIβ, with little effect on topoisomerase IIα. Conversely, VM-26 and merbarone appear to preferentially inhibit topoisomerase IIα, requiring concentrations 3-fold and 8-fold lower respectively, than those required for equivalent inhibition of topoisomerase IIβ (Drake *et al.*, 1989).

The future development of more highly specific topoisomerase II poisons relies on getting a better understanding of the drug-topoisomerase II interaction and the reasons why this interaction leads to cell death. Part of this understanding is to determine the inhibitory effect of currently used and potentially important new drugs on each individual isozyme of topoisomerase II. Having purifed to near-homogeneity the human topoisomerase IIα isozyme, it has been possible to carry out a number of studies on the inhibitory effects of novel drug compounds on this isozyme in collaboration with other groups. Two of these studies are discussed here showing one compound which appeared to have no effect on the topoisomerase IIα isozyme activity and another similar drug which strongly inhibited it.

GR63178A (Figure 65) is a water-soluble analogue of the pentacyclic pyrolloquinone mitoquidone and is currently being clinically evaluated as an anticancer drug. Its mechanism of action is unknown but may be related to its quinone group or planar ring system, which are similar to those found in the anthracyclines, doxorubicin and daunomycin. As these latter drugs have been shown to stabilize the cleavable complex formed by topoisomerase II, the effect of GR63178A on the decatenation activity of topoisomerase IIα was measured. The effect of GR54374X (Figure 65), the major metabolite of GR63178A, was also measured in a decatenation assay.

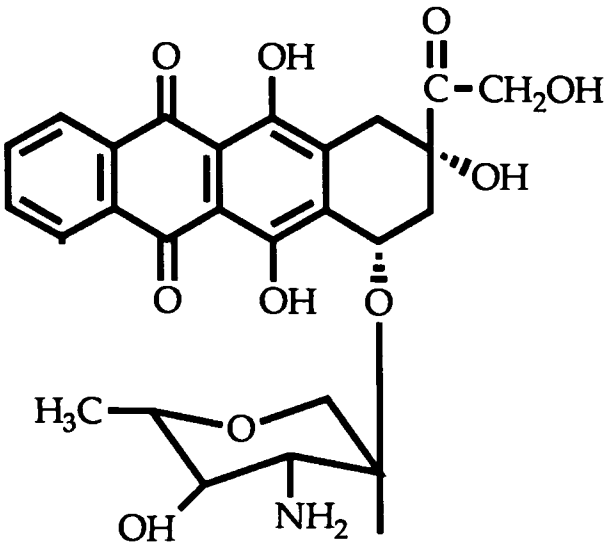
Neither GR63178A nor GR54374X gave any inhibition of topoisomerase IIα-catalysed decatenation of kDNA (result published in Cummings *et al.*, 1992). A maximum drug concentration of 100μg/ml was tested for both parent drug and its metabolite. The similarities in structure to some other topoisomerase II poisons makes it significant that these novel compounds have no effect on topoisomerase IIα activity and it will be important in the future to also test them against topoisomerase IIβ. It was also reported in Cummings *et al.* (1992) that GR63178A did not appear to bind to DNA or to induce DNA damage through free radical formation. Hence, although GR63178A shares chemical features in common with well-established classes of cytotoxic anticancer drugs it is unlikely that it owes its *in vivo* anti-tumour activity to DNA binding, topoisomerase II inhibition (at least not through the α isozyme), or generation of free radicals and reactive oxygen species. This work was done in collaboration with Dr. J. Cummings of the ICRF Medical Oncology Unit, Edinburgh (U.K.).

A new drug with similarities to the anthracyclines that has proved interesting in clinical trials is the anthrapyrazole, CI-941 (Figure 65), which is a novel DNA-binding drug developed as a non-cardiotoxic alternative to doxorubicin (Talbot *et al.*, 1991). Phase II studies suggest that CI-941 may be one of the most active drugs yet developed against advanced breast cancer, with a response rate of 63%. The cardiotoxicity of currently used anthracyclines is thought to result, in part, from the formation of free radicals due to a reduction of the drug to its semiquinone form with subsequent generation of reactive oxygen species. The heart is particularly vulnerable to these reactive radicals owing to a relatively low level of the detoxifying enzymes, superoxide dismutase, catalase and glutathione peroxidase (Judson, 1991).

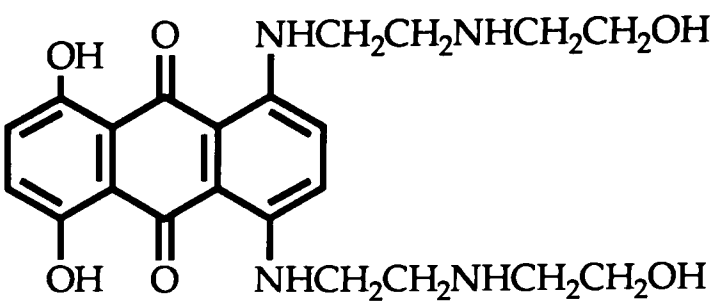
The structure of CI-941 is closely related to the anthacenedione, mitoxantrone (Figure 65), which has demonstrated useful clinical activity with reduced cardiotoxicity but is clearly less potent than doxorubicin as a single agent and has a reduced spectrum of activity (Judson, 1991). CI-941 has a chromophore modification of the anthracenedione nucleus, where an additional pyrazole ring has been fused in place of one carbonyl group (Figure 65). The planar conformation and cationic nature of the anthracyclines, essential for intercalative binding to DNA, is thus maintained in CI-941. CI-941 has *bis*-hydroxyethylaminoalkyl side chains identical to mitoxantrone, replacing the glycone structure of doxorubicin (Figure 65).

A standard decatenation assay was carried out comparing the inhibitory effect of similar doses of CI-491 and m-AMSA, a reasonably specific topoisomerase II poison, on purified topoisomerase IIα enzyme. Reactions were incubated at 30°C for 45 minutes before stopping and running on a 1% agarose gel (Figure 66). Results clearly showed that whilst m-AMSA only had a partial inhibitory effect at the highest concentrations used (50 and 100μM), CI-491 was extremely potent causing a complete inhibition of decatenation at 20μM. It would appear then that this compound, unlike GR63178A, is an extremely potent inhibitor of topoisomerase IIα protein. It will be important to find out whether this drug has other cellular targets or whether its potent anti-tumour activity does indeed result from its interaction with topoisomerase IIα. This work was done in collaboration with Dr. S. Houlbrook and Dr. D. C. Talbot at the MRC Radiobiology Unit, Harwell (U.K.).

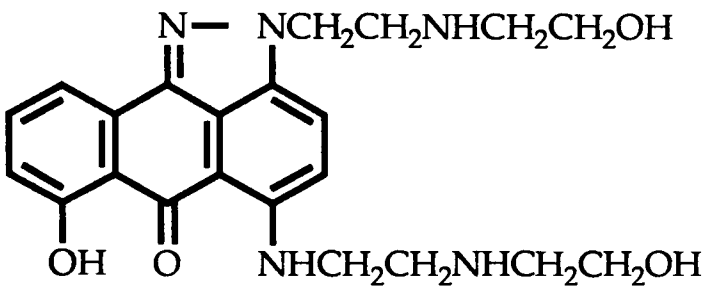
These examples demonstrate the potential use of highly purified human topoisomerase II isozymes to screen for novel topoisomerase II poisons. With an increasing understanding of the differential expression of the two isozymes and their different roles in DNA processes, it will be vital to look at the activity of these new compounds against both isozymes independently. In this way, the potential chemotherapeutic advantage of each new drug can be more accurately assessed.



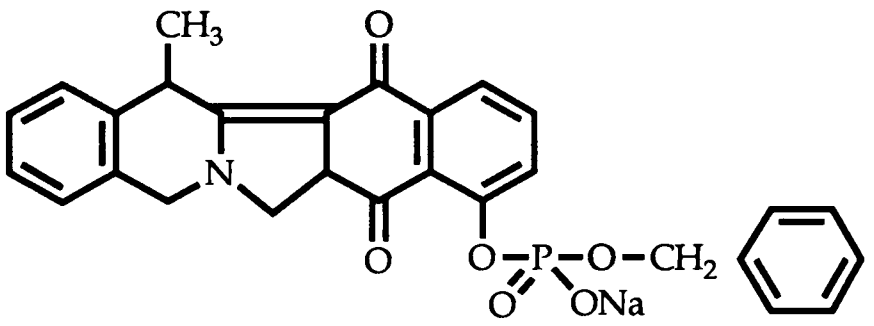
Doxorubicin



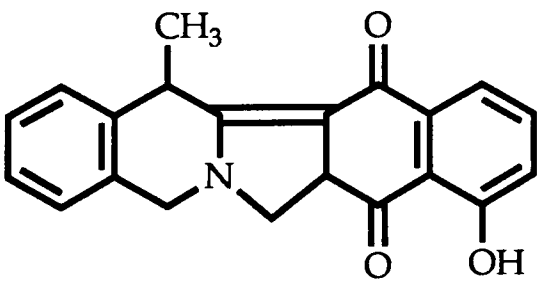
Mitoxantone



CI 941



GR 63178A



GR 54374X

Figure 65 Chemical structures of doxorubicin, mitoxantrone and three related novel drug compounds being tested as potential topoisomerase II poisons. CI-941 is an anthrapyrazole compound and GR63178A is a pentacyclic pyrolloquinone. GR54374X is the major metabolite of GR63178A.

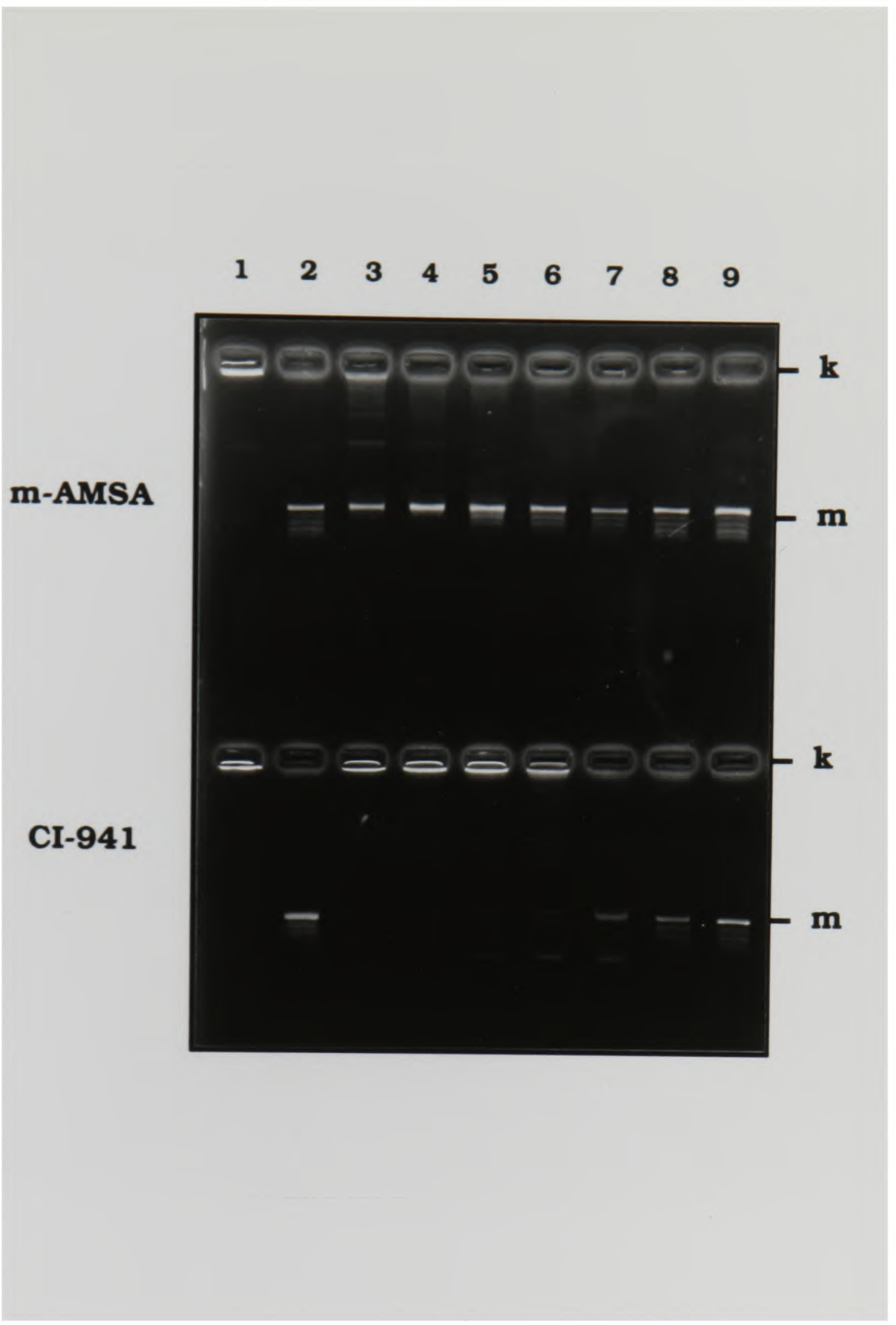


Figure 66 Inhibition of topoisomerase IIα-mediated decatenation by CI-941 compared to m-AMSA. Decatenation reactions were carried out in the absence (lane 1) or presence (lanes 2-9) of purified topoisomerase IIα protein. m-AMSA (top panel, A) or CI-491 (bottom panel, B) were included at 0 (lane 2), 100 (lane 3), 50 (lane 4), 20 (lane 5), 10 (lane 6), 5 (lane 7), 1 (lane 8) and 0.1 (lane 9) μM. Following incubation at 30°C for 45 minutes, samples were electrophoresed on a 1% agarose gel, stained with ethidium bromide and photographed under UV-illumination.

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