CHARACTERISATION OF NUCLEAR SUB-STRUCTURES

BY

SHAILENDRA BHANUBHAI PATEL

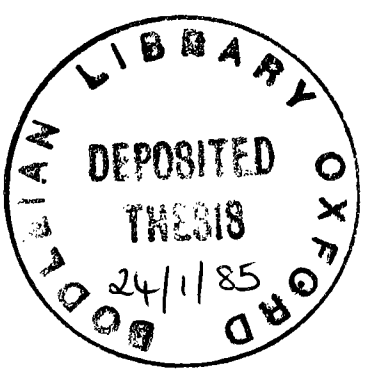
WOLFSON COLLEGE

OXFORD UNIVERSITY

submitted for the degree

of Doctor of Philosophy

Trinity term, 1984



This thesis is dedicated to

my family,

for the immense love and understanding

they have given me

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ABSTRACT

When living cells are lysed in non-ionic detergents and 2M NaCl, structures are released that resemble nuclei, termed nucleoids. Nucleoids contain tenaciously attached DNA, RNA and protein. The nature of the interactions of these components is poorly understood.

It is known that the DNA is attached to these structures in a looped configuration, and newly synthesised DNA is found closely associated with the attachment sites. Therefore, the speculation that these attachment sites have a functional signification other than for structural purposes has been seriously considered.

To investigate these possibilities, the proteins were characterised for DNA-binding activity, and the presence of any enzymatic activity. Some of the nucleoid proteins are derived from the cell surface, and specific phosphorylating and methylating activities were also detected. The significance of these findings remains to be determined. The study of the DNA-binding activity is hampered by the fact that these proteins are not readily solubilised away from the nucleic acids. However, DNA-binding proteins are present in nucleoids. No specificity for DNA sequences was demonstrable, using the protein blotting technique. In the course of these studies, a new technique was devised to enable sequence-binding proteins to be identified.

Examination of the DNA close to the attachment sites shows it to be enriched in transcriptionally active genes, and in a given population of cells, some genes are closer to the attachment sites than others. This supports the idea that genes are specifically arranged within the nucleus of any cell, and that this position is of functional significance. Direct examination of the most closely adherent DNA to these structures did not reveal any one DNA sequence that may mediate attachment.

ACKNOWLEDGEMENTS

I thank my supervisor, Peter Cook, for allowing me a free hand most of the time and Professors Harris and Abrahms for their financial assitance and encouragement.

In particular, I would also like to thank Dean 'Denis' Jackson for his invaluable help in proof-reading this thesis, and for the collaboration on several mind-blowing, but unpublished experiments. Denis single-handedly obtained thesis photographs when many had failed.

This work was supported by an award from the E.P.A. Cephalosporin Fund, and greatly encouraged by liberal alcohol supplied on Friday nights by Peter and Cornie. I would also like to thank Dr. Stephen Goss for many conversations, intellectual and otherwise, along the "gallery of wuz 'ere", and Mary Williams for several attempted murders of the various non-curricular cell lines that I tried to establish.

I would also like to thank the Brazilian World Cup squad for the best summer during this period and Mary Mason for her invalauble help in managing the glassware every morning, and Mike Simpkins for managing the coffee finances.

Finally, I would like to thank my family for letting me pretend to be an ostrich for three years.

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INTRODUCTION

For many years microscopists have recognised the existence of sub-structures within cell nuclei. Initial studies were confined mainly to preparations manually extracted from lower eucaryotes, which have larger nuclei facilitating isolation[2,3]. With improving methodology and concomitant advances in the understanding of chromatin structure[15], greater importance has been attributed to these structures.

The nucleus is a complex organelle yielding a variety of sub-structures, depending upon the method of extraction, termed nuclear envelopes[5], pore-lamina complexes[6,7], nuclear ghosts[8], nuclear matrices[9,10], chromosome scaffolds[11], and nucleoids[12] [summarised in fig. 1]. The most common feature of these methods is extraction with a non-ionic detergent and a highly ionic solvent e.g Triton X-100 and 2M NaCl, except for the preparation of the nuclear envelopes, which are made in a similar way to most membrane preparations.

Nuclear envelopes are comprised of the two nuclear membranes, with membrane proteins, and a rim of material adherent to the inner nuclear membrane. Extraction of these envelopes with detergents and high concentrations of salt produces nuclear pore-lamina complexes, the 'pores' are thought to be responsible for the nucleo-cytoplasmic traffic and the lamina to reflect some residual structure of the nucleus. Similar extraction of isolated nuclei results in the production of nuclear matrices that retain the nuclear morphology, and are composed of pore-lamina complexes and a trabecular core containing a variable amount of nucleic acid, due

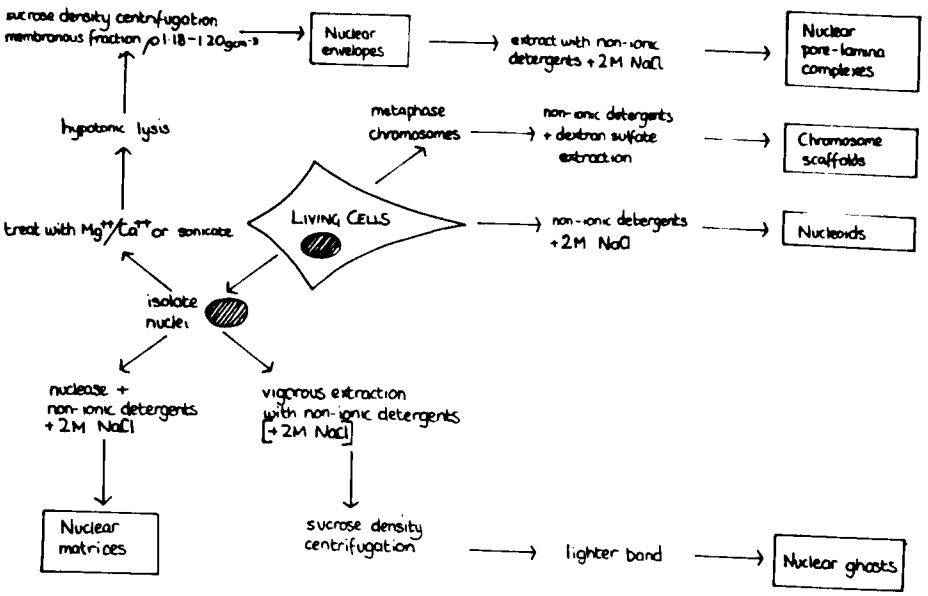


Fig. 1 Isolation of nuclear sub-structures A schematic representation of the procedures used in isolating the various sub-structures is shown above. Note the similarities in extraction procedures.

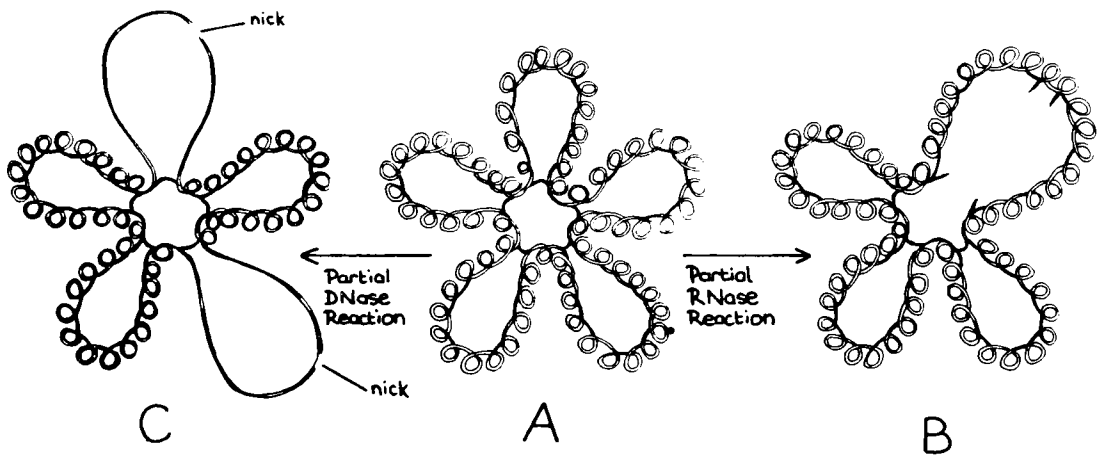


Fig. 2 Supercoils in E.coli nucleoids The model for the folded E.coli chromosome is depicted above. RN'ase treatment of the folded, supercoiled chromosome (A) removes the domains (B) and partial DN'ase treatment or irradiation removes the supercoils (C). Adapted from Pettijohn and Hecht(38).

to the use of nucleases during extraction. Extraction of cells with detergents and high concentrations of salt results in the production of nucleoids. All the DNA, nuclear RNA and a large fraction of the cytoskeletal elements are found tightly associated with these nucleoids. Nuclear ghosts are isolated by detergent extraction of nuclei and purification of the lighter bands on sucrose density gradients [see fig. 1]. It is unclear how these ghosts relate to these other structures, but they may be 'empty' matrices. Extraction of isolated chromosomes with detergent and high salt concentration results in the production of chromosome scaffolds. These may also retain all the DNA and RNA. All these sub-structures are proteinaceous and have a small amount of lipid associated with them. Even after extensive nuclease treatment, a residual amount of RNA and DNA is invariably present.

There has been a considerable amount of speculation about their role in nuclear biology. Why should a small amount of DNA and RNA be so tightly attached to these structures? Whilst a number of studies have indicated their involvement in the processes of DNA replication, transcription, RNA metabolism and nucleo-cytoplasmic interactions, none have been definitive, and some have suggested that any such interactions may be due to artefacts as a result of their extraction procedures. An interpretation is that living cells are made up of intricate skeletons, permeating both the nucleus and the cytoplasm, onto which are attached cellular processes responsible for the biological activites. Perhaps these sub-structures are remnants that may allow us to explore the intimate behavior of the biological molecules. Intricate cellular skeletons may be seen in the electron micrographs of cells extracted <u>in situ</u> on EM grids[16,17], showing the presence of a filamentous network

connecting the cytoplasm and the nucleus. These may form the basis for the complex compartmentalization known to exist in living cells.

STRUCTURAL ATTACHMENTS

Non-random organization of the chromosomes in the nucleus was proposed over sixty years ago[18]. Since then a variety of reports have suggested that the organisation of the chromosomes in both interphase and mitosis may be non-random e.g. the localisation of the X-chromosome with respect to the nuclear membrane [ref 20 and refs therein], and the apparent non-random association of the chromosomes in chromosome spreads[21,22,60]. Many microscopists have noted the association of the chromosomes with the nuclear membrane[23,24,25], the most consistent being the Barr body of the inactivated X-chromosome[26]. All of the above provide only circumstantial evidence of spatial orientation of the nuclear elements[27].

On a more molecular basis, models have been proposed to account for the amount of condensation required to pack the DNA into the nucleus and to propose that the DNA within the nucleus may be attached at sites along the DNA[28]. Such ideas were encouraged by the bacterial chromosomal attachment model of Jacob et al[30], a model that has led many to consider similar models for the organisation of the DNA in eucaryotic cells[29,31,32]. The bacterial model proposed that the DNA was attached to the membrane to enable the equal separation of the daughter chromosomes. The DNA was believed to be looped via attachments to the membrane. Such models have since been shown to be very useful in understanding the bacterial chromosomal organisation[33]. Comings and Okada proposed a similar model for the organisation of eucaryotic DNA[29], a model that has gained popularity with

others[34,35]. Because many of the problems facing eucaryotic studies are highlighted by studies carried out in procaryotic systems, consideration of these studies, based on investigation of the bacterial nucleoid is relevant here.

E.coli nucleoids

When E.coli cells are treated with lyzozyme to remove their cell walls and extracted with Triton X-100 and 1M NaCl, compact structures called nucleoids can be isolated by sedimentation[36,37]. These nucleoids contain protein and the entire chromosomal DNA and RNA. The DNA is negatively supercoiled and arranged into 40-50 loops, each of about 100kbp[36,37]. Both the RNA and the protein appear to be involved in maintaining the compactness. RN'ase and protease treatments result in the loss of compactness, but not the supercoiling[38,42]. The looped nature of the nucleoid is demonstrated by the ability to remove supercoils by DN'ase nicking, or irradiation without affecting the supercoiling in the other loops [fig. 2]. Each loop is therefore termed a domain, as it does not communicate freely with other loops. Much of the RNA in the nucleoid is nascent tRNA, rRNA, and mRNA[39,40]. These nucleoids make excellent templates for *in vitro* transcription and do not require the addition of any exogenous RNA polymerase for synthesis, though only elongation of pre-existing RNA chains is seen.

This has led many to question whether these structures are artefacts, the DNA precipating onto a core, mediated by transcriptional complexes[41]. This would also explain the RN'ase sensitivity of the loops. Such claims are reinforced by experiments that show that autoradiographic grains due to *in vivo*

pulse-labelled RNA are distributed all over the intact cell, but are confined mainly to the core in nucleoids isolated from these cells[42]. Artefactual association would be less likely if the domains seen *in vitro* also existed *in vivo*. Attempts to demonstrate this have produced equivocal results[43], largely because of the difficulties in measuring *in vivo* domain numbers. That some domains exist *in vivo* has been shown quite elegantly by Pettijohn and workers[45]. They detected tortional tension within living *E.coli* cells that was sequestered into domains. Tortional tension can be used to influence the unwinding of the DNA, and when coupled with domains, may be used to exert selective control[33]. Interestingly, the number of gyrase molecules present in the *E.coli* chromosome is very similar to the number of the DNA domains, i.e one loop-one enzyme[46]. This enzyme is able to alter the degree of supercoiling in a closed molecule of DNA, via an ATP-dependent reaction[47].

Whilst tortional tension can be detected in living *E.coli* cells, none has so far been demonstrated in living eucaryotic cells[43]. However, DNA can be extracted from higher cells such that it is supercoiled, as demonstrated by its biphasic sedimentation pattern in gradients containing varying concentrations of ethidium bromide[12,48]. Because genetic and visco-elastic studies indicate that chromosomal DNA is a very long linear molecule, the isolated DNA must be constrained to remain supercoiled. Supercoiled DNA has now been shown to be present in HeLa nucleiods[12] and chromosome scaffolds[51].

Since treatments such as X-irradiation, or nicking with DN'ase I introduce single-strand breaks into the DNA, any supercoiling present will be released. This can be detected by comparing the

amount of dye bound in treated and untreated nucleoids by fluorimetry, which can distinguish between bound and free dye. Hence comparision of lightly irradiated with unirradiated nucleoids shows that the nucleoid DNA is supercoiled[34]. It is estimated that a nucleoid contains 50,000 loops, each of about 200kbp in size, and one supercoil every 90-180bp. Or the equivalent of 10-20kbp of 'open' DNA per loop [1 supercoil can unwind 10bp of DNA]. Interestingly, this is the level of supercoiling found to occur naturally in DNA isolated from procaryotes. In theory, 10% of the genome can therefore be 'open'.

Using fluorimetry, Cook and Brazell[34] showed that the supercoils in HeLa nucleoids were not affected by RN'ase, or low concentrations of chaotropic agents such as urea, sarkosyl, or potassium thiocyanate, but were affected by protease and DN'ase. Furthermore, the supercoiling was stable to pH of 11, and temperatures of up to 95 degrees C[34]. They concluded that the mechanism holding the DNA in these loops was very stable [their proteases were thought to be contaminated with DN'ases][34]. As yet, it is not possible to determine the effects on the loop-sizes, or to draw the conclusion that the loops are held in place by a mechanism resistant to RN'ase treatment [cf. with the studies on E.coli[38]]. Since no tortional tension can be detected in vivo in HeLa cells[45], there is no evidence to indicate that the DNA in higher cells is organised in domains as in procaryotes. Because of this, it has been suggested that all the supercoiling detected in the isolated DNA is normally accounted for by the interaction of the histones with the DNA in vivo[49]. The studies of supercoiled DNA in the other nuclear sub-structures is sufficiently poor to warrant no disscussion here.

The looped model of the DNA has led many to speculate as to its purpose. By extrapolation from the bacterial model[30], replication was one of the first to be considered[53,54]. This has subsequently been extended to consider replication and transcription, RNA processing[85,86,94], and the packaging of the DNA into the nucleus[28].

Attachment sequences

DNA could be bound to the sub-structures via specific sequences recognised by proteins, by DNA-DNA quadruplex interactions, or by mechanisms that do not rely upon specific recognition of the DNA code. The idea that the attachment may be mediated by specific sequences is appealing and many have attempted to demonstrate the presence of these sequences. Assuming that the loop size is 100kbp and that the total genome contains 10^7kbp, then there must be a minimum of 100,000 loops and as many attachment sequences. Razin et al[52], using chromosome scaffolds isolated from mouse L cells, found that when these scaffolds were digested with restriction endonucleases, 96% of the DNA was released from the cores. The remaining 4% of the DNA was purified and shown to be enriched in satellite and moderately repetitive sequences, as demonstrated by solution hybridisation and caesium chloride density gradient centrifugation. Interestingly, they found that the average size of the residual DNA was 3-4kbp, giving a loop size estimate of 75-100kbp. Jeppesen and Bankier[59] used two methods for producing residual DNA associated with Hamster cell scaffolds; either by digesting the scaffolds with micrococcal nuclease or DN'ase I. About 0.1-0.5% of DNA remained. This residual DNA was indistinguisable from total DNA as judged by pyrimidine 'finger printing', though it was enriched in repetitive sequences as judged

by solution hybridisation. They argued that the 'attachment sequence', if present, would not be unmasked by their protocol. A recognition sequence can be as small as 10-20bp [e.g. cf. lac and lamda repressor sites] and the level of digestion they obtained would not be enough to show a sufficient enrichment of these sequences. Bowen, using HeLa scaffolds[61], found that the 2% of DNA, remaining after digestion with Hae III, showed a fifty-fold enrichment of a 1.7kb Hae III fragment which is known to contain repetitive elements[62]. However, on further digestion with micrococcal nuclease, about 0.5% of the DNA remained, but this DNA did not show enrichment of the same fragment. Instead, it resembled total DNA in sequence. Enrichment of repetitive sequences has also been found for DNA associated with bovine nuclear matrices[63], and rat liver nuclear matrices[64], the latter showed an enrichment of rDNA sequences.

So why are the repetitive sequences found enriched above, not considered to be attachment sequences? Appealing theories for genetic control demand the use of such sequences[65]. Unfortunately any one sequence does not fulfill all the expected qualities of an attachment sequence. For example, the 1.7kb Hae III fragment de-enriched at higher DNA digestion levels instead of becoming super-enriched. The sequence may be small enough to have escaped detection. Or perhaps the attachment of the DNA is not based on primary sequence recognition.

Regardless of the mode of attachment, a consequence of such an organisation would be that the genes in a loop would be specifically orientated with respect to these sites. The technique of Razin et al[52], using restriction endonucleases to generate residual DNA with defined ends, has been adapted to investigate

whether genes are specifically attached[66]. The relative frequency of specific genes is investigated using 'Southern blotting' and hybridisation, comparing the abundance of a gene in the residual relative to total DNA. This technique of 'detachment mapping' has led to some interesting results. Cook and Brazell showed that the α-globin gene was closer to the sites than the β- or the γ-globin genes[66]. Proviral sequences in cloned cell lines are enriched in the residual DNA, when these sequences are actively transcribed, but not in revertant cell lines where they are silent[67]. Solution hybridisation of this residual DNA with total cell RNA indicates that transcribed segments are closer to these attachment sites[94]. Similar correlations of 'active' genes being enriched in the residual DNA have also been drawn by others e.g for ovalbumin[68], and SV40 genes[69].

However many others have claimed that the residual DNA is not enriched in active genes or repetitive sequences[70,71,72,73]. Basler et al[71] could find no enrichment of satellite or active sequences in residual DNA isolated from rat liver or hamster scaffolds and Ross et al[70] could find no enrichment of the globin genes in matrix DNA isolated from chicken erythroblasts. Since they treat their nuclei or chromosomes with nucleases prior to salt extraction, the nuclease-sensitive 'active' conformation will still be present[180,181], resulting in the the preferential loss of these sequences.

The problem of artefactual association must still be considered. For example, the enrichment of 'active' sequences may result from the insensitivity of these segments,after isolation, to nucleases, perhaps due to the presence of the remnant

transcriptional or replication complexes. Or, like the E.coli nucleoid, the active genes are precipitated onto the sub-structures during preparation. Some of these criticisms have been answered elsewhere[66,67,94]. Nevertheless, these possibilities will always exist until we can study such attachments in situ.

REPLICATION AND THE NUCLEUS

Ever since Jacob et al[30] put forward their model of membrane attachment of the bacterial chromosome for DNA replication, many workers have seriously considered similar models for eucaryotic DNA[76,77,78]. Nascent DNA was shown to be tightly associated with isolated nuclear membranes[14], and this resisted even the most vigorous of extractions[54,55]. Fortunately, autoradiography of cells and nuclei did not show a localisation of the grains solely with the membrane, but throughout the nucleus[74,75]. Inevitably, the biochemical isolation of the other nuclear sub-structures led to suggestions of their involvement in DNA replication. Sites of synthesis were moved to the nuclear matrices, and again pulse-labelled DNA was found to be enriched with these[58].

The first convincing evidence came from the work of Djikwel et al[76]. They uniformly labelled the cellular DNA with ^{14}C isotope and then, pulse-labelled replicating DNA with ^{3}H-Thymidine. After the preparation of the matrices from these cells, they digested the matrices with DN'ase 1 and compared the ratio of the two isotopes in the residual DNA remaining associated with the matrices. Even when 95% of the ^{14}C label had been solubilised from the matrices, only 60% of the ^{3}H label had been removed i.e the newly synthesised DNA was enriched in the matrices. This DNA was shown to be newly synthesised by the presence of 'Okazaki' fragments and by its sensitivity to S_1 nuclease. One criticism has been that only sites close to the outside of the nucleus would be exposed to label and the replication would therefore appear to take place at these sites. The use of Arabinosyl cytosine [ara-C] should therefore

allow this to be investigated. Ara-C slows down the rate of DNA synthesis and therefore longer pulse times can be used to allow uniform distribution of the label throughout the cells. Djikwell et al found that the ara-C treatment did not significantly alter their results. From their results, Djikwell et al[76] favoured the looped model of DNA organisation, as described by Dingman[32], and placed the attachment site of the replicating DNA before the replication fork.

Similar findings were subsequently made by Pardoll et al[77], using 3T3 cell matrices, and McCready et al[78], using HeLa nucleoids. Interestingly, McCready et al, using autoradiography, were able to show that the newly replicated DNA was initially associated with the sites and, on pulse-chase, could be shown to move away from these sites[78]. To rule out the possibility that these isolated sub-structures have an affinity for replicating DNA, all three groups[76,77,78] showed that the isolated sub-structure showed no affinity for single-stranded DNA <u>in vitro</u>, whether added during isolation or after. Vogelstein et al[79] have recently shown that, even after labelling the nuclear DNA <u>in vitro</u> by 'nick translation' and then extracting nuclear matrices, no affinity for the labelled DNA is demonstrable. Note that, since 'active' DNA in the nucleus is sensitive to nucleases[180], none were used during extraction of the matrices, as such treatments are known to preferentially release replicating DNA.

Attachment sites for DNA replication may be necessary for the same reasons as for prokaryotic DNA replication, namely for separation of daughter chromosomes. Having such localised sites may also allow for rapidity and high fidelity of synthesis e.g. by metabolic channeling of the precursors[83]. Attachment is also

necessary to maintain supercoils in linear DNA. Supercoiling may facillitate recognition of replication origins by proteins. It is known that palindromic sequences in isolated supercoiled plasmids adopt hair-pin loop configurations, although these structures do not appear to exist *in vivo*[80]. Should the spatial organisation of the DNA turn out to be of importance for gene expression, then the inheritance of the differentiated trait may also be mediated by such attachments.

TRANSCRIPTION AND THE NUCLEAR SUB-STRUCTURES

It has been known for a long time that the RNA within the cytoplasm and the nucleus exists as an ordered structure, complexed with proteins, called ribonucleoproteins [RNP][87,88,89,90,91]. Procedures for the isolation of RNPs invariably involve vigorous disruption of the nucleus for solubilisation, usually by sonication[132]. Current trends indicate that these RNPs are thought to be attached to the nuclear sub-structures. The tight association of RNA with chromatin has been known for a long time[92,93]. That this association may not entirely be due to DNA-RNA interactions is suggested by experiments that remove greater than 90% of the DNA, but remove less than 50% of the RNA[85]. Earlier studies showed the clear association of the RNPs with isolated nuclear nuclear matrices[85,97] and nucleoids[98,100]. Characterisation of the RNA associated with the nuclear matrices has shown it to be heterogeneous and both newly synthesised and processed transcripts have been shown to be present[85]. It has therefore been suggested that these anchor sites could therefore play a role in RNA processing[95].

Both spliced and unspliced globin transcripts have been found to be associated with matrices[70,182], as have adenovirus transcripts in infected HeLa matrices. In influenza virus-infected chicken embryonic fibroblasts, both the plus and the minus strands of the single-stranded RNA virus are found to be associated with nucleoids[98]. Because of the presence of the newly synthesised RNA in these structures, RNA synthesis has also been proposed to take place here[95,97]. There is a correlation between the

transcription of a gene and its closeness to the attachment sites. Perhaps all genes that are actively transcribed are close to these sites[94], the attachment mediated by the transcriptional complex at the growing end. This is similar to the proposal for the association of the RNA in E.coli nucleoids[38]. Interestingly, the E.coli nucleoids contain a large amount of RNA polymerase[41]. However, although it has been demonstrated that eucaryotic RNA polymerase-transcriptional complexes can be found associated with chromatin extracted with medium salt concentrations[183], no endogenous RNA polymerase activity has yet been found in nucleoids from HeLa cells[100]. There are now many correlations between 'active' chromatin structure and its sensitivity to DN'ase 1, or association with acetylated histones, or HMG 14 and 17 proteins, or the state of methylation of the gene[101]. To this one could add the closeness of the gene to the attachment sites. At this stage it is not possible to determine which is the chicken and which is the egg. Other ways of controlling gene expression with which the nuclear sub-structures may be involved are selective transcription and the transport of the messages out to the cytoplasm. It is thought that only some of the genes are transcribed at any given time, though recent evidence suggests that perhaps all genes are transcribed, but that control could be exerted by selective rate increases[103]. Using solution hybridisation of DNA to RNA, about 1-2 transcripts of globin genes per 6 HeLa cells were found to be present, though globin gene expression is not known to be a characteristic feature of HeLa cells[103].

Selective transport to the cytoplasm may also be important. How such transport is brought about is still largely unknown, though the 'pores' in the nuclear membrane have been implicated to play a major part[104]. Studies on isolated nuclei have shown

that specific RNA efflux can be demonstrated which is size-dependent and closely mimicks the *in vivo* situation[105,106,107]. The nuclear envelope contains nucleotide tri-phosphatase activity[107], and specific receptors for insulin[108,109]. The addition of insulin to the nuclei affects the RNA efflux patterns[106], as do temperature changes[109].

Unfortunately, none of sub-structures have been shown to play a major role in transport, save the fact that all the RNA is 'tied down' in the nucleus, so by association, they may do so. Since replication of DNA takes place at fixed sites in the nucleus, perhaps DNA modification such as methylation also takes place there. Somatic recombination, known to take place in lymphocyte differentiation, mating alleles of yeasts, antigenic variation in trypanosomes and fruit fly biology[102], may also be associated with the nuclear sub-structures.

PROTEINS OF THE SUB-STRUCTURES

Nuclear envelope and the lamina-pore complex

Controversy has existed for a long time concerning the relationship between the endoplasmic reticulum [E.R.] and the outer nuclear membrane. Microscopic contiguity has been demonstrated in many[111,112], but not all cells[113]. Isolation of the nuclear envelopes has always been difficult, due to the presence of the closely adherent shell of material on its internal surface[113]. Comparision of the protein species of the E.R. and the N.E. show that there are similarities and differences[114]. These differences are amplified if both membranes are extracted with non-ionic detergents[114].

Extraction of the N.E. results in the production of the pore-lamina, which is greatly enriched in three species not found in the E.R. These species are called lamins N1, N2 and N3[114], or A, B, and C[115]. They constitute about 25% of the total N.E. protein and are found universally[113,114,117]. Their molecular weights, vary between 50kd to 75kd partly as a result of inaccuracies of determinations based on gel electrophoresis and partly because these may reflect species differences. Because of the considerable enrichment of these proteins in the pore-lamina fraction, initial hopes were pinned on these proteins forming the basis for the 'pores', controlling the RNA efflux. Subsequent studies have been unable to confirm this, and there is evidence to suggest their non-involvement[115]. The chicken erythrocyte has few pore-complexes, as seen under the electron microscope, but there is no difference in the relative amounts of lamins[116].

Similarly, careful studies correlating the presence of the pore-complexes and polypeptide species failed to show a constant relationship[117].

A number of groups have raised antisera to lamins, and from antibody staining of the N.E. or the pore-lamina preparations, both found that the adherent material stained, but not the pores. The antisera raised against N1 showed cross-reactivity to N3, suggesting that these proteins may be homologous[81]. In isolated nuclear envelopes, N1 is present as polymers, N2 as trimers and N3 as monomers, these interactions mediated by mercapto-ethanol reduceable bonds[119]. Peptide degradation products of N1 and N3 show them to share considerable homology, but N2 appears to be quite distinct[119]. Surface iodination of intact nuclei results in iodination of N1 and N2, these are therefore believed to be exposed to the cytoplasmic face, whereas N3 was only iodinated if the nuclei were disrupted[118]. Electrophorectic analysis of isolated pore complexes from Xenopus laevis showed that N2 was considerably enriched, and hence the role of these lamins in pore structure is not entirely ruled out[115]. Interestingly, N3 exists as a phosphorylated species, and is postulated to play a role in RNP transport[119].

Whilst the structural comparisions between E.R. and the N.E. have not proved exciting, functional studies have turned out to be quite enthralling. Initial studies showed that the N.E. contained enzymes similar to those found in the E.R. such as NADH-cytochrome c reductase[120], epoxide hydratase, UDP-glucoronyl transferase, and also contained cytochrome P450[121,122,123]. These mixed function oxygenases are known to participate in the metabolism of steroids and hydrocarbons. Note that neither the

E.R. nor the N.E. contain Na-activated ATP'ase[120], a characteristic of the mitochondrial and the cellular membrane. There are also significant differences between the E.R. enzymes and those of the N.E.[124]. For example, phenobarbitone pre-treatment of the cells resulted in a far greater induction of the E.R. enzymes than the N.E. enzymes[123]. But if the cells were pre-treated with 3-methyl-cholanthrene, the N.E. enzymes were greatly induced over E.R. ones[123]. Furthermore, the types of enzymes induced appears to be significantly different. Using benzo<a>pyrene as the substrate, it was found that the products from the E.R. were distinguishable from those of N.E. incubations[124]. The cytochrome P450 is also distinguishable between these two membrane preparations by its spectral emission patterns[124].

Since metabolites of benzo<a>pyrene such as dihydrodiols, quinones, phenols and oxides have been shown to be highly mutagenic[124], the metabolism of these types of compounds by the N.E. may be of considerable importance. The N.E. metabolism produces far more benzo<a>pyrene 7,8 dihydrodiol 9,10 oxides which have been shown to covalently bind to DNA[126]. These compounds tend to be short-lived, and since water molecules can compete effectively for their reactive sites, the proximity of these enzymes to the DNA may be significant. Furthermore, these enzymes appear to be induced by tumor promoters such as 3-methyl-cholanthrene. The DNA and RNA associated with the N.E. may therefore have a higher susceptibility to mutagenesis. If 'active' genes are closer to these sites these may be where most of the mutagenesis takes place. This may, in part, explain the relative cytotoxicity of many carcinogens over their mutagenic ability. This possibility can be tested experimentally, using

detachment mapping to show that genes closer to these sites may be more vulnerable.

Protein kinases

Other distinguishing features between the E.R. and the N.E. have included the activites of the endogenous protein kinases[127]. Distinct and different proteins are phosphorylated by the E.R. kinases and the N.E. ones. These kinases are cyclic AMP-independent, although the one involved in RNP transport is ATP-dependent[106]. One of the proteins phosphorylated by the N.E. kinases is N3[127]. Protein phosphorylations are regarded as important since they may be sites of control, but whilst many proteins have been found to undergo cell-cycle specific phosphorylations[128,129], none have been shown to be sites of control. Kinase activity has also been found to be associated with rat liver matrices[130], though no specific proteins were found to be phosphorylated.

Cytoskeletal elements

The proteins of the nuclear matrices have also been studied. Gel electrophoresis of matrix proteins shows a great many species, of which the major proteins are the lamins, actin, cytoskeletal elements and vimentin. The amounts of these vary considerably between the tissue of isolation. Hence more keratins are found in HeLa matrices than rat liver matrices. The filamentous proteins, especially the keratins, also show considerable heterogeneity between different tissues[131]. The origin of these proteins is likely to be both from the nucleus and the cytoplasm. It has been shown that cytoplasmic filaments tend to collapse onto the nucleus during preparation[184], and hence upon isolation of the matrices

from these nuclei, these proteins remain associated with the residual structures. A greater amount of filamentous material is present if the residual structures are isolated by direct lysis of the cells as in the isolation of the nucleoids.

Ribonucleoproteins

As has already been disscused, the RNP and DNA are intimately associated with these structures. Comparision of the proteins of nuclear matrices and RNPs suggests that many of these proteins are common to both[135]. Furthermore, all of the proteins found in the matrices can also be found in nuclei[135]. Hence it is proposed that if the nuclei are sonicated the RNPs detach from the residual sub-structures, but if the nuclei are detergent and salt extracted, the RNA with residual proteins remain attached. Further evidence for such involvement has come from the finding that the poly-A binding protein, present in the isolated RNPs, is also present in nuclear matrices[136]. Cross-linking studies *in vivo* have shown that two proteins of 41.5kd and 43kd are intimately associated with RNA extracted from HeLa matrices, or RNPs isolated from HeLa nuclei[137]. It is thought that these proteins may mediate the attachment of the RNA to the matrix.

Because of the attachment of the DNA to these structures, DNA-binding proteins have also been investigated. Comings and Wallack isolated matrix proteins using SDS, renatured them with urea, and showed that these proteins contained DNA-binding species. These showed a preference for A-T rich DNA, and it was speculated that these proteins may play a role in the attachment of the G-bands of the chromosomes to the matrix[138]. Bowen et al[139], using protein blotting, were able to identify the molecular weights

of many of these species. Lebkowski and Laemmli[140] have further shown that the DNA-binding properties of scaffold proteins is affected by Cu-chelating agents. DNA polymerase[83], polyoma T antigen[139], SV40 T antigen[142], and adenovirus proteins[143] have been shown to copurify with nuclear matrices.Specific steroid-binding has also been demonstrated, as has insulin binding[108,109]. More recently, it has been shown that heat shock proteins[147] may also be found tightly associated with nuclear matrices[146]. It has been previously shown that these proteins could be found in the nucleus[148], but their functions still remain to be determined. These proteins are now regarded as 'stress proteins', since they are inducible by heat, treatment with certain poisons such as amino acid analogues and oxidative phosphorylation inhibitors[147,149,150].

MATERIALS AND METHODS

MATERIALS

All radiochemicals were purchased from Amersham International [UK], DN'ase I and RN'ase A were bought from Worthington, and restriction endonucleases Eco R1, HIND III, Bam H1, Hae III, PVU II were obtained from Bethesda Research Labs.[USA], DNA polymerase 'large fragment' from New England Biolabs [USA], proteinase K, micrococcal nuclease and yeast tRNA from Boehringer Corp.[FRG], thymidine, phenylmethylsulphonyl fluoride and Tris base from Sigma Co.[USA] and all other chemicals from British Drug Houses [UK]. Spinner medium and amino acid-free minimum essential medium for cell culture was obtained from Gibco [UK]. Nitrocellulose filters were brought from Schleicher and Schuell or from Sartorius. Specially pure formamide was brought from Fluka [Swiss].

CELL CULTURE AND SYNCHRONY:

Mycoplasma-free HeLa cells, originally from Dr. R.T. Johnson [Cambridge], were grown in Spinner cultures, without antibiotics, as previously described[12]. The cells show considerable heterogeneity karyotypically, ranging from 6-24 pg of DNA per cell with a mean of around 12 pg. HeLa cell synchrony, using thymidine-nitrous oxide for M phase cells, or double thymidine for G_1/S phase cells was obtained as described by Warren[151]. S phase synchrony, in initial experiments, checked by ^3H-thymidine incorporation and autoradiography, and was found to be >85%. Mitotic synchrony was routinely tested by lysis of cells in nucleoid lysis mix [see later], stained with ethidium bromide and

viewed under U.V. fluorescence for their characteristic appearance. Bacterial strains were grown in nutrient broth using conventional procedures. A list of the strains used with relevant characteristics is shown in table 1.

Table 1

Bacterial strain	characteristic	Ref.
294/pKB280	Lambda repressor over-producer	170
JL 475	Lex A protein over-producer	171
JL 472	Lex A protein over-producer	171
W3110	Phage Lamda Lysogen	Lab. Strain
LK 133	Human γ-actin plasmid	172
RR1/pHPT2	Mouse HGPRT gene cDNA plasmid	173
HB101/pHu4A	Human histone 4A gene plasmid	174
HB101/pXLr101	Xenopus laevis rDNA plasmid	175
HB101/pXLo31	Xenopus laevis 5S DNA plasmid	176
MC106/pB0.7	Human 'Alu' repeat DNA plasmid	177

RADIOISOTOPE LABELLING OF CELLS:

When labelling for short periods [<1h], the cells were concentrated forty-fold in culture medium. For longer periods, the cells were maintained in their original culture volume. Uniform labelling of DNA was achieved by growing cells for 20h in ^{3}H-thymidine [40-60 Ci/mmol] at 0.05 μCi/ml or in ^{14}C-thymidine [90-140 Ci/mmol] at 0.01μCi/ml. For protein labelling L-[4,5-^{3}H] leucine [130-190 Ci/mmol], L-[methyl-^{3}H] methionine [70-90Ci/mmol] or L-[^{35}S] methionine [>800 Ci/mmol] was used. After a

pre-incubation in the appropriate amino acid-free medium for 15 min at 37 deg.C, the cells were resuspended in fresh medium and labelled at 10μCi/ml and incubated, with regular agitation, for up to 1h at 37 degrees C. For longer periods of labelling, unlabelled amino acid at one hundredth its normal concentration was present [see fig. 3]. When labelling with [^{32}P]-orthophosphate, cells were depleted of their phosphate pools by incubation in phosphate-free medium for 0.5h prior to labelling. Carrier-free phosphate label was added at 100μCi/ml for short periods or at 10μCi/ml for longer periods. All labelling was stopped with addition of ice-cold PBS, the cells washed and harvested by centrifugation.

CELL IODINATION

Cell surface iodination was carried out using a slight modification of the method of Hubbard and Cohn[152]. 5 x 10^7 cells were washed three times in ice-cold PBS with 20mM glucose, resuspended in 1ml of this solution and left on ice. To this was added 32ml of 16mg/ml lactoperoxidase solution and 6ml of freshly diluted glucose oxidase [7.2ml of 1000 u/ml in 2ml PBS]. 1mCi of carrier free ^{125}I was added and the cells incubated on ice for 15 min. The reaction was stopped with cold medium, and the cells washed thoroughly to to remove unbound radio-isotope. Cell viability, tested by dye exclusion, remained constant at 85 % throughout this procedure.

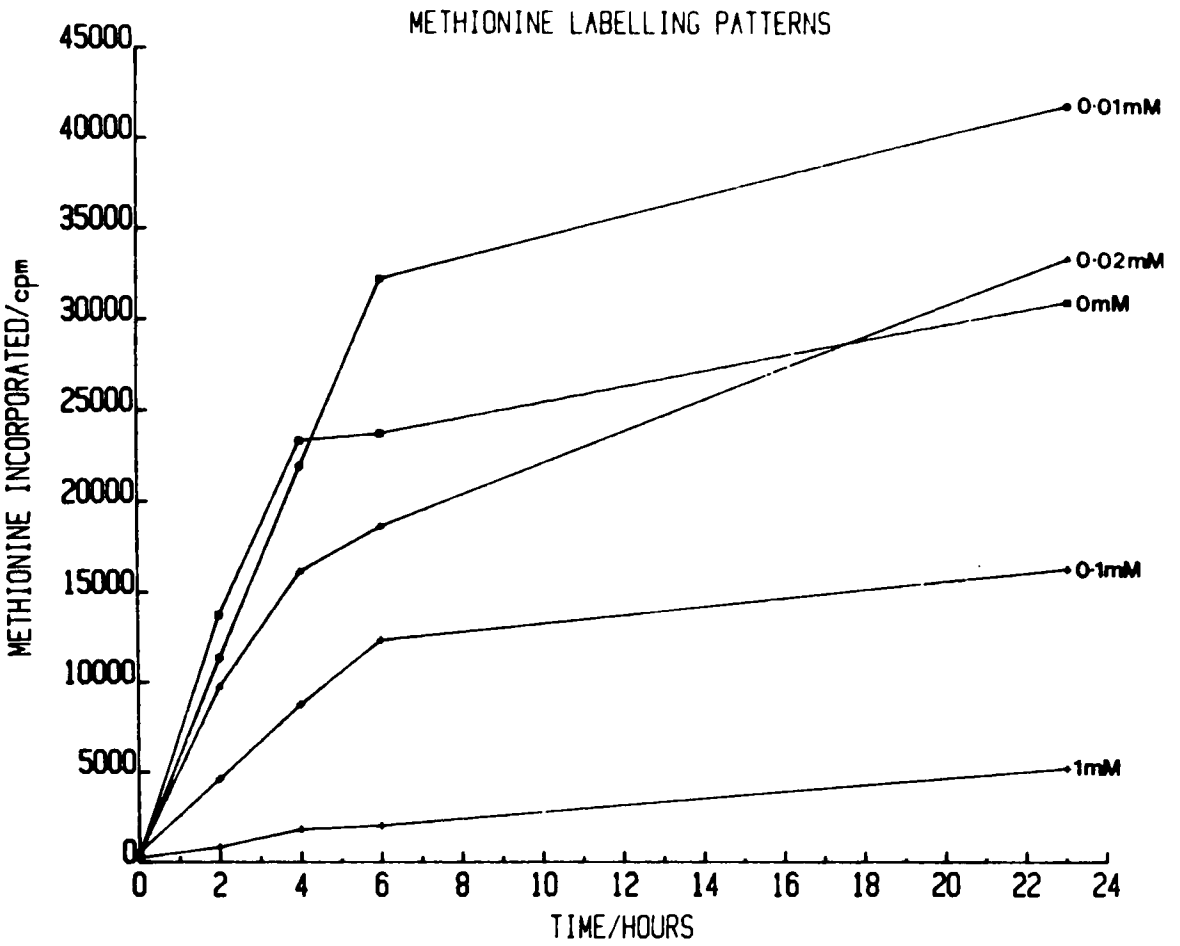


Fig. 3 Incorporation of labelled methionine into Hela cells HeLa cells, growing at 0.4 x 10^6/ml, were countinously labelled at 1µCi/ml of 35S-methionine [specific activity >800Ci/mMol] in methionine-free medium, with unlabelled methionine added as indicated. Normal methionine concentration is 1mM. For maximal incorporation, unlabelled methionine was present at 0mM [short term labelling], or 0.01mM [long term labelling].

HEAT SHOCK:

The cells were concentrated forty-fold and incubated at 45 degrees C for 10 min with constant agitation. Medium, at 37 degrees C, was added to restore the original culture volume and the cells grown at 37 degrees C for appropriate times. The cell labelling procedures were as described.

CELL FRACTIONATION

HeLa nucleoids were isolated using a modification of the original procedure[12]. 80 x 10^6 cells were lysed in a final volume of 6ml, loaded onto 25ml of 15% sucrose-salt shelf on a 5ml 30% sucrose-salt shelf and centrifuged at 7K rpm for 25 min in a SW27 rotor in a Beckman ultracentrifuge. For mitotic nucleoids, the spinning conditions were altered to 12K rpm for 25 min. The nucleoid lysis mix, sucrose-salt solutions are exactly as previously described[12], except that all solutions contained the protease inhibitor phenylmethyl sulfonyl fluoride [PMSF] at a final concentration of 0.1mM. Nucleoids were also isolated, using a quicker method. 50µl of cells were lysed with 150µl of lysis buffer, and layered onto a gradient of 15% sucrose-salt [0.6ml], on a cushion of 30% sucrose-salt, and spun, in a Beckman microfuge, for 4 min, at 4 deg. C. The nucleoids were isolated by puncture of the side of the tube using a needle.

HeLa nuclei were isolated as described by Krokan et al [153], except HEPES buffer was replaced by Tris buffer.

For partial purification of the 5S gene-binding protein, HeLa cells were homogenised, spun at 100,000g for 30 min, the supernatant collected, made 30% with ammonium sulphate, and the

precipitate collected, dialysed against 0.1mM PMSF, and lyophilised. This was used as the crude extract.

IN VITRO PHOSPHORYLATION OF NUCLEOIDS

Nucleoids were isolated, as described above, diluted 10-fold in TE buffer with added $MgCl_2$ and PMSF at 10mM and 0.1mM respectively to yield a final concentration of 0.2M NaCl, and 2 x 10^6/ml of nucleoids. The reaction was initiated with the addition of [γ-^{32}P]-ATP, at a final concentration of 2mCi/ml, and incubated for 30 min at 37 deg. C. The reaction was stopped by the addition of 2x concentrated sample buffer, and an aliquot separated by SDS-PAGE. The gels were not stained, as too little protein is loaded to obtain a staining pattern. To determine the amount of radioactivity incorporated, an aliquot was spotted on glass fibre filters, extracted with 5% Trichloro-acetic acid, then with ethanol, dried and counted.

IN VITRO METHYLATION OF NUCLEOIDS

Nucleoids were diluted 10-fold with TE buffer, pelleted, and resuspended in TE buffer with $MgCl_2$ and PMSF at 10mM and 0.1mM respectively. After brief digestion with RN'ase and DN'ase at 15µg/ml each, the nucleoids were pelleted, and resuspended at about 10 x 10^6/ml in TE buffer, with 10mM $MgCl_2$, 0.1mM PMSF, and [^{3}H-methyl]-S-adenosine at 0.1mCi/ml. The reaction was incubated at 37 deg. for 2h, stopped by the addition of sample buffer, and an aliquot subjected to SDS-PAGE.

ISOLATION OF PROTEINS:

Nucleoid proteins were isolated as follows. About 40 x 10^6 nucleoids were diluted ten-fold with 10mM Tris-HCl, 0.1mM EDTA, pH 7.5 [TE buffer] and pelleted at 10,000g for 5 min. The pellet was resuspended in a final volume of 10ml of TE buffer and SDS was added to a final concentration of 1%. The solution was left to stand overnight at room temperature and then spun at 100,000g for 90 min to pellet out the viscous nucleic acids. The supernatant was dialysed extensively against 0.1mM PMSF, lyophilised and stored dry at -20 deg.C. The dry protein was accurately weighed, rehydrated in sample buffer[154] at 12 mg/ml and heated at 100 degrees C for 3 min. Actual protein concentration was determined on samples using the dye-binding assay and was found to be in good agreement with the weight. HeLa nuclear extract was made by resuspending HeLa nuclei at 40 x 10^6/ml in sample buffer, heated at 100 degrees C for 3 min and spun at 100,000g for 120 min to pellet out the nucleic acids. The supernatant was used as the extract. The protein concentration was about 12-15 mg/ml as determined by the dye-binding assay [see below].

Total cell extract from bacteria was made by incubating PBS-washed cells, from a 100ml of late log-phase culture, in a final volume of 2.5ml of 100mg/ml lyzozyme in TE bufer for 30 min on ice. 2.5ml of twice strength sample buffer was added, the solution made homogenous and heated at 100 degrees C for 3 min. The nucleic acids were pelleted out as above and the supernatant used as total cell extract sample.

PROTEIN CONCENTRATION DETERMINATION:

Protein concentration was determined using the dye-binding assay[155] [Biorad Inc. kit]. The assay can be performed on samples containing SDS and mercaptoethanol[156] and is useful in cases where the proteins are not readily soluble in conventional aqueous buffer. Calibration was performed using bovine γ-globulin protein supplied with the kit.

NUCLEIC ACID ISOLATION:

Cellular DNA was isolated using a modified procedure of Gross-Bellard et al[157]. To isolated nucleoids, sarkosyl was added to a final concentration of 1%, heat-inactivated RN'ase A added at a final concentration of 50µg/ml and the solution incubated at 37 degrees C for 2h. Proteinase K was added to a final 125µg/ml and the solution incubated at 56 degrees C for 3h. The NaCl concentration was raised to 0.4M and the solution extracted twice with phenol, once with ether and dialysed over-night against TE buffer, with one change. The dialysed sample was retreated with RN'ase A, proteinase K and phenol extracted. The solution was concentrated by butan-2-ol extraction, ether extracted once and dialysed, with several changes, against TE buffer at 4 degrees C for 48h. This DNA was essentially free of RNA as judged by gel electrophoresis. Where required the DNA was banded in CsCl gradient by spinning in a 951.4mg/ml CsCl solution [final] with ethidium bromide at 0.01mg/ml for 40h at 100,000g. The DNA band was isolated, and the ethidium bromide removed by extraction with isopropanol, dialysed and stored at -20 degrees C.

Plasmid DNA was isolated by a scaled up method of Birnboim and Doty[158], followed by CsCl-EtBr gradients [0.88g/ml CsCl [final]

with 0.3mg/ml ethidium bromide] spun in a Ti50 rotor [Beckman Ultracentrifuge] at 43K rpm for 48h and the denser plasmid band isolated and treated as above. The DNA was usually stored at -20 degrees C, but where supercoiled DNA was required the DNA was stored at 4 degrees C. All DNA concentrations were determined by their u.v. absorbance profiles using extinction coefficients determined by Warburg and Christiansen[159].

DETACHMENT MAPPING:

For detachment mapping experiments, procedures similar to those of Cook and Brazell[66] were followed. HeLa cells were uniformly labelled with ^{3}H-thymidine [as described above] and nucleoids isolated. These nucleoids were diluted 10 fold with the 10mM Tris-HCl [pH 7.5], 0.1mM EDTA [to reduce the salt concentration to 200 mM] and divided equally into separate tubes. Various concentrations of restriction endonucleases were added to each tube, incubated at 37 degrees C for 15 min and the digestions stopped by the additon of EDTA to a final concentration of 10mM. For a 20-fold range of enzyme added, the level of DNA detached from the 'cages' does not parallel the amount of enzyme used. Hence about 150 units/ml of EcoR1 resulted in 95% of the DNA being released from the cages, whereas 15 units/ml and 1.5 units/ml resulted in approximately 80 and 40% release respectively after a 0.5h incubation. In subsequent experiments, only one level of digestion was performed, by incubating nucleoids with EcoR1 at 100 units/ml for 30 min to 1h. This results in approximately 1-2% of the DNA still remaining associated with the cages.

After digestion all samples, except one, were spun at 15,000g for 10 min to pellet the cages. The latter sample, not spun, was

used as a source of 100% DNA [this sample was partially digested with EcoR1 to facilitate purification of DNA]. The supernatants, containing released DNA fragments, were generally discarded. The pelleted cages were carefully rinsed with 10mM Tris and resuspended in 10mM Tris, 2mM EDTA, 1% sarkosyl and 0.2mg/ml heat-inactivated RN'ase A. After incubation at 37 degrees C for 2h, proteinase K was added at a final 0.125mg/ml and the incubation continued at 56 degrees C for 12-16h. The 100% DNA sample was similarly treated. After raising the NaCl concentration to 0.3M, the samples were phenol extracted twice, concentrated approximately 2.5 fold with butan-2-ol, ether extracted and dialysed, with two changes, against TE buffer at 4 degrees C 36h. The 100% DNA sample was optically scanned to determine the DNA concentration. Aliquots of each sample were counted for tritium radio-activity, and using the 100% DNA sample as standard, the DNA concentrations of the rest of the samples determined. The [^{3}H] content of the samples was found to be more reliable than the optical scans, since the optically-determined DNA concentrations for the cage-associated DNA were never in good agreement with the ethidium bromide stain of the subsequent gel. The extra nucleic acids indicated by the optical scan are presumably due to very small RNA fragments since digestion of these DNA samples with RN'ase T2, and subsequent scanning, results in a better estimation of the DNA concentrations [unpublished observations].

Known amounts of the DNA samples were ethanol precipitated, digested to completion with Eco R1 and separated on agarose gels. The gels were stained with ethidium bromide, photographed and blotted onto nitrocellulose filters [see below]. In some experiments Bam H1 was used instead of EcoR1 for the inital digestion. After purification the DNA was digested to completion

with the corresponding enzyme. In some case, the second digestion was performed using Hae III, and the samples were separated on 2% agarose gels. Note that in this latter case the initial detachment was performed using EcoR1, and the subsequent digestion of the purified DNA with Hae III. Since Hae III has more recognition sites than EcoR1, there is no need to cut the DNA with Eco R1 as well since only a few Eco R1 sites have been cut in the first place.

GEL ELECTROPHORESIS:

Denaturing protein gels were run essentially as described by Laemmli[154] except that the TEMED concentrations used in the upper and lower gels were 0.036% and 0.025% respectively and the persulfate concentrations 0.25% and 0.1% respectively. Where gels with urea were run, only freshly made urea [Aristar grade, BDH] at a final concentration of 4M was used. Gels were electrophoresed at 3.3 mA/cm, with cooling. Where required the gels were fixed and stained with 1% Coomassie Brilliant Blue R250 or 1% Kenacid in acetic acid:methanol:water mixture [5:5:1], and destained in the latter solution.

Agarose gel electrophoresis was carried out using the TEA buffer system [40mM Tris, 2mM EDTA, 20mM Na acetate, adjusted to pH 8.3 with glacial acetic acid] The samples were suspended in TEA buffer containg 2% ficoll and 0.01% bromocresyl purple. Routinely 3mm thick, 0.66% agarose gels [for 1kb-20kb separation] were run, though occasional variations of 0.7% and 0.8% agarose gels were also used to prevent monotony. 2% agarose gels were used to resolve 0.2-2kb fragments of DNA. Agarose gels were electrophoresed for 16h at 2.6mA/cm^{2}at room temperature, stained

with ethidium bromide, photographed and processed accordingly. 4% acrylamide gels, using TEB buffer system, were also used to separate small nucleic acid fragments.

LECTIN-BINDING TO PROTEIN GELS:

After electrophoresis, protein gels were fixed and stained with Coomassie blue, a described above, and photographed. For lectin binding[169], the stained gels were washed in PBS, 0.4M NaCl for 18h, with several changes, incubated with radio-labelled Concanavalin A, or wheat germ agglutinin in PBS and 0.4M NaCl for 18h and then washed thoroughly in PBS and 0.4M NaCl over 18h with several changes to remove any unbound lectin. Prior to drying, the gels were shrunk in the fixative stain [see above] as this prevented the gels from cracking up during drying. Radio-labelled lectin was kindly supplied by Dr.M.E.Bramwell.

DNA-BINDING ASSAY

DNA-binding assays were carried out essentially as described by Riggs et al[99]. Nucleoid proteins were isolated as described above, using SDS and dialysis and lyophilisation. The dried proteins were resuspended in 8M urea, 10mM Tris, 0.1mM EDTA, 50mM NaCl pH 7.5 at aproximately 10mg/ml and dialysed against the same buffer except that it contained less and less urea to none over 36h at room temperature. Protease inhibitor PMSF at 0.1mM was also present. The dialysed solution was spun on a bench centrifuge to remove any suspension and used for DNA-binding. The suspension was found to be cytoskeletal elements by SDS-PAGE.

DNA was labelled by nick-translation as described below. An aliquot of protein was added to the binding buffer, 10mM Tris,

0.1mM EDTA, 50mM NaCl, 1x Denhardt's solution, 5% DMSO pH 7.5 and the binding initiated with the addition of the labelled DNA in a final volume of 50µl. The mixture was incubated at 20 deg. C for 20 min and passed through a nitrocellulose filter. The contents of the incubation tube were washed out with 1ml of binding buffer, and passed through the filter. The filter was washed with a total of 15ml of the binding buffer under gentle positive suction, dried and the retained radioactivity measured.

'SOUTHERN' BLOTTING AND HYBRIDISATION:

3mm thick agarose gels, after fragment separation and staining, were then briefly rinsed, incubated in 0.5M NaOH for 45 min and neutralised with 1M Tris-HCl, 3M NaCl [pH 7.5] for a further 45 min, rinsed in 12 x SSC and blotted onto nitrocellulose filters for $48h^{160}$. All times were doubled for 6mm thick gels. About 300ml of buffer was used for a gel measuring 13cm x 14cm. The blotted filters were rinsed once in 2 x SSC, blotted dry, baked for 16h at 65-72 degrees C in a hot-air oven, and stored under vacuum at room temperature. When required the filters were carefully rehydrated in prehybridisation buffer [50% formamide, 3 x SSC, 3 x Denhardt's solution and 500mg/ml yeast tRNA] and prehybridised in 5ml of this buffer in a sealed bag at 42 degrees C overnight. All the prehybridisation buffer was expelled, hybridisation mix added [1-1.5ml of 50% formamide, 3 x SSC, 3 x Denhardt's, 600mg/ml tRNA, 10% dextran sulfate and radioactive probe at 10ng/ml] and the bag resealed and incubated for 18-36h at 42 deg. C. The bag was massaged 3 times a day to ensure even distribution. After hybridisation the filters were washed 3 times in 200ml of 0.1 x SSC/0.1%SDS at 55 deg. C. The latter temperature, the stringent temperature, was determined using the

following equation:

$$Tm = 81.5 + 16.6[\log M] + 0.41\ [\%\ G + C] - 0.72\ [\%\ \text{formamide}]$$ [161,162]

where Tm is the melting temperature of DNA, M is the monavalent salt molarity, G + C represents the base composition of the DNA and the percentage formamide is that used in the hybridisation. For HeLa DNA % G+C was taken to be 49[163], and for 3 x SSC the equation reduces to:

$$Tm = 96.52 - 0.72[\%\ \text{formamide}].$$

Hence for 50% formamide Tm is 60.5 deg. C. Hence if the hybridisation is carried out at 42 deg. C, then the stringent temperature for washing in 0.1% SSC/0.1% SDS must be 53.4 deg. C. The washed filter was sealed wet in a thin plastic bag and autoradiographed at -70 deg. C using pre-flashed[164] Fuji-RX X-ray film and intensifying screens. After autoradiography the filters were stored, still wet, at 4 deg. C.

They are kept moist as subsequent re-use, after removal of the old probe, is greatly facilitated. Prior to re-use, the filters were washed once in 200ml of 90% formamide, 0.1% SDS, 0.1 x SSC [NB Tm = 8.02 deg. C] at room temperature for 30 min. The filters become very fragile during this period and great care is to be excercised to prevent tears developing. The formamide solution is discarded, making sure that the filters are retained in the container and lie perfectly flat with no crinkles or folds. Gently 2 x SSC is poured in and the filters rinsed. The filters lose their fragility at this point and are then treated for prehybridisation and hybridisation as above. The filters may be

re-used up to 4 times thus without serious deterioration in quantity or quality of the result.

PROTEIN BLOTTING :

Protein blotting was carried out essentially as described by Bowen et al[139] with the following changes. Protein gels [see above] were soaked in the transfer buffer [10mM Tris, 50mM NaCl, 2mM EDTA, and 0.1mM DTT pH 7.5] for 0.5h prior to blotting [0.25cm^2/ml], with occasional agitation. Blotting was carried out, using the apparatus described in fig. 4, for 36h with one 21 change of buffer. Longer soaks of the gels prior to blotting were found to remove significant amounts of the low molecular weight proteins [see results]. After blotting the filters were kept moist and presoaked in binding buffer [10mM Tris-HCl, 1 x Denhardt's solution, 0.1mM EDTA, 50mM NaCl, pH7.5] as described by Bowen et al[139] for 5 min. Each filter is then sealed individually in a plastic bag with the binding buffer containing radioactive nucleic acid. About 0.2mlcm^{-2} of binding buffer containing 0.4ngcm^{-2} of radioactive nucleic acid is used. The bag is sealed and incubated for 1h, with constant agitation, at room temperature. Each filter was then washed in a total of 1l of binding buffer, with four changes [without Denhardt's solution] for 1h, dried, and autoradiographed using pre-flashed X-ray film and intensifying screens at -70 degrees C. The salt concentration in the washing or the binding buffer was 50mM, unless otherwise stated. Routinely, three track strips of gels were blotted onto each filter, one of these three tracks containing protein molecular weight standards. The use of radioactive protein markers help identify the molecular weight of proteins that are imaged on the autoradiograph.

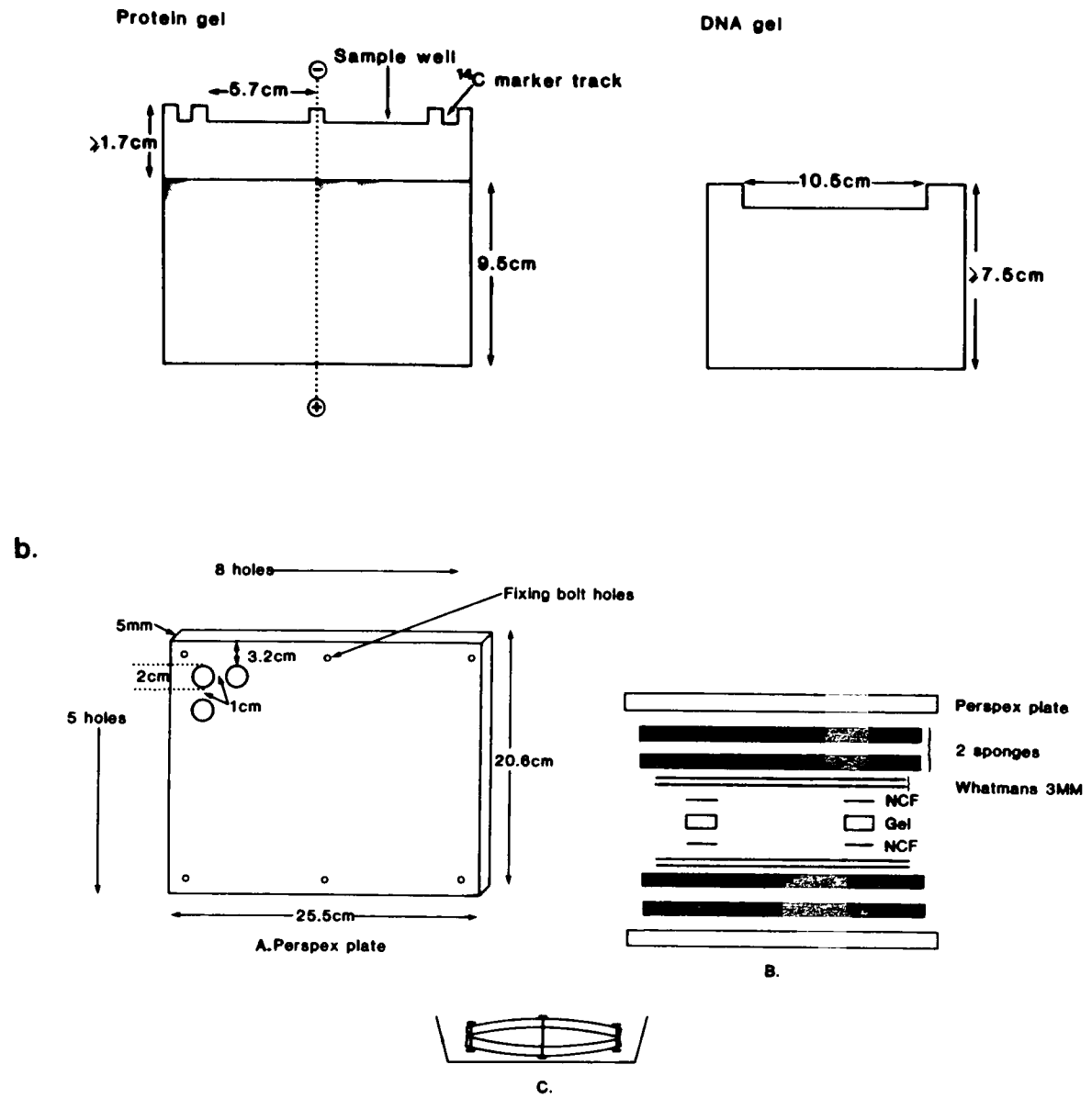


Fig. 4 Protein blotting procedures The apparatus and dimensions of the gels used in blotting are illustrated above. Fig. 4a shows the sizes of protein and DNA gels used in blotting. The perspex blotting apparatus is illustrated in 4b. The gel is sandwiched between nitrocellulose filters and compressed using the perspex plates [fig. 4b,B].

'SOUTHERN CROSS':

The technique of the 'Southern Cross'[165] is illustrated in fig. 5 Protein blotting is carried out exactly as described above, except that the blotted filter replica is presoaked in the transfer buffer [10mM Tris, 0.1mM EDTA, 5% DMSO, 2mM $CaCl_2$ and 200mM NaCl] for 5 min and placed on the agarose gel at right angles,with the protein-blotted face of the filter down [fig. 4C]. The agarose gels, after electrophoresis, was soaked in the transfer buffer for 30 min with agitation prior to transfer of the labelled fragments through the protein blotted filters. This transfer is carried out at room temperature for about 18h [estimated buffer flow through the filter was 0.5 $ml/h/cm^2$], the filter then rinsed in the transfer buffer, dried and autoradiographed as usual.

When the DNA, or RNA fragments in the agarose gel are unlabelled, the filter, after the cross, is prepared for hybridisation with the appropriate probes to detect bound DNA or RNA. For DNA, the rinsed filter is dipped in 0.4M NaCl for 20 secs, then in 0.5M Tris/1.5M NaCl [pH 7.5] for 20 sec, blotted dry and baked for 2-16h at 65-70 degrees C, and prehybridised and hybridised as described above. For RNA, the filter is baked after transfer and treated accordingly. Using unlabelled DNA enables one to cross supercoiled DNA with the proteins to detect differences in affinities for supercoiled, linear and relaxed molecules of DNA.

IN VITRO NUCLEI ACID LABELLING:

DNA was 'nick translated' essentially as described by Rigby et al[166] using [^{32}P]-dATP [specific activity > 2000 Ci/mmole] to a specific activity of 2 - 8 x 10^8dpm/μg, using a kit supplied by Amersham International [UK]. End labelling of restriction

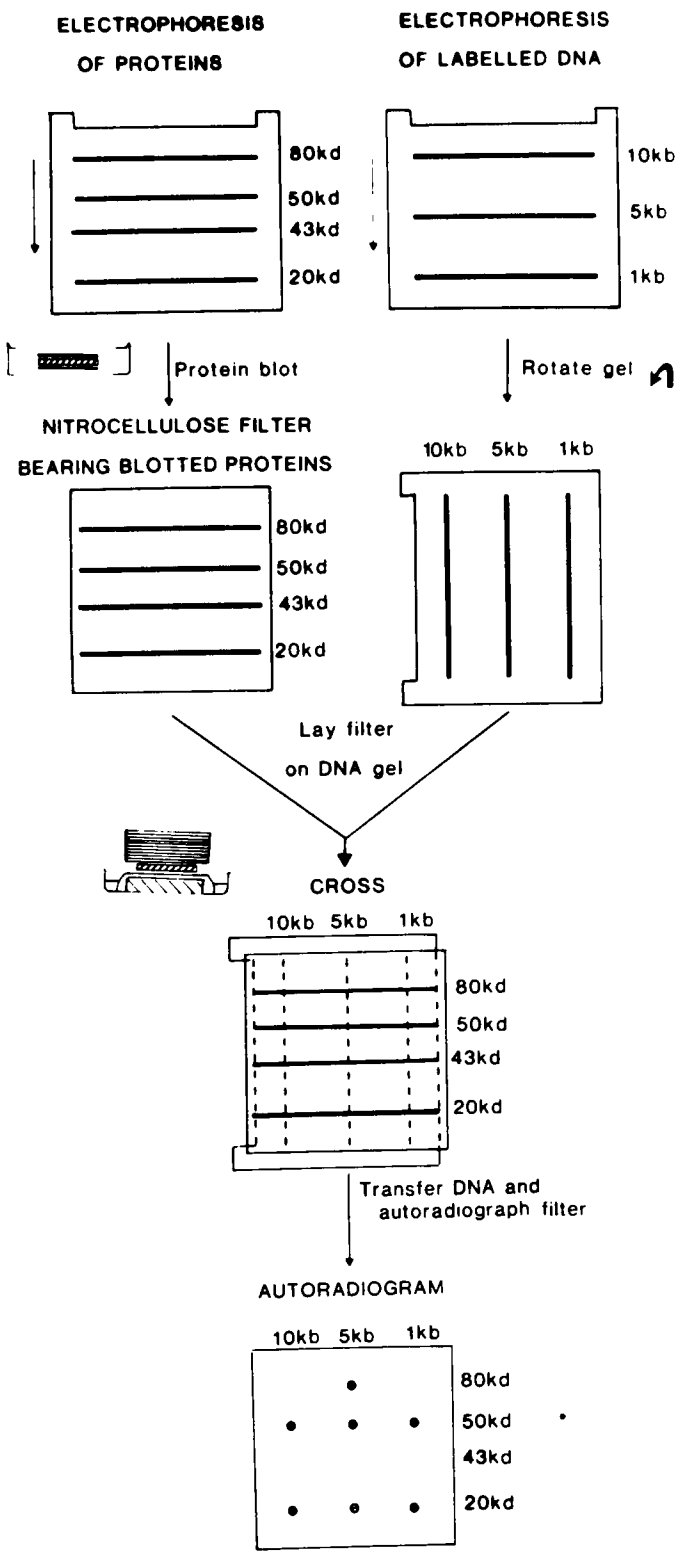


Fig. 5 Southern Cross The 'cross' is performed by taking the protein-blotted nitrocellulose filter, placing it at right-angles across the DNA gel, and transferring the DNA through the filter. Each protein band is therefore exposed to each DNA fragment. In the example shown, the 80kd protein specifically retains the 5kb DNA fragment, whereas the 50kd and 20kd bind DNA non-specifically and the 43kd protein none at all.

endonuclease-cut DNA fragments was carried out using the 'fill in' reaction of the Klenow enzyme. The Hind III reaction mixture [20mM Tris-HCl, 7mm $MgCl^2$, 60mM NaCl, 0.1ml DTT pH 7.5] contained DNA at 0.1-0.8mg/ml, Klenow enzyme [New England Biolabs] at 0.04 units/ml, [^{32}P]-dATP [specific activity >2000 Ci/mmole] at 1-10μC/ml and cold dCTP, dGTP and dTTP at 8μM each. Labelled dATP is used as it can be used to 'fill in' ends left by digestion with Eco R1, BAM H1, or Hind III [the choice of label depends upon its presence in the recognition sequence]. After incubation at room temperature for 1h, the reaction is stopped with an equal volume of twice strength TEA sample buffer, resulting in a specific activity of 1-12 x 10^6dpm/mmole ends of DNA. The end-labelled sample is electrophoresed on agarose gels and used in the 'Southern Cross'. The unincorporated label can be discarded from the gel by judicious trimming or by prior gel filtration over a Sephadex G-50 column.

Iodination of nucleic acids was performed using the Commerford reaction[167], as described by Prensky[168], using carrier-free ^{125}I. For RNA a specific activity of 20-50 x 10^6dpm/μg was obtained. Nucleic acids were also end-labelled using the kinase method of Williams and Lloyd[178]. Briefly 1μg of RNA was dissolved in 2ml of 99% formamide and 1mM MgCl. The sample was heated at 100 deg. C for 10 - 20 min. . This results in the fragmentation of the RNA producing 5'-hydroxy termini which are then end-labelled using T4 polynucleotide kinase and [^{32}P]ATP by addition of 20μl of kinase buffer [manufacturers' conditions] and 4 units of the enzyme, the mixture incubated at 37 degrees C for 1h. The reaction was stopped by heat inactivation and the labelled product separated by column chromatography, resulting in specific activities of 1-2 x 10^7dpm/μg. DNA was similarly labelled.

U.V. IRRADIATION OF DNA

Supercoiled plasmids, at 0.2mg/ml in 10mM Tris, 0.1mM EDTA, pH 7.5, were irradiated with u.v. light at 1500J/m^2 and the degree of intactness before and after irradiation compared by gel electrophoresis. This could easily detect differences of 50% change in the forms of linear, relaxed and supercoiled circles. The samples were stored for no more than 24h prior to use. Where the initial samples contained closed circular DNA, no linear DNA was detected after irradiation.

DENSITOMETRY OF AUTORADIOGRAPHS

Autoradiographs were scanned by a Joyce-Loebl densitometer and the size of the signal calculated by area determination. For nucleic acid hybridisations the signals are not directly proportional to the amount of hybridisation, hence comparisions of signals were only made where the sizes of the signals were within 50% of each other.

NUCLEOID PROTEIN STUDIES

Cell surface proteins

The close association of the cytoskeleton with the remnant nuclear structures suggests that the two may be contiguous. Recent electron microscopic studies have shown that such contiguities do exist[184].The cytoskeleton is also known to interact with many cell surface proteins. Since the isolation of nucleoids results in the inward collapse of the filaments, these proteins may bring with them cell surface proteins. In order to investigate this possibility, nucleoid proteins were isolated from cells whose surfaces had been previously radio-labelled using the glucose oxidase-lactose peroxidase method[152]. The protein patterns were compared with total cell extracts of these cells [fig. 6]. The radio-iodination does not label all the cell surface proteins, but only a sub-set[152]. Comparing the Coomassie stained and autoradiographic patterns, the labelling does not reflect the abundance of the proteins. There are many similarities between nucleoid and total cell extract proteins, but not all the cell surface labelled proteins are found associated with the nucleoids. A major cell surface protein [arrowed, . see fig. 6] is not detectable in the nucleoid fraction. Included in this figure is the labelling pattern obtained after phosphorylation in vivo. Note that a few of the surface proteins may also be phosphorylated. An interpretation of this result is that these cell surface proteins associate with the nucleoids via the cytoskeleton. These cell surface proteins may be 'receptors'. It is known that nuclear matrices and nuclear envelopes contain specific steroid and insulin receptors[108,109], and that some of these receptors do remain

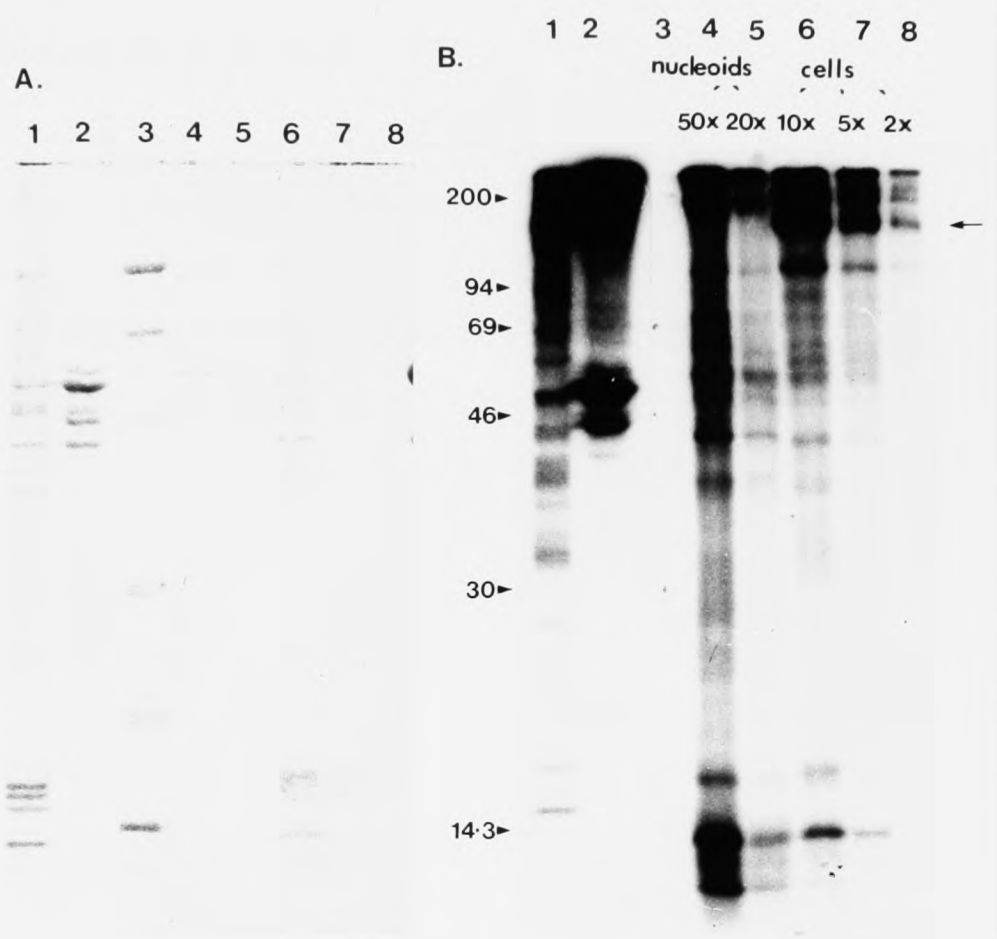


Fig. 6 Distribution of cell surface proteins in nucleoids HeLa cells were radio-iodinated as described in Materials and Methods. Total cell or nucleoid proteins were isolated, separated by SDS-PAGE, stained with Commassie blue (fig. 6A) and the gel autoradiographed (fig. 6B). Tracks 1 and 2 contain total cell extract and nucleoid protein respectively, isolated after phosphorylation in vivo with labelled phosphate (see Materials and Methods). Tracks 4 and 5 contain nucleoid proteins and tracks 6, 7 and 8 total cell extract, isolated after cell-surface iodination. The relative cell equivalents of proteins loaded onto the gel are as indicated in fig. 6B. Hence track 4 contains nucleoid proteins from 50 cell equivalents compared with track 8 that contains 2 cell equivalents. Track 3 contains protein molecular weight standards of 200, 94, 69, 46, 30 and 14.3kd. The arrowed band (fig. 6B) is absent in nucleoids (compare tracks 4 and 8).

associated with the cytoskeleton, even after a Triton X-100 wash, as do some enzymes[189]. It remains to be seen whether these receptors can be shown to be present in nucleoids.

Nucleoid glycoproteins

Another method of characterising the proteins is to study sub-species such as glycoproteins which are found in both the cytoplasm and the nucleus[191,192]. This can be accomplished by staining SDS-PAGE separated proteins with radio-labelled lectin. The plant lectins concanavalin A [con A] and wheat germ agglutinnin [WGA] bind to glycoslated proteins containing terminal mannose and glucose [con A], and terminal sialic acid [WGA][169]. Nucleoids contain a variety of glycosylated species as shown in figure 7. Lectin-binding can be used to study the effects of mercaptoethanol on the migration of the proteins after SDS-PAGE. Nucleoid proteins run with or without mercaptoethanol do not show any significant changes as judged by the Coomassie pattern [fig. 8A]. However, after staining these gels with con A, several differences are seen [fig. 8], indicating that some of these proteins contain disulphide bonds. Figure 8B also shows the con A stain of a cytoplasmic extract, sharing many similarities with the nucleoid proteins. There is at least one protein [44kd] that is entirely nucleoid in distribution [asterix, fig. 8B]. It may be that the proteins that are similar are derived from the cell surface and those that are confined to the nucleoid from the nuclear membrane. Whilst the above studies allow differentiation between cellular localisation, no indication of their function is obtained. There are proteins that are present in the nucleoid only. To date, the ultra-structural localisation of any of these proteins is not known [except those of the lamins]. Even the examination of lamina-pore

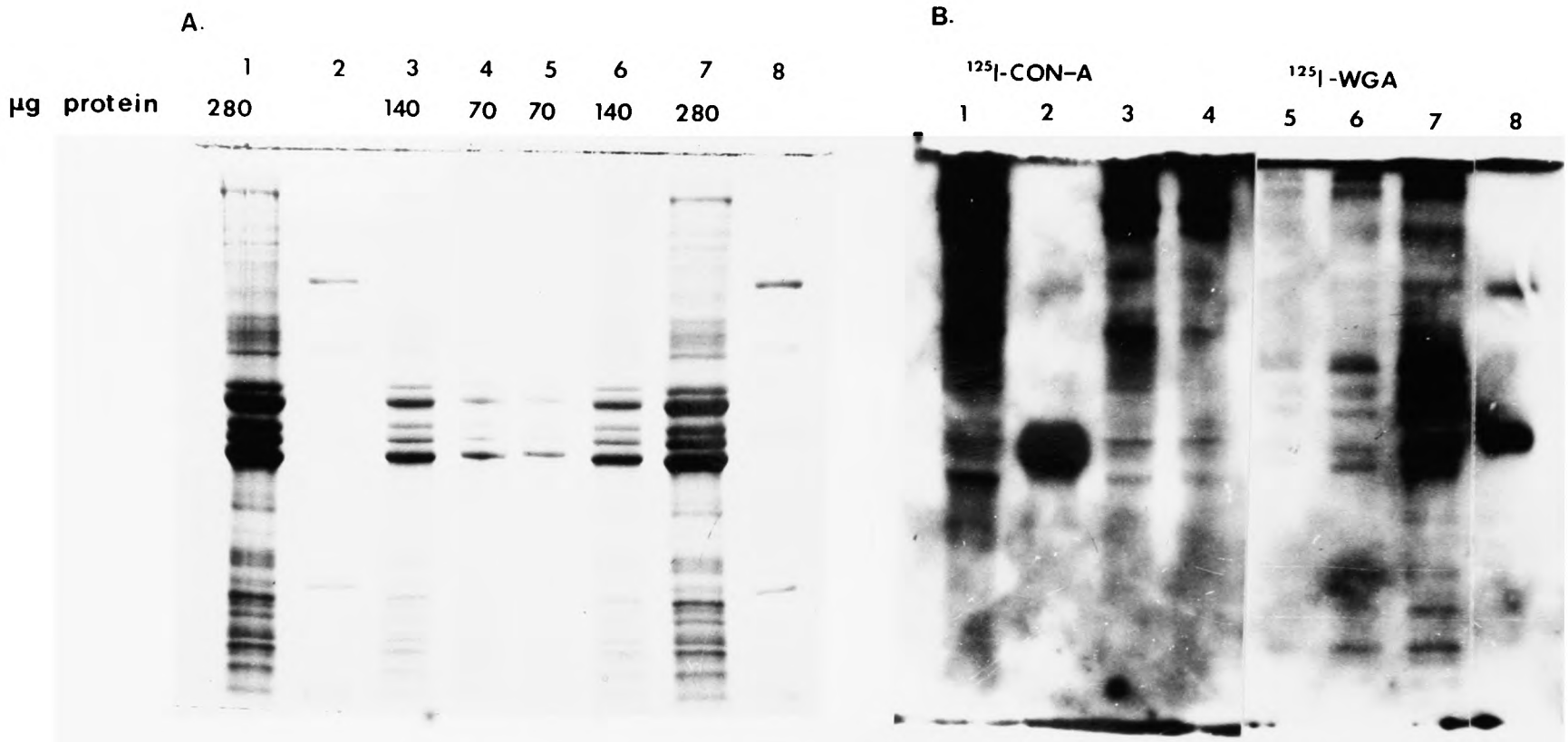


Fig. 7 Lectin binding to HeLa nucleoid proteins HeLa nucleoid proteins were separated by SDS-PAGE, fixed and stained with Commassie (fig. 7A), and then with radiolabelled lectins (fig. 7B), as described in Materials and Methods. The gel was stained with radiolabelled concanavalin A (tracks 1 - 4), or wheat germ agglutinnin (tracks 5-8) and autoradiographed (fig. 7B). Tracks 2 and 8 contain marker proteins of 94, 69, 46, 30 and 26 kd. A variety of differences in the lectin staining patterns are seen, indicating that nucleoid proteins are glycosylated with different terminal sugars.

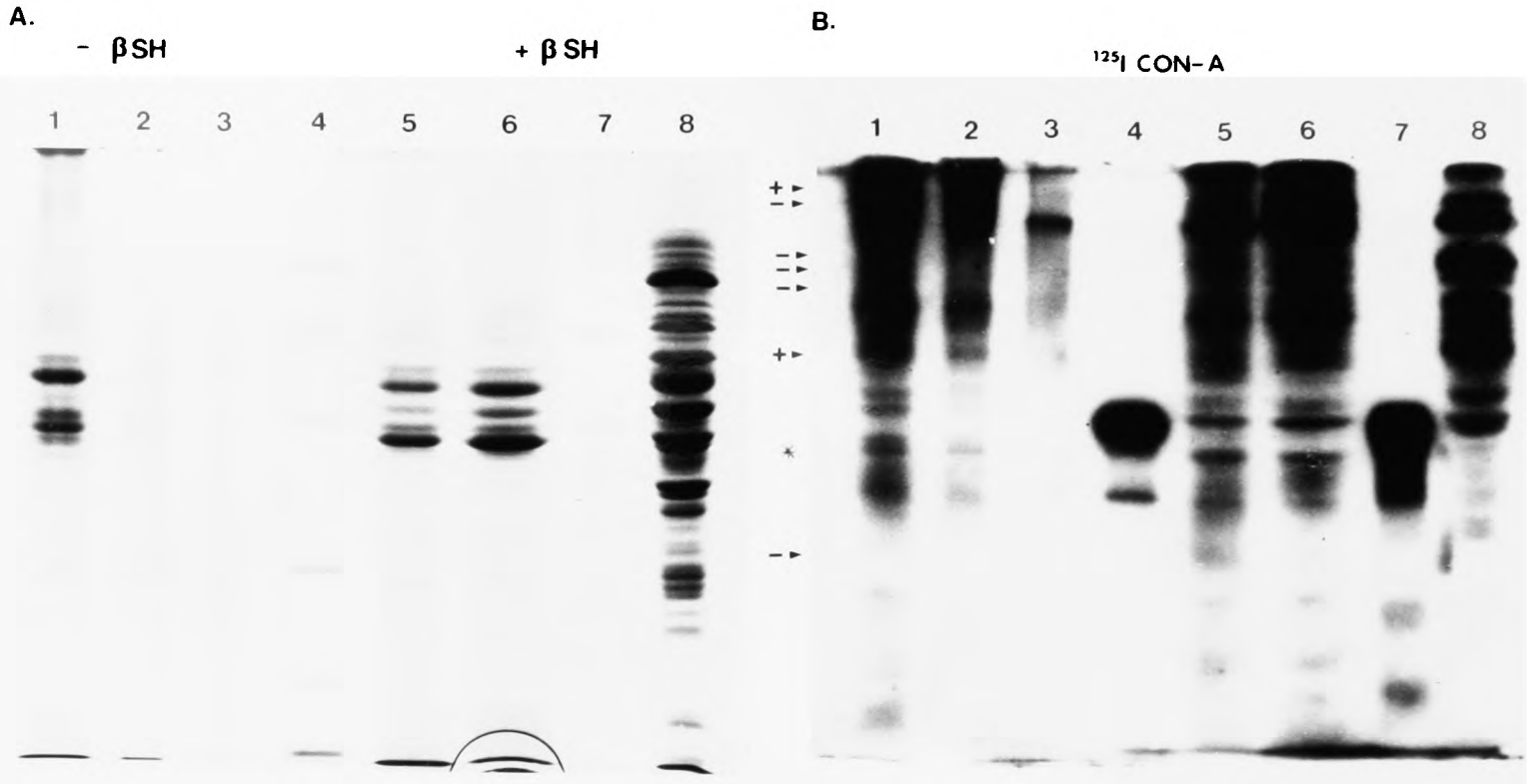


Fig. 8 Effects of mercaptoethanol on the migration of protein species HeLa proteins were dissolved in sample buffer with (tracks 5-8) or without (tracks 1-4) mercaptoethanol. After electrophoresis the gel was stained with Commassie (fig. 8A) and then with radio-iodinated concanavalin A (fig. 8B) as described in Materials and Methods. Tracks 1, 2, 3, 5 and 6 contain nucleoid proteins and track 8 cytoplasmic extract. Tracks 4 and 7 contain protein markers of 94, 69, 46, 30, 26 and 14.3 kd. Arrows by the side of track 1, fig. 8B, indicate the loss (minus) or gain (plus) of a band after the addition of mercaptoethanol. The asterisk indicates the presence of a concanavalin staining band that does not appear to be present in the cytoplasmic extract and therefore is presumed to be a nucleus specific glycoprotein.

complexes by SDS-gel electrophoresis and electron microscopy has not revealed the proteins responsible for the pore structure[117].

Enzymatic characterisation of these sub-structures is also sparse. DNA polymerase activity has shown to be present in nuclear matrices[84,190], as has endogenous kinase activity[130]. Using the assay mixture for in vitro replication as previously described[185], no such activity could be demonstrated in nucleoids isolated from G_1/S phase or mid-S phase synchronised cells [results not shown]. One reason for the lack of synthesis may have been the use of the nucleoid DNA which is intact, whereas most polymerase assays employ nicked or gapped DNA. This possibility was not explored.

Kinase activity in nucleoids

Incubation of the nucleoids with labelled ATP and Mg++ results in acid-precipitable incorporation of the label [fig. 9]. Electrophorectic examination shows that significant amounts of the label have been incorporated into three proteins [46, 51, and 57kd, fig.10]. This phosphorylation was inactivated by heat, protease and in the presence of EDTA, but not by pre- or post-treatment of the nucleoids with nucleases [fig. 10]. The phophorylation is also present in 400mM NaCl, although slightly diminished [track 15, fig. 10]. There appears to have been some proteolytic activity in track 7 in fig.10. Since the intensity of the lower molecular weight proteins is increased in this instance, these species may be the degradation products of the major proteins [cf. intensity of 33kd protein, fig. 10]. This possibility was not followed up. The 57 and 46kd proteins are phosporylated to the same extent. Proteins phosphorylated *in vitro* are also phosphorylated *in vivo*,

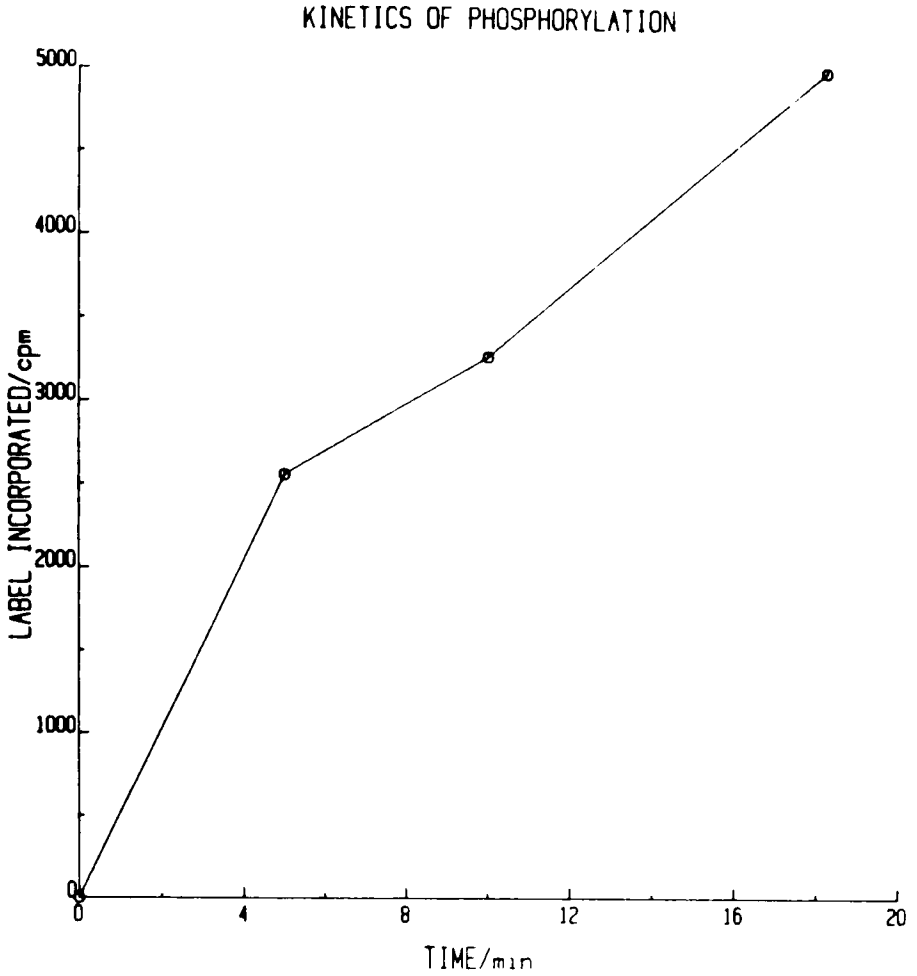


Fig. 9 Graph of phosphate incorporation into nucleoids in vitro HeLa nucleoids were isolated as described in Materials and Methods. 5µl of nucleoids [20 x 10^6/ml in 1.95M NaCl] were diluted into a final assay volume of 50µl containing 10mM Tris, 10mM $MgCl_2$, 0.1mM PMSF pH 7.5 and 0.1µCi of [α-32P]-ATP, and incubated at 37 degrees C. Reactions were stopped by the addition of an equal volume of 2x concentrated sample buffer, and aliquots spotted onto glass fibre discs, and the acid-precipitable radioactivity determined. The time course of phosphate incorporation was plotted.

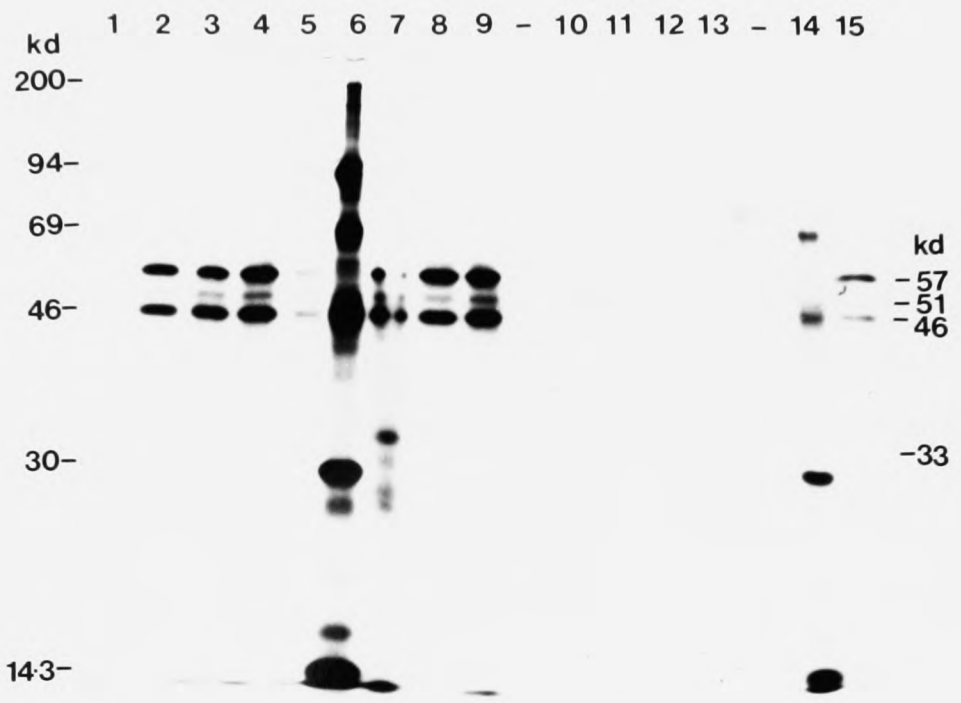


Fig. 10

Phosphorylation in vivo of nucleoid proteins by endogenous kinase HeLa nucleoids were phosphorylated as described in fig. 9, and aliquots separated by SDS-PAGE, fixed and autoradiographed. The gel was not stained since too few nucleoids are loaded [10^4/track] to give an adequate stain. Tracks 1-4: the time course of the reaction 0, 5, 10 and 20 min respectively. Track 5: the effect of pre-digesting the nucleoids with RN'ase A prior to addition of label. Track 7: RN'ase was added after 30' incubation with label [less sample loaded accidently]. Tracks 8 and 9: As 5 and 7 except DN'ase I was used. Track 10: the effect of treating the nucleoids with proteinase K. Track 11: 2mM EDTA replaced 10mM $MgCl_2$ in the assay. Track 12 and 13: the effect of incubation at 0 and 65 deg. C respectively. Track 15: incubation in 400mM NaCl. Proteins of 57, 51, and 46 kd are appreciably phosphorylated, though minor bands are also detected. Note that in track 7 there appears to have been some proteolytic activity. This has lead to enhancement of the lower molecular bands [esp. 33kd band]. Track 6 and 14 contain marker proteins of 200, 94, 69, 46, 30 and 14.3 kd.

and this is not affected by pre-treating the cells with cycloheximide, actinomycin D or thymidine. However, the relative ratios of the major phosphorylated species differ from that obtained *in vitro* [figs. 6 and 11]. The 57kd protein is phosphorylated to a greater extent than the 46kd protein. Phosphorylation of minor species is also seen. The extra bands [arrowed, fig. 11] are due to contamination of the nucleoids resultant from using the quicker microfuge technique to isolate the nucleoids. This technique was used so as to minimise contamination of equipment, as large amounts of radioactivity were being handled. Interestingly, the *in vivo* pattern of phosphorylation is not changed by heat shock treatment of the cells [fig.12]. Kinase activity has also been reported in nuclear matrices[130], though no distinct protein species were found to be phosphorylated.

The sizes of the labelled proteins correspond to those of vimentin [57kd], and two cytokeratins [46kd and 51kd][186]. Vimentin is a cytoskeletal protein known to be phosphorylated prior to mitosis[16,187]. Comparison of HeLa scaffold and matrix proteins, by 2-D gel electrophoresis, shows them to be very similar, except that a 57kd protein is relatively enriched in the scaffold fraction[188]. A 51kd protein, another cytokeratin, is also modified in the scaffold fraction as judged by the change in its iso-electric point[188]. These results would suggest that these filament proteins may be important in the interphase-mitosis transition. Whilst it would be interesting to monitor the kinase activity, associated with the nucleoids, around the cell cycle, this was not investigated. More activity would be expected to be present in late G_2 than in any other phase of the cell cycle.

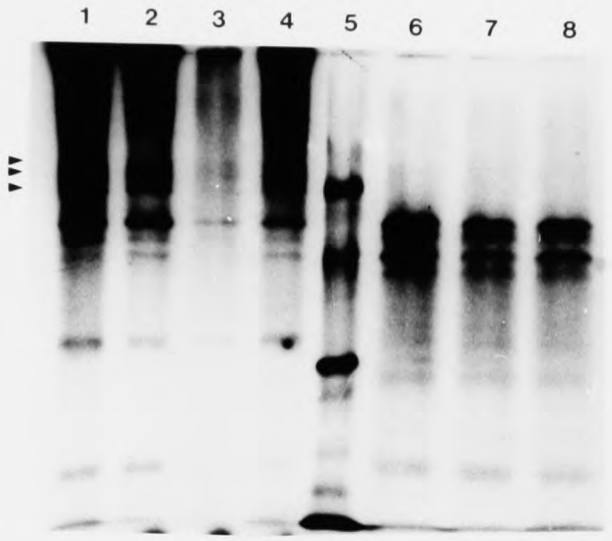


Fig. 11

Phosphorylation in vivo and in vitro of HeLa nucleoid proteins

Phosphorylation in vivo was carried out as described in Materials and Methods. Briefly the cells were pre-incubated in phosphate-free medium for 15 min, incubated in phosphate-free medium containing labelled phosphate at 100μCi/ml for 30', washed and nucleoids isolated, using a technique suitable for isolating nucleoids on a bench microfuge [see Materials and Methods; whilst this technique is quicker, it leads to some partial extraction of the cells]. The nucleoids were washed in 10mM Tris, 0.1mM EDTA, 0.1mM PMSF, pelleted, resuspended in sample buffer and the proteins separated by SDS-PAGE, fixed and autoradiographed. Drugs, where added, were present during both the pre-incubation and incubation steps. Patterns obtained in vivo in the presence of nil [track 1], 2.5mM thymidine [track 2], 4μg/ml actinomycin D [track 3], or 50μg/ml cycloheximide [track 4]. Less material was loaded in track 3 by accident. Track 5 contains molecular weight markers of 69, 46, 30 and 14.3kd. Tracks 6 to 8: phosphorylation obtained in vitro as described for figure 10. Track 6: no treatment, tracks 7 and 8: the effect of treatment with RN'ase A prior to or post-incubation with label. Note that, whilst species that are phosphorylated in vitro are also phosphorylated in vivo, the relative amounts are different. The extra bands [arrowed] are due to the partial extraction.

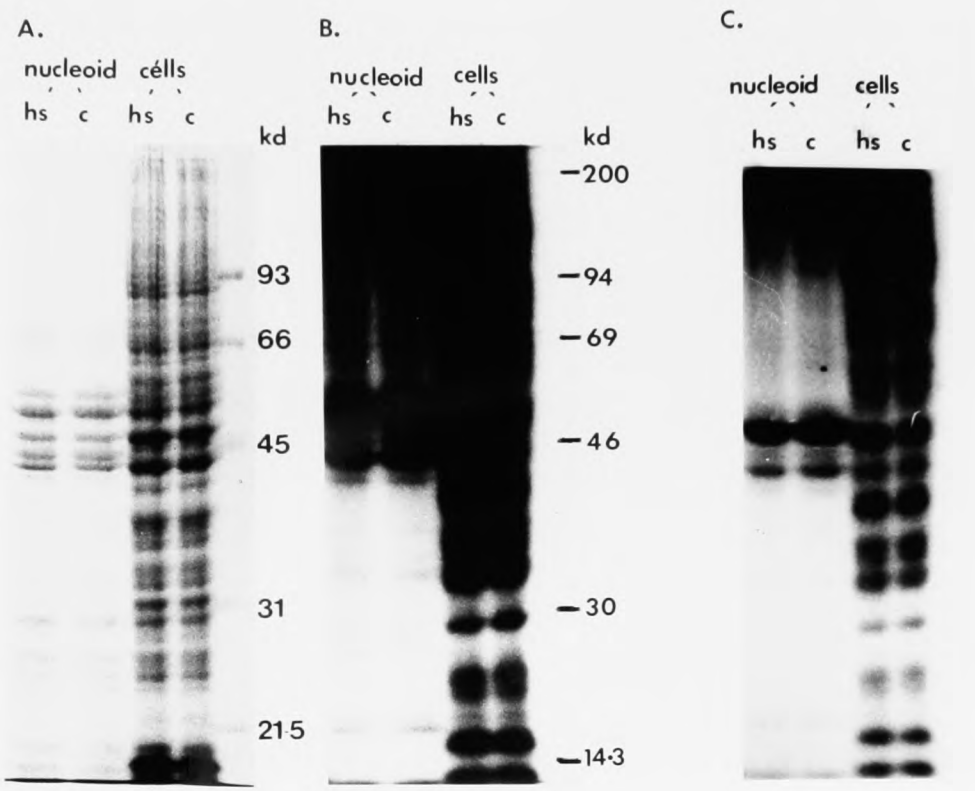


Fig. 12 Effect of heat-shock on phosphorylation HeLa cells were labelled with phosphate as described in figure 11 except the pre-incubation step was 30 min long and the final phosphate label concentration was 100μCi/ml. After the pre-incubation, the cells were either heat-shocked at 45 degrees C for 10 mins or incubated at 37 degrees C for 10 mins, and then labelled. Total cell extract, or nucleoid proteins were separated by SDS-PAGE, stained [fig. 12A] and autoradiographed [24h, fig. 12B and 6h, fig. 12C]. Tracks 1 and 2: nucleoid proteins, and tracks 3 and 4 total cell extracts from heat shocked (hs), or control (c) cells respectively. Heat shock treatment does not appear to change the phosphorylation patterns.

Nucleoid Methylase

The nucleoids also contain a protein methylase. Incubation of isolated nucleoids with Mg++ ions and labelled S-adenosyl methionine [SAM] results in incorporation of the label into a sub-set of nucleoid proteins [major bands at 66 and 81kd, arrowed, fig.13]. This incorporation does not reflect the Coomassie pattern, and is Mg++ ion dependent, sensitive to heat and proteases. Whilst this pattern was found to be present on several occassions, some experiments failed to show any label incorporation. Unfortunately, SAM readily decomposes and this was thought to be the reason why methylase activity was not detected on these occassions.

Nucleoid fragility

Perhaps proteins mediating the tight association of DNA with nucleoids might be solubilised after DN'ase treatment. In the course of such experiments, it was noted that incubation of nucleoids in buffer alone, Mg++ ions, or RN'ase apparently solubilised some nucleoid proteins, whereas treatment with DN'ase resulted in little solubilisation. HeLa cells were labelled with ^{35}S-methionine, and nucleoids isolated [see materials and methods]. The nucleoids were diluted 10-fold, and incubated with no additions, 10mM $MgCl_2$, DN'ase, or RN'ase added, at 37 deg. C for 30 min [fig. 14]. The samples were then spun at 100,000g and the supernatants and pellets analysed for protein, by SDS-PAGE and autoradiography. This effect is highly reproduceable. No significant differences were found when ^{3}H-leucine replaced ^{35}S-methionine, whether as a pulse-label [fig. 15], or long-term label [fig. 16]. More proteins were solubilised in the absence of

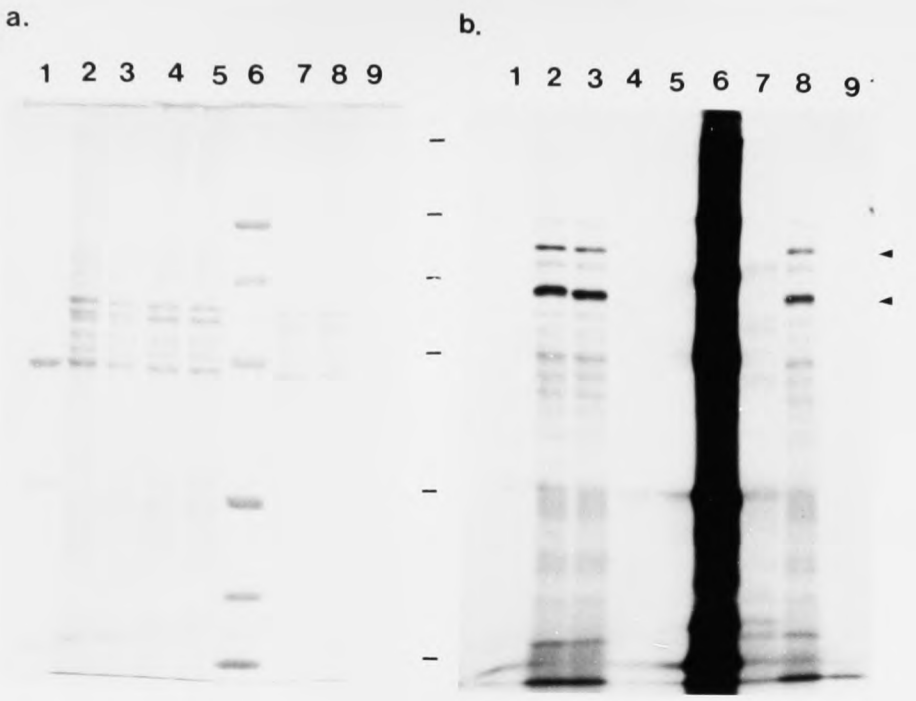


Fig. 13 In vitro methylation of nucleoid proteins HeLa nucleoids were isolated as described in Materials and Methods, diluted with 10mM Tris, 10mM $MgCl_2$, 0.1mM PMSF pH 7.5 to be 2.5 x 10^6/ml, pelleted and the pellets treated briefly with 30µg/ml DN'ase and 15µg/ml RN'ase final to allow adequate suspension. To 50µl of nucleoids, 5µCi of [3H]-methyl-S-adenosyl methionine was added and incubated at 37 degrees C for 2h. The nucleoids were pelleted and the pellets resuspended in 50ml of sample buffer and the protein separated by SDS-PAGE, and stained (a), and autoradiographed (b) as described above. Tracks 2 and 3: nil else added, tracks 4 and 5: incubated in the presence of 50mM EDTA, track 7: the nucleoids were heated for 5' at 65 degrees C prior to labelling, track 8 is the same as track 2, and 3. Track 9: the effect of proteinase K on labelling. Tracks 1 and 6 contain actin and protein standards respectively. Fig. 13b is an autoradiograph of the gel in fig. 13a. The bars alongside fig. 13b represent molecular weight markers of 200, 94, 69, 46, 30 and 14.3kd. A variety of proteins are methylated, of which the 66 and 81kd bands are prominent [arrowed]. The 46kd cytokeratin may also be methylated.

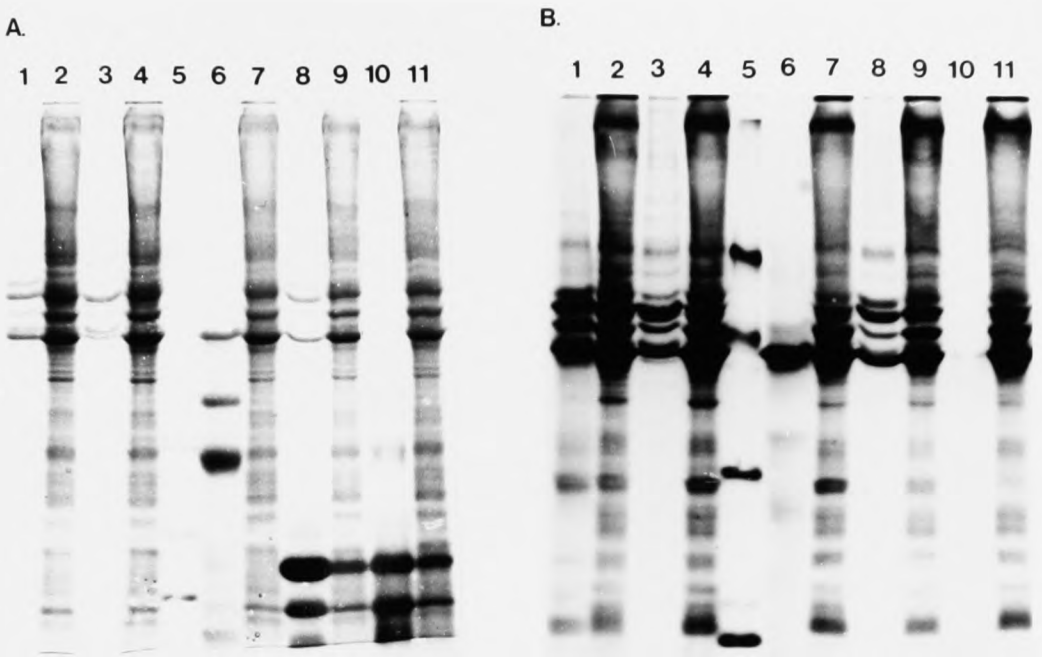


Fig. 14 Stability of nucleoid proteins HeLa cells were pulse-labelled with 35S-methionine for 10 min [see materials and methods], and nucleoids were isolated and diluted with 10mM Tris, 0.1mM EDTA, pH 7.5 such that the final NaCl concentration was 100mM. The nucleoids were then incubated with no additions [tracks 1 and 2], 10mM $MgCl_2$ [tracks 3 and 4], DN'ase I at 0.1mg/ml and 10mM $MgCl_2$ [tracks 6 and 7], RN'ase at 0.1mg/ml [tracks 8 and 9], or all [tracks 10 and 11]. Track 5 contains marker proteins of 69, 46, 30 and 14.3 kd. After incubation, the nucleoids were centrifuged and the pellets and the supernatants collected. The pellets were rinsed in TE buffer and resuspended in sample buffer, whereas the solubilised proteins were precipitated by the addition of trichloro-acetic acid to a final 10%, the precipitate collected, rinsed twice in 70% ethanol, and resuspended in sample buffer. The pellets [tracks 2, 4, 7, 9, 11] and the supernatant proteins [tracks 1, 3, 6, 8, 10] were heated and separated by SDS-PAGE. Fig. 14B is the autoradiograph of the Commassie-stained gel in A. Note that more proteins are solubilised by nil, $MgCl_2$, or RN'ase treatment [cf. tracks 1, 3, 6, 8] than by DN'ase treatment alone.

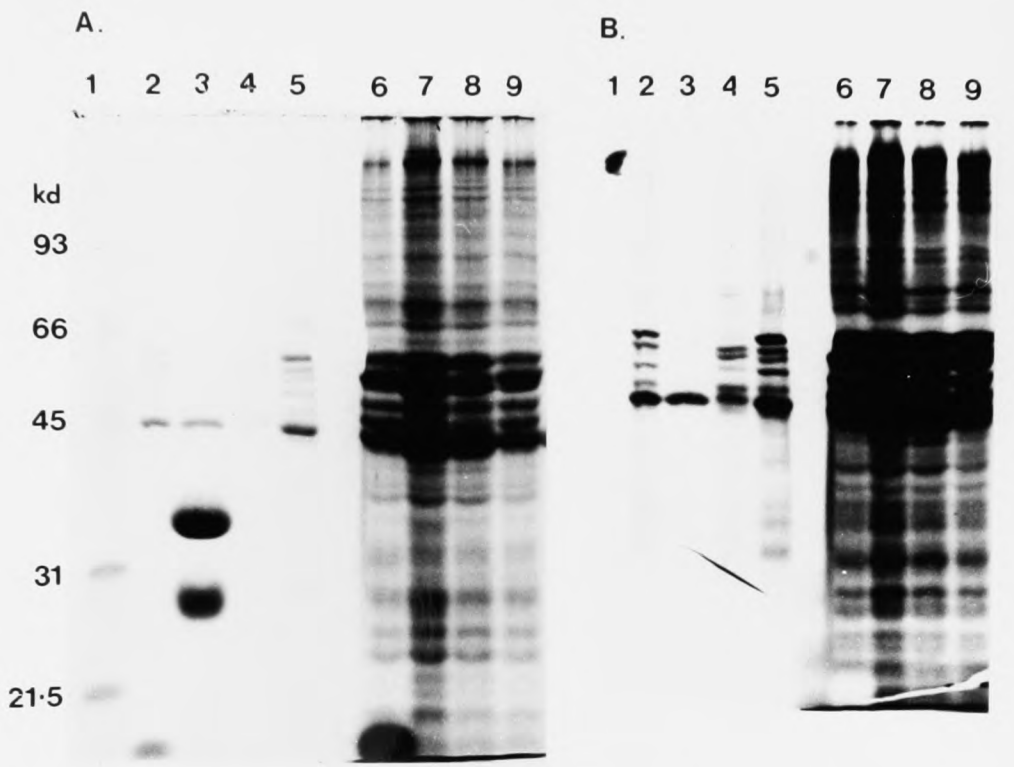


Fig. 15 Stability of nucleoids Legend is as for fig. 14, except that 3H-leucine was used as the label. Tracks 2, 3, 4, and 5 contain the supernatant proteins and tracks 6, 7, 8, and 9 the pellets after RN'ase, DN'ase, $MgCl_2$, or no treatment respectively. No differences were noted if the label is leucine, instead of methionine. Fig. 15A is the Commassie stain and 15B the autoradiograph of the gel. Track 1 contains marker proteins, as indicated.

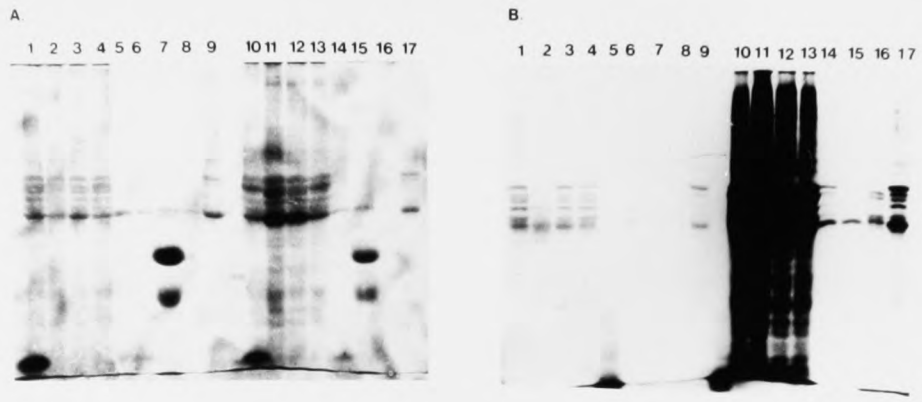


Fig. 16 Stability of nucleoids The cells were either pulse-labelled [tracks 1-9], or labelled for 24h [tracks 10-17] with leucine, and nucleoids treated as described in fig. 14. Tracks 1, 2, 3, 4, and 10, 11, 12, 13, contain pellets after RN'ase, DN'ase, $MgCl_2$, or no treatments respectively. Tracks 6, 7, 8, 9 and 14, 15, 16, 17 supernatant proteins after RN'ase, DN'ase, $MgCl_2$, or no treatment respectively. Track 5 contains actin as marker protein. Fig. 16A is the Commassie stain, and 16B the autoradiograph of the gel.

treatment, or if RN'ase or Mg++ alone were present. The DN'ase treatment solubilised very few proteins. The major protein seen at about 46kd is actin, that appears to depolymerise spontaneously, and is present in all the supernatants [cf. tracks 2, 3, 4, and 5, fig. 15].

The DNA in the nucleoid, once it has 'spilled' out [see fig. 17], would be liable to shear, as well as decrease the sedimentation rate of the nucleoid. Any handling will lead to fragmentation, and apparent solubilisation of the proteins.

Electron microscopic observations suggest that most of the DNA is naked, in good agreement with the above studies that show that few proteins are released, even after >90% of the DNA has been solubilised by DN'ase treatment. Scanning electron micrographs of DN'ase-treated nucleoids show them to be compact, and the overall morphology is well maintained [fig. 18].

Conclusions

Nucleoids contain protein kinase activity that is specific for cytoskeletal elements, and methylase activity that shows specificity for a sub-set of proteins. However, it is unclear whether these enzymes are normally present in the cytoplasm, or located in the nucleus. Since the biological activities of their substrates is also unknown, what differences these activities make to cellular function is also unclear.

Clearly, some of the nucleoid proteins are derived from the cell surface, as shown by the cell-surface iodination experiments. It is equally likely that some of the cytoplasmic proteins may also be present in nucleoids. Are these proteins present in the

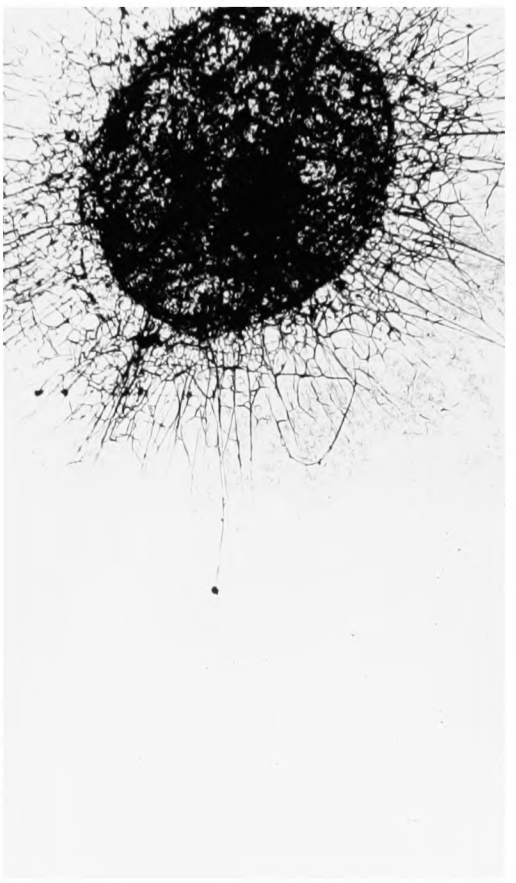


Fig. 17 Electron micrograph of HeLa nucleoids HeLa nucleoids were prepared and spread onto electron microscopy grids and stained using the Kleinschmidt procedure[78]. The DNA spills out of the nucleoid forming a skirt. The bar indicates 5μ. The microscopy was kindly performed by Dr. McCready.

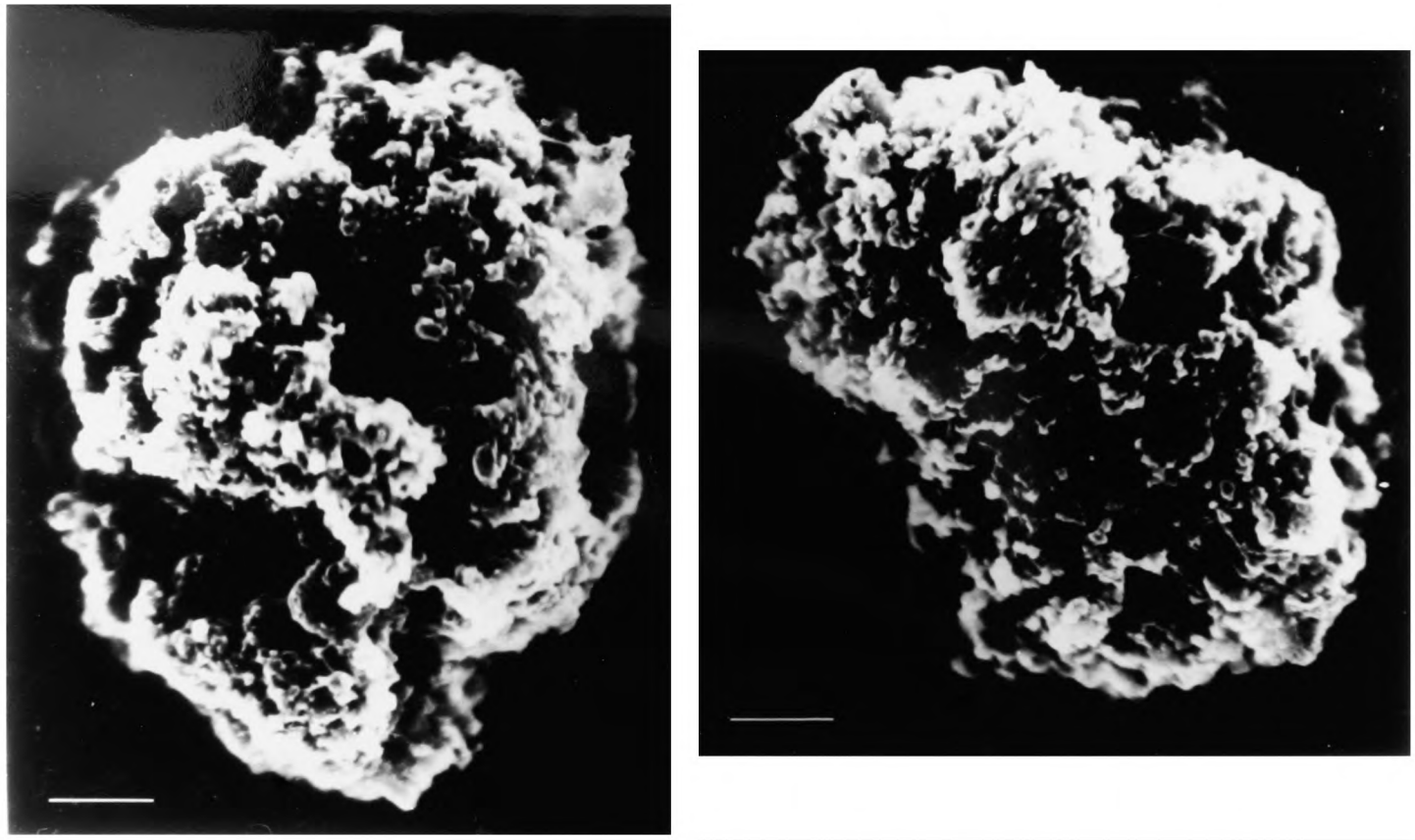


Fig. 18 Electron micrographs of nucleoids HeLa nucleoids were digested with DN'ase I and prepared for the scanning electron microscope. Figs. 18A and B show two different views of the same nucleoid as observed under scanning EM. The bar indicates 5μ. Microscopy was performed with the kind assistance of Miss Bergin.

nucleoids by virtue of an underlying sub-structure, or is this contamination? It is my belief that the cell-surface proteins are associated with the nucleoids via the cytoskeleton. Unfortunately, the techniques used to isolate cytoskeletal filaments, and their associated proteins are so similar, that independent confirmation may be difficult to obtain.

What is clear is that the majority of the DNA in the nucleoids is naked, and few, if any proteins are solubilised by DN'ase I. Since the only protein associated with the DNA is likely to be at sites where the DNA is attached, it may be possible to isolate both the sequence of DNA, and the proteins responsible for this interaction.

DNA-BINDING STUDIES

Filter-binding assay

Preliminary studies showed that nucleoid proteins contain DNA-binding proteins [figure 19]. HeLa nucleoid proteins, isolated as described in materials and methods, were renatured by dissolving them in 8M urea, 10mM Tris pH 7.5, 0.1mM EDTA and 50mM NaCl, and the urea dialysed away gradually over 36h at room temperature. This protein solution was used in a filter-binding assay as described in materials and methods. An aliquot of protein solution was mixed with radio-labelled DNA, incubated at 20 deg. C for 30 min. and passed through a nitrocellulose filter. The filter was washed by suction, dried and the retained radio-labelled DNA measured. The effects of varying the DNA or protein concentration are shown in figure 19. No DNA was retained in the absence of nucleoid protein or if bovine serum albumin was used alone. At fixed protein concentration, saturation was not obtained at protein:DNA ratio of 14:1 [wt/wt]. Identification of these DNA-binding proteins may be acheived using a new technique described by Bowen et al[139]. Briefly, the proteins are separated by SDS-PAGE, blotted on to nitrocellulose filters, renaturing the proteins in the process. The blotted filters are incubated with radio-labelled DNA, washed, dried and autoradiographed [see materials and methods for details]. Bowen et al[139] were thus able to identify DNA-binding proteins in HeLa nuclear matrix and scaffold preparations. This technique was therefore applied to investigate DNA-binding proteins in HeLa nucleoids. The procedure was tested using HeLa nuclear proteins since they are rich in DNA-binding proteins.

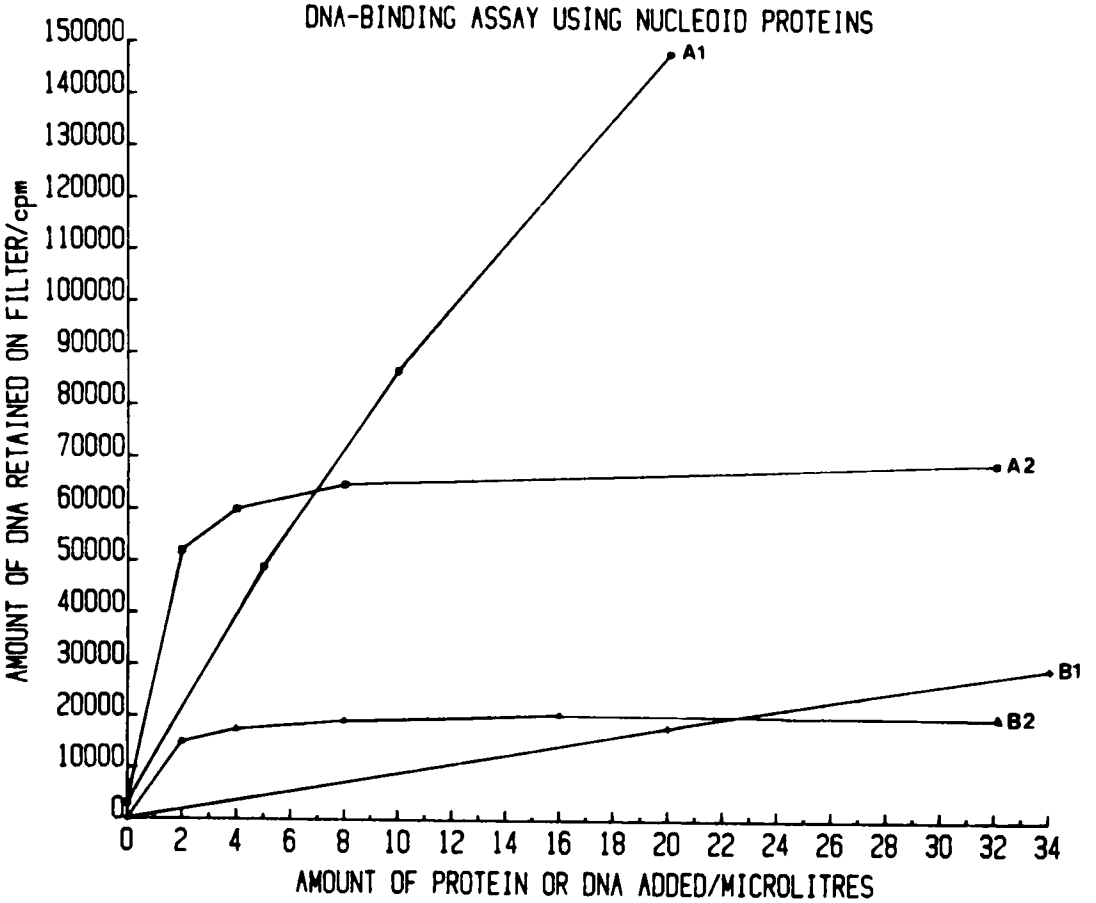


Fig. 19 Kinetics of DNA-binding to HeLa nucleoid proteins Nucleoid proteins, dissolved in urea, were renatured by dialysis as described in Materials and Methods. Nick-translated DNA was added to various amounts of protein (curve A2,B2) or variable amounts of DNA were added to a fixed amount of protein (curve A1,B1), incubated at 20 degrees C for 15 mins. The mixture was filtered through nitrocellulose filters and washed with 15ml of binding buffer under suction, and counted for radioactivity. A1: fixed protein added at 5.4µg, DNA varied at 0.25ng/µl. A2: fixed DNA at 5ng, protein varied at 2.7mg/ml. B1: fixed protein at 3.48µg, DNA varied at 0.25ng/µl. B2: DNA fixed at 5ng, protein varied at 0.8mg/ml.

Protein blotting

The blotting method [materials and methods] was modified slightly since significant amounts of low molecular weight proteins were lost from the gels during the soaking stages [fig. 20]. Hence all gel strips were soaked for 0.5h only, prior to blotting onto nitrocellulose filters. Initial experiments showed that the presence of urea in the gel made no significant difference to the DNA-binding pattern obtained subsequently [unpublished observations and see later]. Although not tested formally, it was estimated that the efficiency of transfer of proteins 96kd and less is about 90 per cent. Fig. 21 shows the gel strips stained after blotting. Significant amounts of proteins >120kd remain untransferred out of the gels. Gels were routinely stained after transfer to check blotting. Although the filters can be stained after blotting, good staining patterns are not obtained. However, an indication of the transfer is obtained by comparing the intensities of the ^{14}C-marker proteins after autoradiography and were found to be in good agreement [except for lyzozyme which can sometimes be lost preferentially during the soaking step as discussed above].

Salt washing procedures

In order to identify DNA-binding proteins with varying affinities for DNA, filters were incubated with radio-labelled DNA at 0.05M NaCl and washed in higher concentrations of NaCl to eliminate non-specific binding. Since DNA remains attached to nucleoids isolated in 2M NaCl, it was expected that some DNA-binding proteins may be thus identified. Fig. 22 shows the pattern obtained with HeLa nucleoid proteins incubated at 0.05M NaCl and washed in the various concentrations of NaCl as indicated.

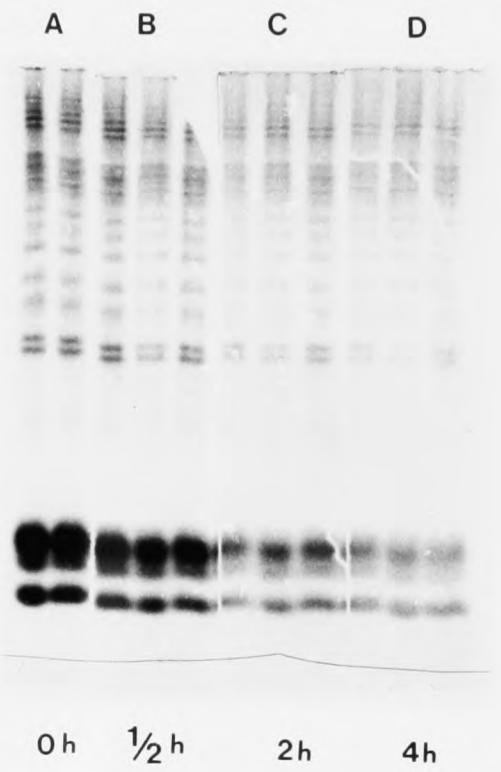


Fig. 20 Effect of soaking gel strips prior to blotting HeLa nuclear extract was separated on SDS-PAGE, and the gel divided into strips and soaked in the transfer buffer for the indicated times. The gel strips were fixed and stained for protein, photographed and the negative scanned using a Joyce-Loebl densitometer. [see Materials and Methods]. 40% of the core histones were lost after 4h of soaking [cf. strips A and D].

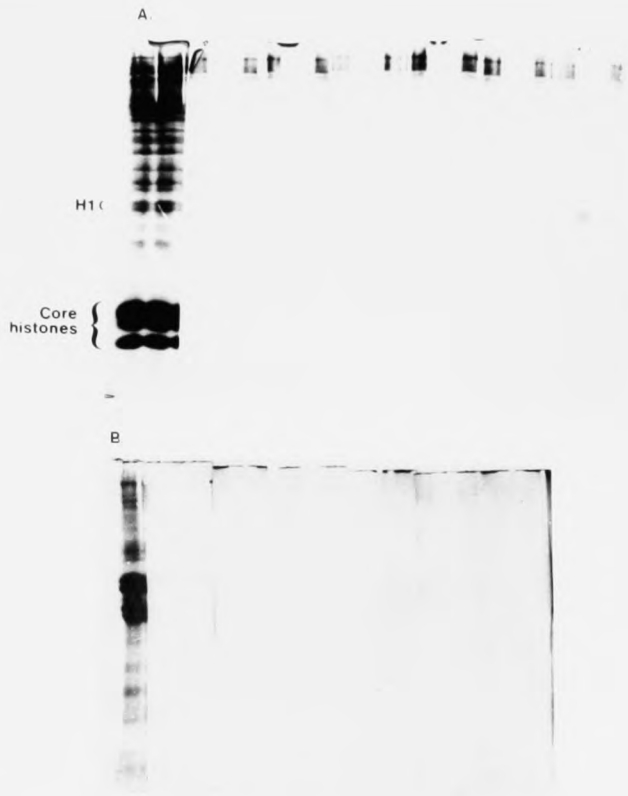


Fig. 21 Efficiency of blotting Gels were divided into strips, blotted, and stained after blotting, as described in Materials and Methods. A strip was stained without blotting for each gel to check the electrphoresis, and for comparision to the blotted strips. Gel A shows the results of blotting nuclear extract, and gel B that of nucleoid proteins. Proteins >120 kd do not transfer out of the gel efficiently [see gel A]. All blotting procedures were tested thus.

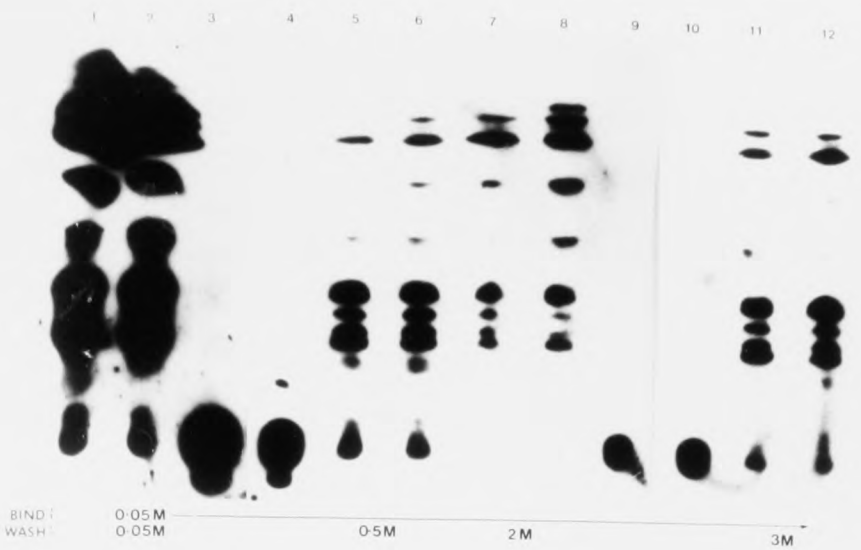


Fig. 22
Effect of salt-washing on DNA-binding to HeLa nucleoid protein HeLa nucleoid proteins were blotted onto nitrocellulose filters and pre-incubated, for 15 min, in 10mM Tris, 0.1mM EDTA, 50mM NaCl, 1x Denhardt's soln., and incubated in the same buffer, with 2.6ng/10ml/filter of nick-translated HeLa DNA for 2h at room temp [see materials and methods]. The filters were washed in 5 x 200ml, total 5h, at room temperature, in binding buffer and NaCl as indicated, dried and autoradiographed. Tracks 1, 2, 5, 6, 7, 8, 11 and 12 contain nucleoid proteins and the rest core histones. The salt concentration for incubation and washing are shown below the tracks. Binding to core histone is seen, even after washing in 3M NaCl [see text].

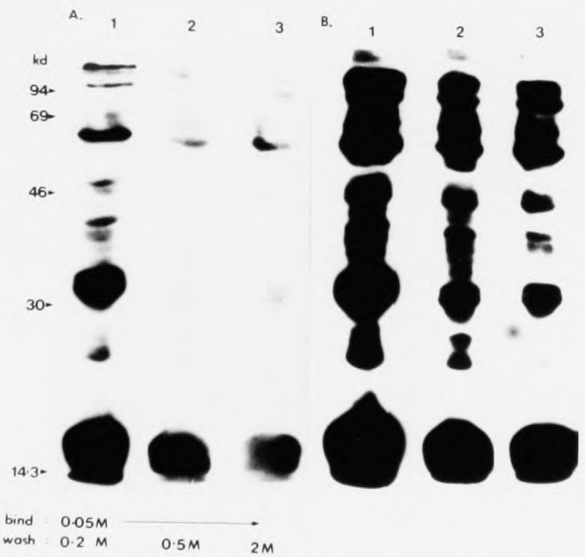


Fig. 23 Effect of salt-washing on DNA-binding to HeLa nuclear extract HeLa nuclear extract was blotted onto nitrocellulose strips, incubated with labelled HeLa DNA, and washed in various NaCl concentrations as indicated [see Materials and Methods]. Fig. B is a longer autoradiograph of A. Whilst a significant amount of DNA is washed off by the higher salt concentrations, no differences in the pattern is visible. Note that the DNA binding by the histones [around 14.3kd] is still present, even after a 2M NaCl wash.

Even after washing in 3M NaCl, significant amounts of radio-labelled DNA still remain bound. Though the intensities are reduced, the binding pattern is little affected. Core histones, which in solution at 2M NaCl do not bind any DNA, continue to do so, even after a 3M NaCl wash [compare tracks 3, 4, 9, fig. 22]. Whilst some differences in binding are present, even on the same filter [compare tracks 5 and 6, or 7 and 8, fig. 22], such inconsistencies are rare. In all subsequent experiments, duplicates of each filter were compared and only consistent results are shown.

Similar results were obtained for HeLa nuclear proteins [fig. 23]. In neither experiments was it possible to identify proteins with different affinities for DNA. Since the salt washing procedures reduce the amount of DNA remaining bound to the filters, it may be that the washing procedures were ineffective. Washes up to 5h long made no difference. Perhaps the increased salt concentration alters the protein-nitrocellulose filter binding-complex and the DNA becomes resistant to washing. To test this possibility, DNA-binding was carried out at the various salt concentrations [figures 24]. No significant DNA-binding was obtained when the NaCl concentration was 0.5M or higher. Nick-translation produces single-stranded DNA, which is known to bind to nitrocellulose filters with greater affinity at high salt concentration. However, no extra binding was demonstrable when single-stranded DNA was used [fig. 25].

Effect of competition

Although DNA is retained by some proteins, it is difficult to establish whether this binding is sequence dependent. Bowen et

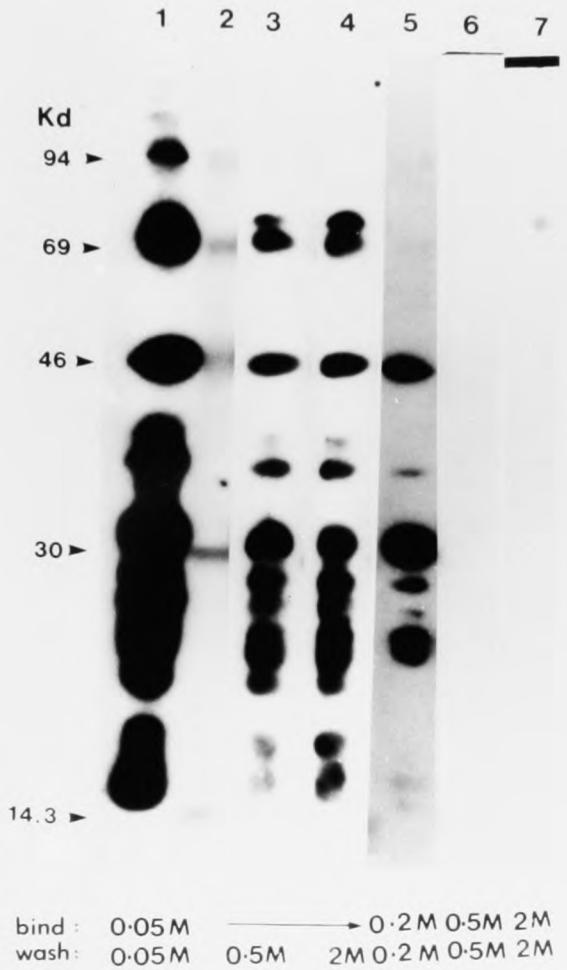


Fig. 24 Effect of binding DNA at higher salt concentrations HeLa nucleoid proteins were blotted as described in Materials and Methods. The binding was carried out at 0.05M NaCl [tracks 1-4] and washed at different salt concentrations as indicated, or DNA-binding and washing carried out at higher salt concentrations [tracks 5-7], as indicated. Note that no binding is present in the presence of 0.5M NaCl [track 6], although significant amounts of DNA remain bound to the proteins when the binding is carried out at 0.05M NaCl, but washed at 0.5M NaCl [track 3]. Note the significant binding in 0.2M NaCl [track 5].



Fig. 25

Effect of single-stranded DNA on binding to HeLa nuclear extract HeLa nuclear extract was blotted as described above and incubated with nick-translated DNA that had been treated with S1 nuclease [to remove 'fold-back' sequences], purified free of the nuclease and made single-stranded. Track 1 contains marker proteins of 94, 69, 46, 30, and 14.3kd. Track 2 and 3: effect of washing the nitrocellulose filter at 50mM and 500mM NaCl after binding in 50mM NaCl. Whilst the intensity of the bands is lower in track 3, no qualitative differences were noted.

al[139] showed that the DNA-binding was present even in a 1000-fold excess of unlabelled E.coli DNA. Therefore, DNA-binding was carried out in the presence of up to 7000-fold unlabelled E.coli DNA. HeLa nuclear extract, blotted onto filters, was incubated with nick-translated HeLa DNA in the presence of unlabelled E.coli DNA [tracks 2-4, fig. 26]. The binding was little changed, but, surprisingly, the reduction in the intensities of the signals was not dramatic, even at an excess of 7000-fold unlabelled E.coli DNA. Labelled E.coli DNA and excess unlabelled HeLa DNA gave similar results [tracks 5-7, fig. 26]. Since the binding of labelled DNA was not dramatically reduced after the addition of 7000-fold unlabelled DNA, it may be that the binding capacity was quite large and effective competitive concentrations were not obtained. The binding capacity could be lowered by blotting less protein. Unfortunately, below a threshold value, about 0.1mg loaded per track for total nuclear extract and HeLa nucleoid proteins, no binding of DNA is obtained. Above this value, the binding capacity seems to be large. Hence the amount of unlabelled HeLa DNA was increased to determine at which point competitive concentrations were achieved. Effective competition is only achieved at an excess unlabelled DNA of a million-fold [fig. 27]. In subsequent experiments tRNA was used as a competitor, because it is both cheaper and has a higher solubility.

Using tRNA as competitor, it is possible to identify proteins that show spcificity for DNA. Figure 28 shows a 50kd protein present in the nuclear extract that has bound DNA in the presence of a million-fold excess tRNA. Note that the binding to other proteins, including the histones, is also effectively suppressed. Unfortunately, no such proteins were detected for HeLa nucleoid extract [fig. 29].

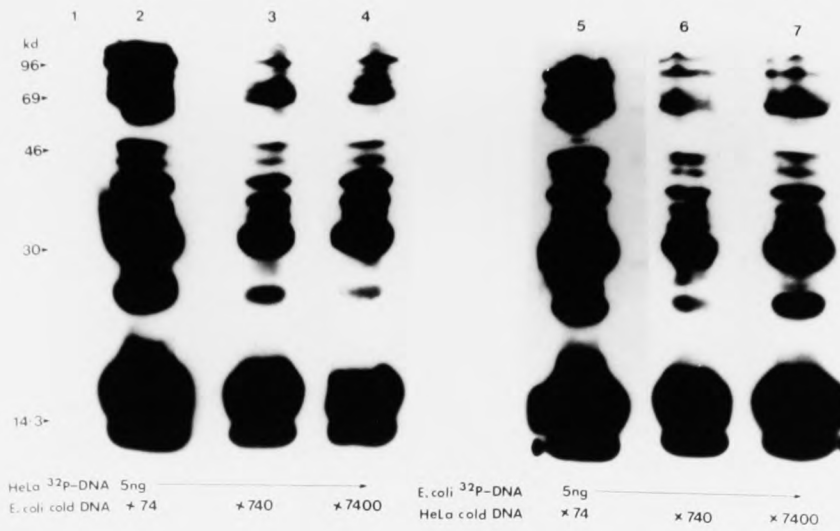


Fig. 26 Effect of competition by heterologous DNA on homologous DNA-binding HeLa nuclear extract was blotted as previously described. Binding and washing was carried out at 0.05M NaCl. During binding of labelled HeLa DNA, various amounts of unlabelled E.coli DNA were added as indicated [tracks 2-4]. Whilst the signal intensity decreases, no significant change in the pattern of binding was noted with increasing competition. Track 1 contains marker proteins. No significant differences are noted if labelled E.coli with unlabelled HeLa DNA is used, [tracks 5-7].

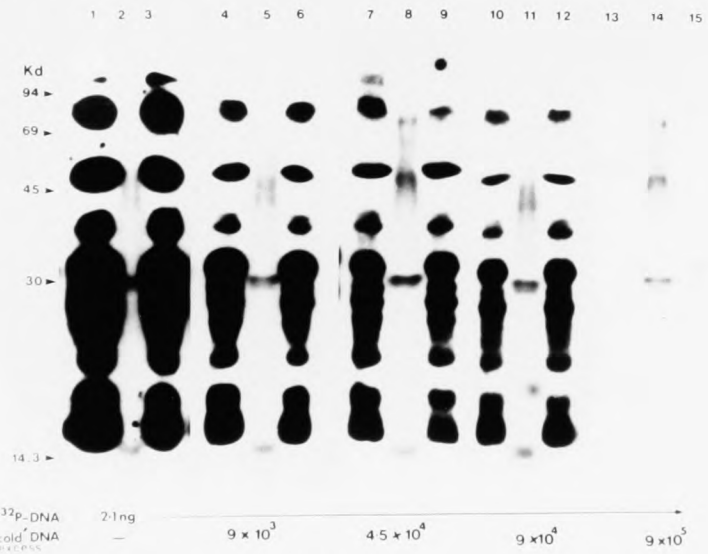


Fig. 27 Effect of homologous DNA on homologous DNA-binding HeLa DNA was bound to HeLa nucleoid proteins, except that increasing amounts of unlabelled HeLa DNA was used as competitor, as indicated. Strips with three tracks are shown to compare consistency of the binding. Binding of DNA is only significantly reduced at about 10^6-fold excess unlabelled DNA [cf. tracks 10-12 with 13-15, and see text]. Tracks 2, 5, 8, 11, and 14 are marker tracks. The decrease in the relative intensities of the bands in the presence of unlabelled competitor is in proportion to their binding in the absence of competition.

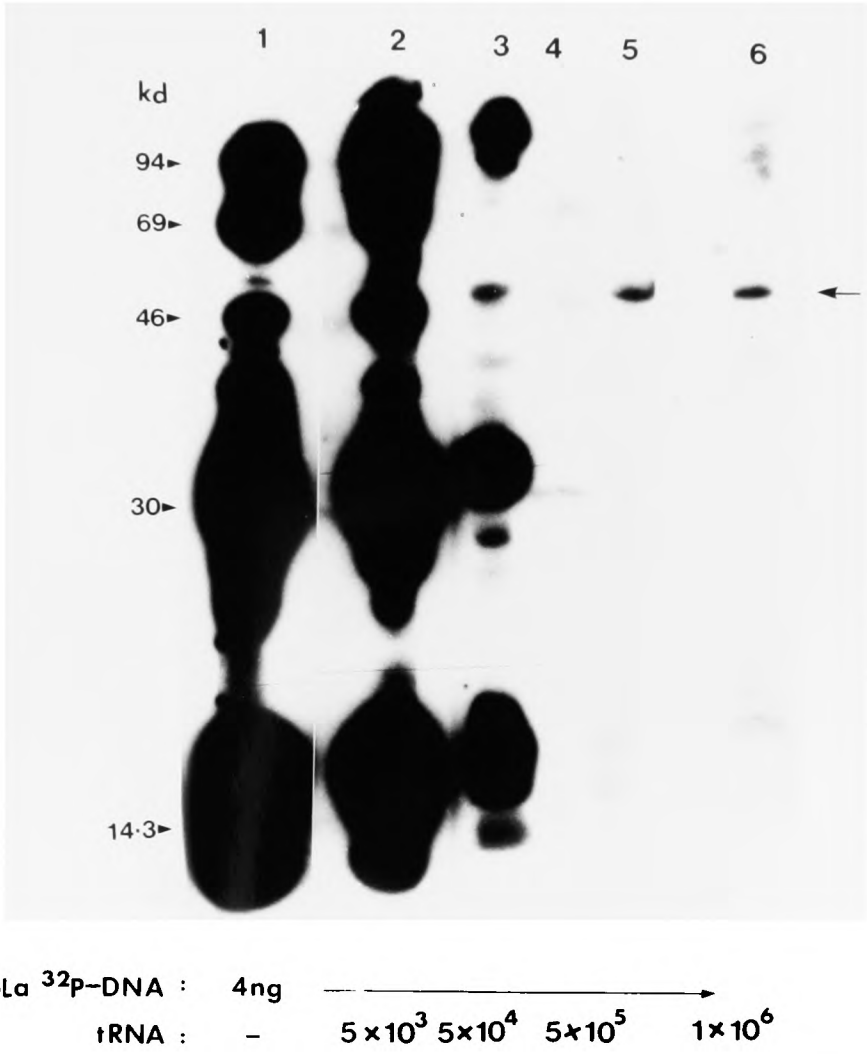


Fig. 28 Effect of competition by tRNA on DNA-binding Legend as fig. 26, except that tRNA was used as competitor. Note that significant reduction in binding occurs at 10^5-fold excess of tRNA. Track 6 shows a band that has bound HeLa DNA, even in the presence of 10^6-fold excess of tRNA [arrowed], and is likely to be DNA-specific.

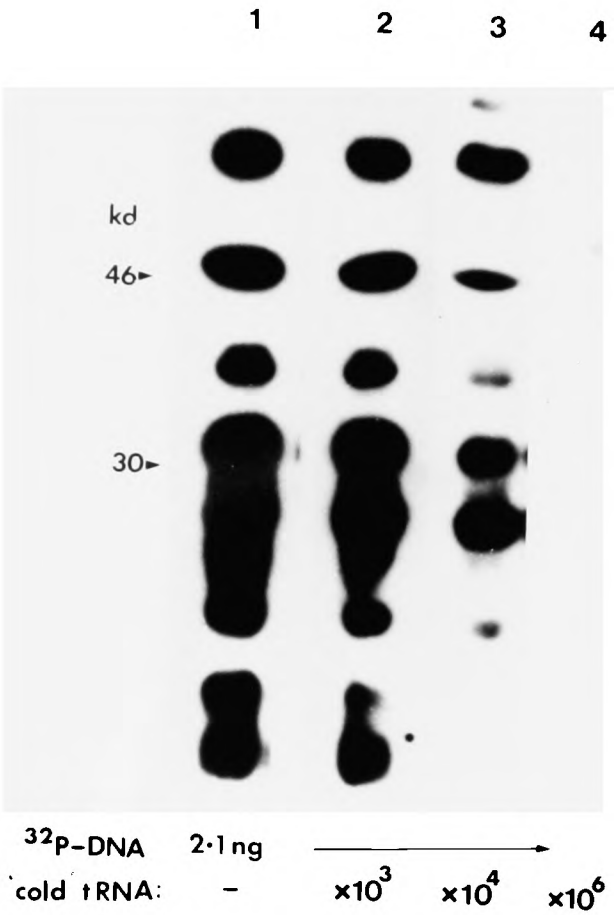


Fig. 29 Effect of competition by tRNA on DNA-binding Legend as fig. 28, except the DNA was bound to HeLa nucleoid proteins in the presence of tRNA, as indicated. DNA-binding was significantly reduced at tRNA excess of 10^6-fold, but no specificity was detected.

This technique was also used to detect RNA-binding proteins by incubating the filters with radio-iodinated RNA [figs. 30 and 31]. For HeLa nucleoids, two low molecular weight proteins are identified that bind to DNA, but not to iodinated RNA. The pattern of RNA-binding to nuclear extract is similar to its DNA-binding pattern.

In general, the above technique allows identification of nucleic acid-binding proteins, but does not allow easy differentiation between proteins of different affinities, or those with sequence specificity. Others have detected sequence specifity using this method[96,139,193]. Bowen et al showed that the lac repressor could specifically retain lac operator sequences, though they reported difficulty with reproduceability[139]. Jack et al bound end-labelled heat shock gene fragments to purified heat shock proteins, eluted off the bound fragments, and found that these proteins showed a preference for a particular DNA fragment[193]. Triadou et al reported specific retention of globin genes by proteins from an inducible cell-line[96]. However, in this latter case, the autoradiography signal is sufficiently weak to suggest that this is not specific retention, but due to threshold effects at low protein concentration. This latter criticsm may also explain the difficulty reported by Bowen et al[139]. Perhaps using simpler systems with purer proteins and simple DNA probes may enable such conclusions to be drawn. Unfortunately, for most eucaryotic systems, the purification, let alone their identificationas sequence binding proteins, is very difficult. In many cases, sequence specificity is only hypothesised to be present. One reason for the lack of such eucaryotic proteins is the difficulty in selective purification of these proteins.

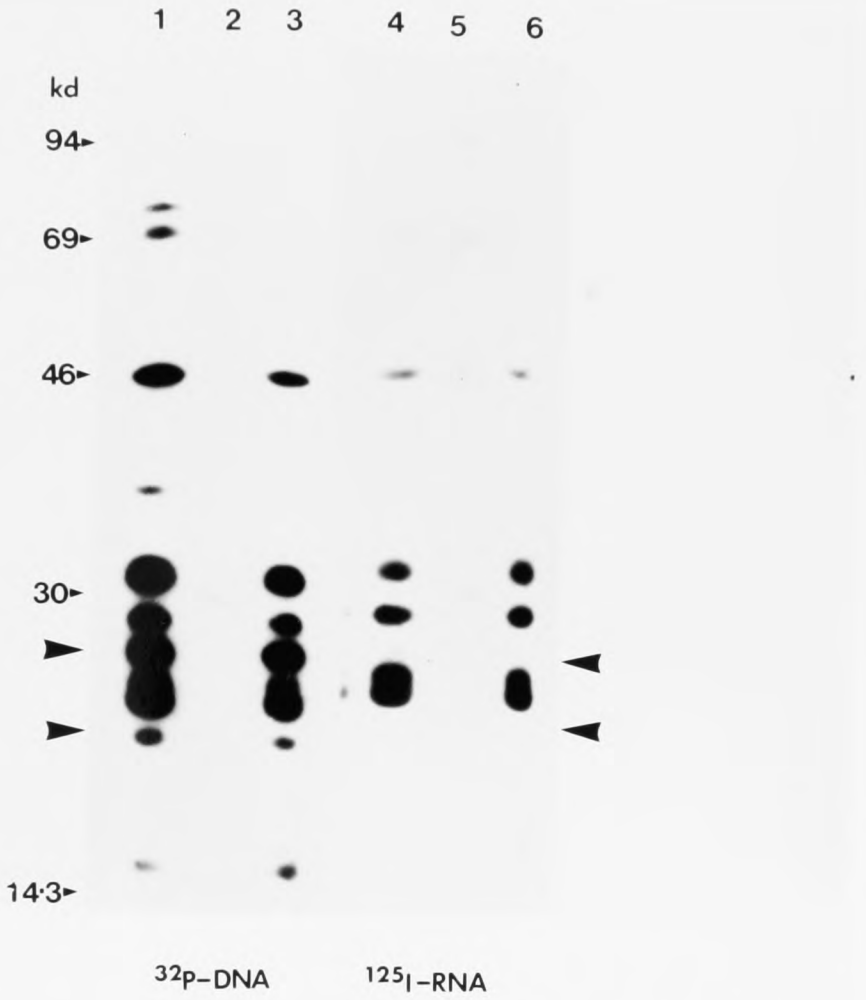


Fig. 30 Comparision of DNA- and RNA-binding to HeLa nucleoid proteins
HeLa nucleoids were incubated with labelled DNA [tracks 1, 2, 3], or labelled nuclear RNA [tracks 4, 5, 6] at 0.05M NaCl, washed at 0.05M NaCl. Tracks 2, 5 are protein markers. Arrow heads indicate proteins that bind to DNA, but not to nuclear RNA.

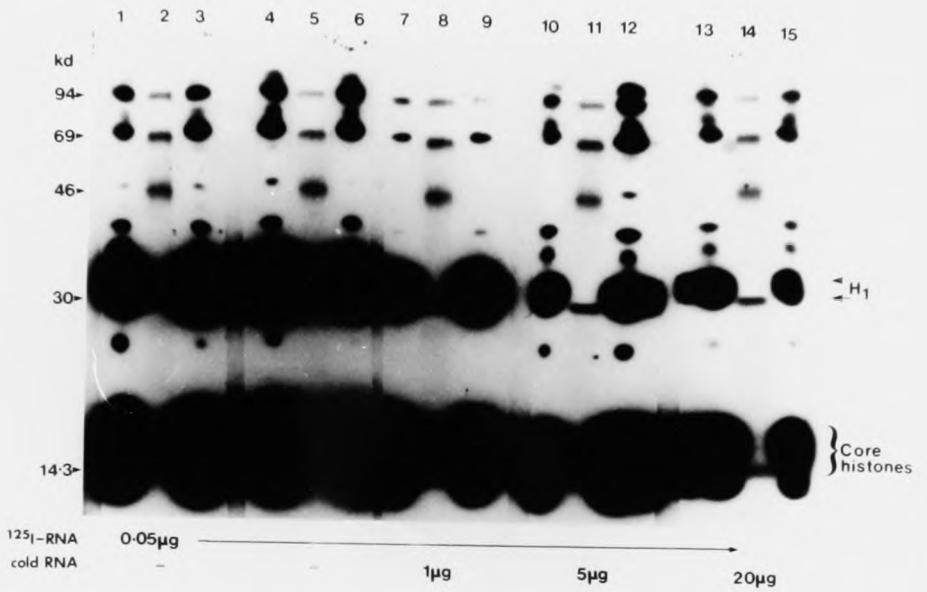


Fig. 31 Effect of binding RNA to HeLa nuclear extract HeLa nuclear extract was bound to radio-iodinated RNA [see materials and methods], and washed in 0.05M NaCl. Tracks 2, 5, 8, 11 and 14 are protein markers. Unlabelled nuclear RNA was also added as indicated.

Using this technique in conjunction with purification procedures may remove some of these obstacles. Carrying out DNA-binding assays at salt concentrations between 50mM and 200mM may identify such proteins [fig. 24]. The above technique was therefore modified to allow easier identification of both the protein and the sequence of DNA it recognises.

SOUTHERN CROSS

Technique

The modified technique is illustrated in figure 5 [see also materials and methods]. A mixture of four proteins is resolved by gel electrophoresis and protein blotted onto a nitrocellulose filter, which is then laid onto an agarose gel containing size-separated end-labelled DNA fragments and blotted such that the electrophoretic polarities of the DNA and the protein are perpendicular to each other. Each protein is therefore exposed to each DNA fragment during the transfer of the DNA through the nitrocellulose filter. The filter is then autoradiographed. In figure 5, the 43kd protein does not bind to DNA, the 50kd and 20kd proteins are non-specific DNA-binding proteins and the 80kd protein shows specifity for the 5kb DNA fragment. The buffers used during transfer are similar to those used for conventional DNA-binding assays.

Lambda repressor binding studies

In order to test this modification, the sequence specific phage λ repressor was used. An E.coli strain was obtained from Dr. B. Meyer that overproduces the repressor to 1% of total cell protein under optimal conditions[170]. Total cell protein extract from the repressor over-producer [E.coli 294/pkB280] was prepared as described in materials and methods, and blotted. The blotted filter was then crossed with end-labelled phage λ DNA cut with Bam H1 and Eco R1 endonucleases, as described above. The crosses were carried out at two salt concentrations, 50mM and 200mM. This would

enable the selection of DNA-binding species with high affinity for the DNA [fig. 32]. At low salt concentrations, a variety of protein species bind DNA. Note that around the 27kd band there is retention of all the DNA fragments at low salt. Whilst the repressor may bind with specificity, its binding is masked by proteins that migrate to a similar position. Raising the salt concentrations leads to the unmasking of the high affinity proteins [figure 32b]. There is now a single autoradiographic spot at the junction of the 27kd protein and the 4.7kb DNA band. The former is the repressor and the latter the DNA fragment that bears the two operator sites. The low molecular weight proteins around 16kd are the histone-like HU proteins of E.coli which are known to have high affinities for DNA.

Effect of Urea on the binding

Protein gels run with or without 4M urea, made no significant difference to the final result obtained [compare a and b, figure 33]. Hence, in subsequent experiments no distinction is made between gels run with or without 4M urea, though most gels were run with urea.

Time of transfer

In the above results, the transfer of DNA out of the gel and through the filter [crossing time] was 18h. However, shorter crossing times can be employed. Figure 34 shows the effect of crossing Lamda DNA, cut with Hind III, for 1.5 and 3h. Hind III enzyme separates the two operator sites into two fragments, hence the retention of two specific autoradiographic spots. Note that the intensities of the two spots is in relation to the known

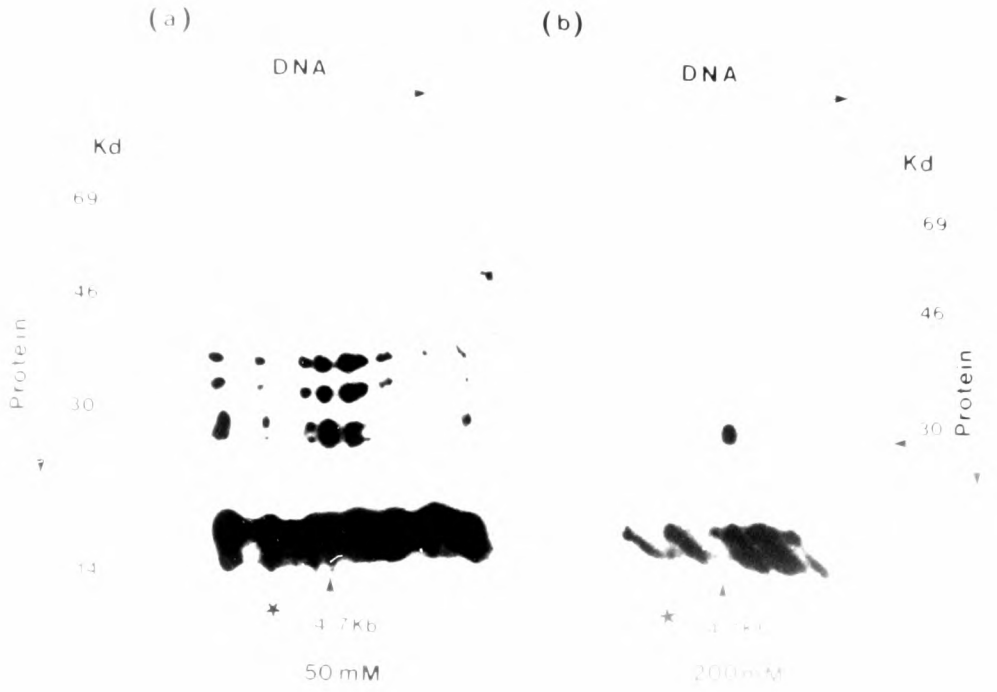


Fig. 32
Effect of salt concentration on DNA-binding to Lambda repressor Total cell protein from the λ repressor overproducer [1.35mg/gel] was crossed with 1.5μg of end-labelled phage λ DNA cut with Bam H1 and Eco R1, at either 50mM NaCl [a], or 200mM NaCl [b] as described in Materials and Methods. The arrow indicates the 4.7kb DNA fragment carrying the repressor binding sites. The sizes of the other fragments indicated by bars are 16.3, 9.0, 5.5, 4.7, 3.8, 3.8, 3.5, 3.2, 2.8, 2.6, 1.7 and 1.2 kb. The asterisk indicates the 9kb band that arises from the cohesive end reannealing of the 5.5kb and 3.5kb bands. Total crossing time was 18h and autoradiography was for 4h.

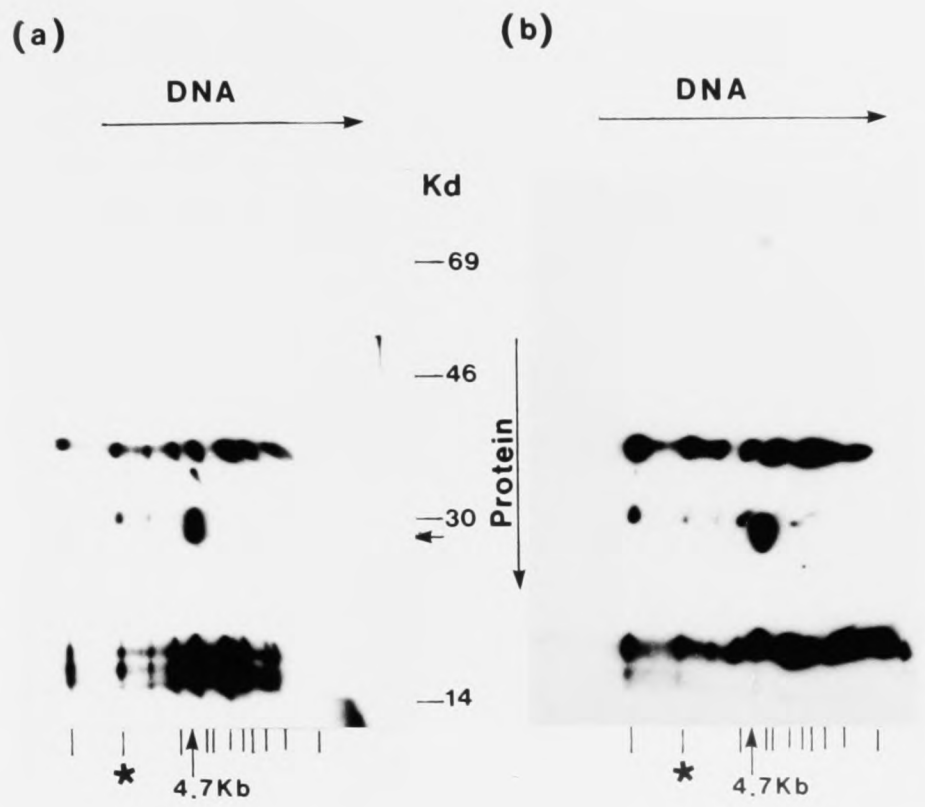


Fig. 33
Effect of 4M urea/SDS or SDS alone on Lambda repressor binding The protocol for crossing was as for figure 32b, except the gel was run with SDS alone [a] or contained 4M urea as well [b]. 2.5µg of DNA was crossed. Note that specific repressor binding to operator sequences is obtained in both cases. Binding to the 40kd protein is sometimes seen, as its affinity for DNA is close to, but less than that of the HU proteins. The autoradiography spot at the 44kd level is spurious [b].

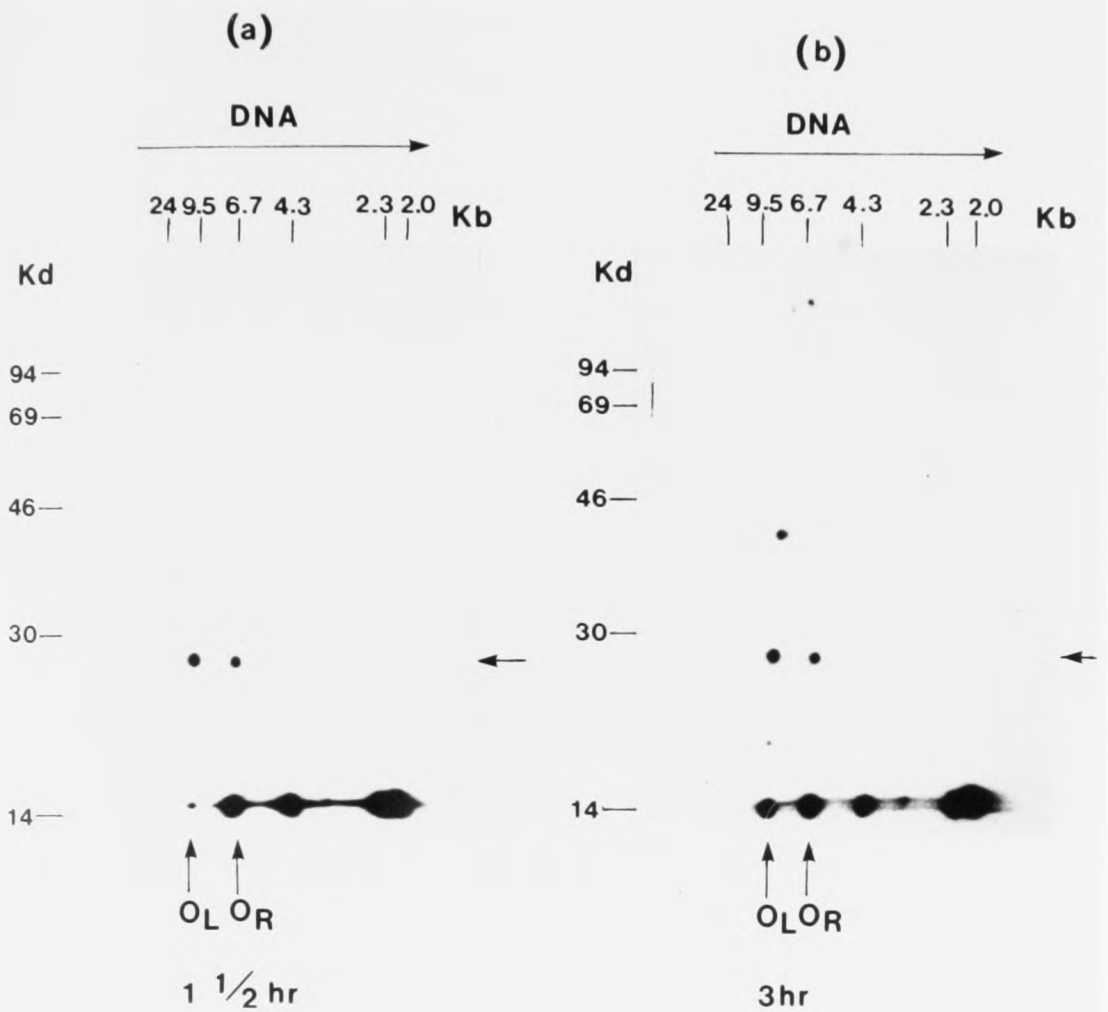


Fig. 34 Effect of crossing time on Lambda repressor binding Total cell protein (0.7mg/gel) from the repressor overproducer was crossed with Hind III-cut Lamda DNA (0.1μg), at 200mM NaCl, for 1.5h [a] or 3h [b] as described in Materials and Methods. Fig. 40[a] and [b] both show specific binding by the retention of the 9.5kb and 6.7kb fragments that carry the operator sequence. The intensity of the 9.5kb spot is also greater than the 6.7kb reflecting the known

affinities by the repressor for these two sites. The short crossing time shows that fragments less than 10kbp are easily transferred through the gel, but the larger 25kbp fragment takes much longer to transfer out of the gel, as would be expected. The 23 kbp fragment is easily detectable after 6h of transfer [fig. 34].

Sensitivity of the cross

To test the sensitivity of the technique, both the concentration of protein loaded onto the gel and the DNA were varied. Figure 35 shows the results of varying the concentration of DNA at fixed proteins loading. The proteins concentration loaded onto the gel was approximately half-maximal to that which could be loaded. Titration of the DNA loaded onto the gel shows that the least amount of λ DNA that must be loaded for detection of specificity is about 0.01μg. It may, therefore, be possible to detect operators at their natural abundance in lysogenic bacteria. The result of such an experiment, crossing DNA from a lysogen with proteins from the repressor over-producer, is shown in figure 36. DNA from a lysogenic strain was cut with Bam H1 or Eco R1. A single spot is reproduceably retained, the size of which matches the size of the fragment expected to carry the. operator sequences [fig. 36]. Other spots are evident in the results shown, but these are spurious and are therefore attributed to background.

There is a threshold value of loading of proteins below which no DNA-binding can be demonstrated [as discussed above]. Figure 37 shows the result of reducing the amount of proteins loaded to 0.27mg. Note that from each gel two replicas are obtained and therefore a maximal of 0.135mg per replica would be theoretically

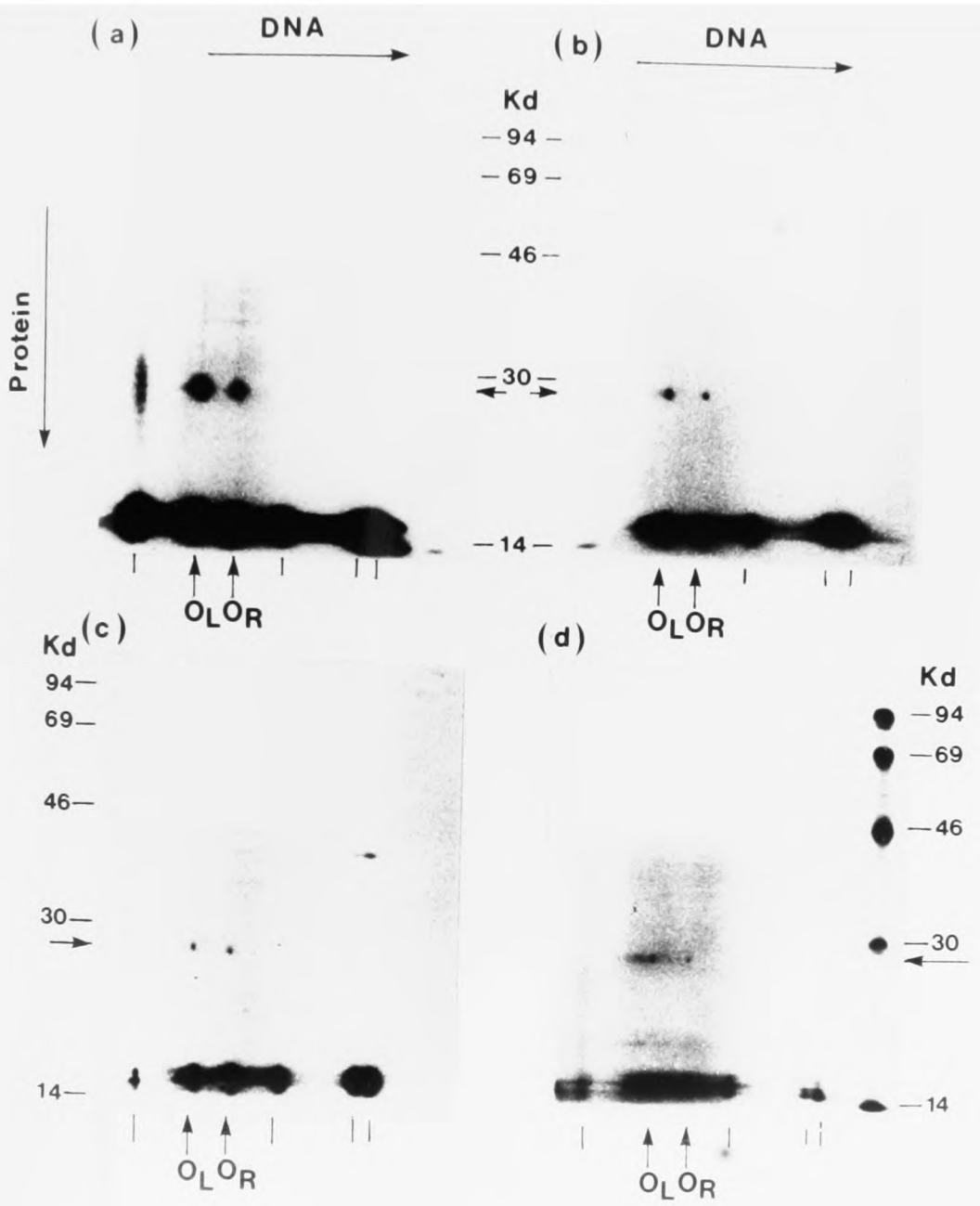


Fig. 35 Effect of decreasing DNA crossed with total cell extract
Total cell protein (0.7mg/gel) from the repressor overproducer was crossed with varying amounts of end-labelled Hind III-cut Lamda DNA; (a) 1µg, (b) 0.5µg (c) 0.1µg and (d) 0.01µg. The crossing time was 6h and autoradiography for 1d (a, b, c) or 7d (d). Specific binding was obtained even with 0.01µg of total DNA loaded onto the agarose gel (see text for discussion).

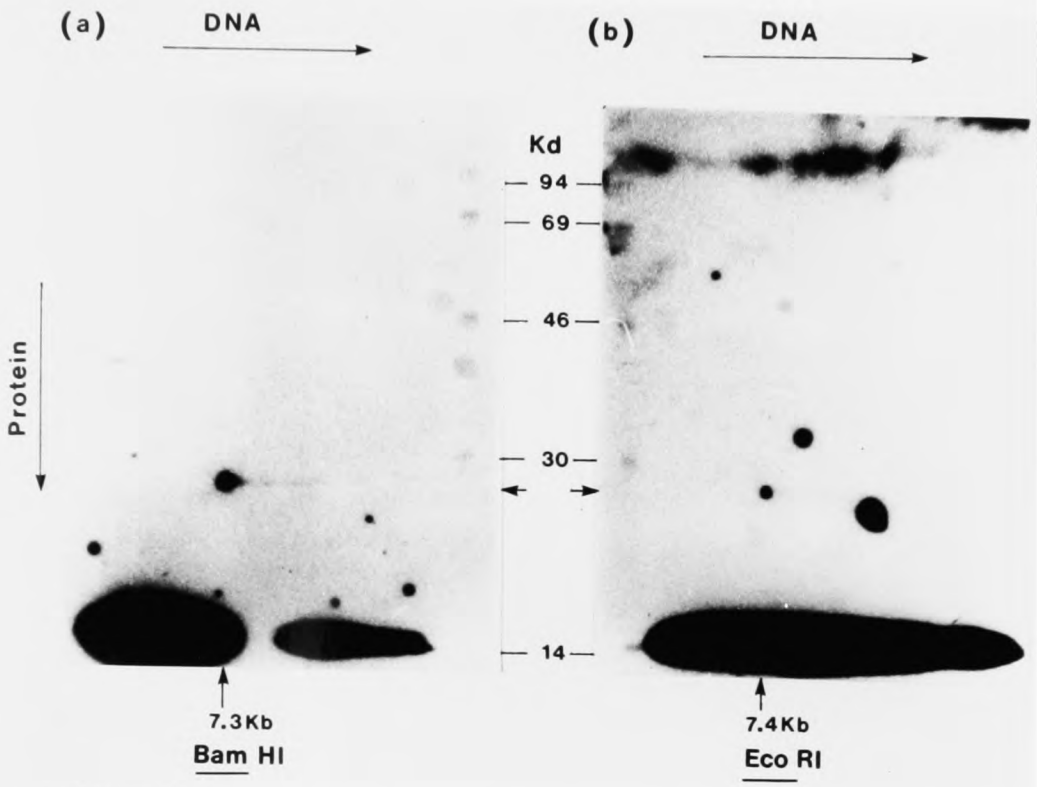


Fig. 36

Detection of operator sites present in DNA from lysogenic strains 1mg of total cell protein from the overproducer was blotted to yield 2 filters, one of which was crossed with 16µg of Bam H1-cut DNA (a), or 16µg of Eco R1-cut DNA (b) from a lysogenic strain of bacterium. Autoradiography was for 1d. The operator fragments are arrowed (7.3kb Bam H1 and 7.4kb Eco R1). Although a variety of other 'hot spots' are seen, these are not reproduceable. The artefact on the 15kd protein band (a) is thought to be due to a bubble during blotting. The specific retention of the repressor fragments indicates that such fragments at their natural abundance can be detected.

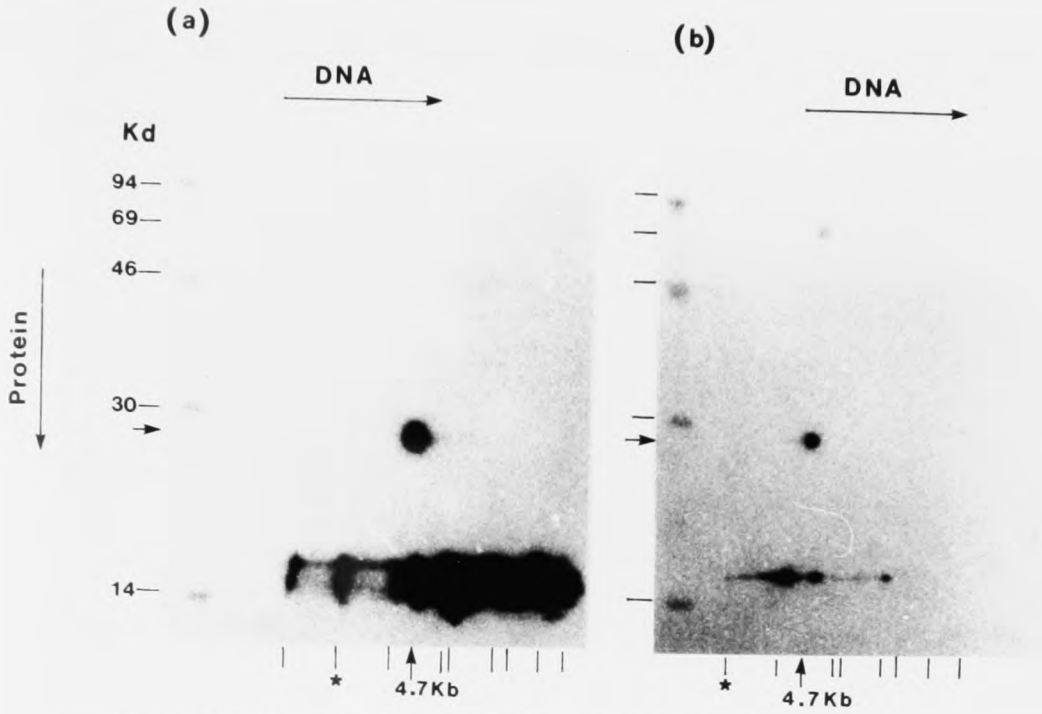


Fig. 37 Effect of reducing protein concentration 1.5μg of λ DNA was crossed with either 1.3mg/gel (a) or 0.27mg/gel (b) of total cell protein from the overproducer for 18h. A positive result is still obtained after 0.27mg protein is used. The lowest amount at which such a clear-cut result was obtained was about 0.2mg (see fig. 38). Autoradiography time was 24h. Note that the intensity of the HU proteins is considerably reduced as total protein is loaded.

expected. A reduction of a 100-fold of protein loaded would have to be achieved for the effective repressor concentrations to be in the range of its natural abundance, at about 0.01% of total cell protein. The present limit obtained is, at best, a ten-fold reduction.

Hence, whilst no enrichment of DNA sequences is required, the repressor needs to be enriched at least ten-fold over its natural abundance to be detected by the cross.

Binding to repressor in the lysogen

Nevertheless, comparisons between DNA-binding of the repressor overproducers and the lysogenic strains were made. About 0.2mg of the repressor overproducer total cell extract was crossed at 50mM and 150mM with phage λ DNA but with Hind III [figure 38a and b], and about 2mg of lysogenic strain total cell extract was similarly treated [figure 38c and d]. Specific binding for the repressor overproducer can only be demonstrated. Raising the salt does not abolish binding of DNA to minor bands in the lysogen extract because the amount of lysogen protein blotted is 10-fold greater than that for the over-producer. At high protein concentrations the level of non-specific DNA binding is also higher. Figure 39 shows the analogous cross of total cell extract from a lysogenic strain with its own DNA at 50mM and 150mM NaCl. No specific binding is noted, as expected.

Binding to Lex A protein

Because this procedure is suited to studying repressors in strains that overproduce them, it was applied to the study of lex A gene product. The lex A gene product controls several bacterial

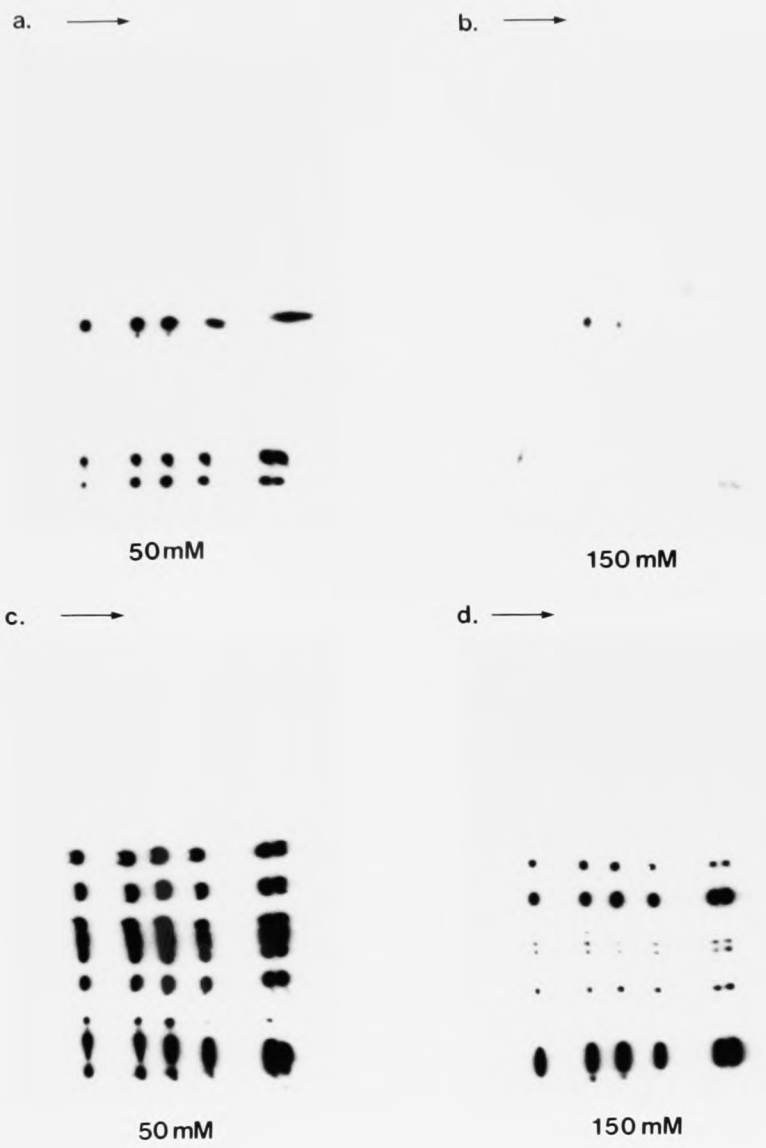


Fig. 38
Comparison of over-producer and lysogenic strain protein binding Total cell protein [0.2mg/gel] from the over-producer (a, b) and [2mg/gel] from a lysogenic strain (c, d) were crossed with Hind III-cut λ DNA at 50mM (a, c) or 150mM NaCl (b, d). No specific binding was detected for the lysogenic strain. The extra spots in (d) are thought to be due to the large amounts of total cell protein loaded onto the gel for blotting.

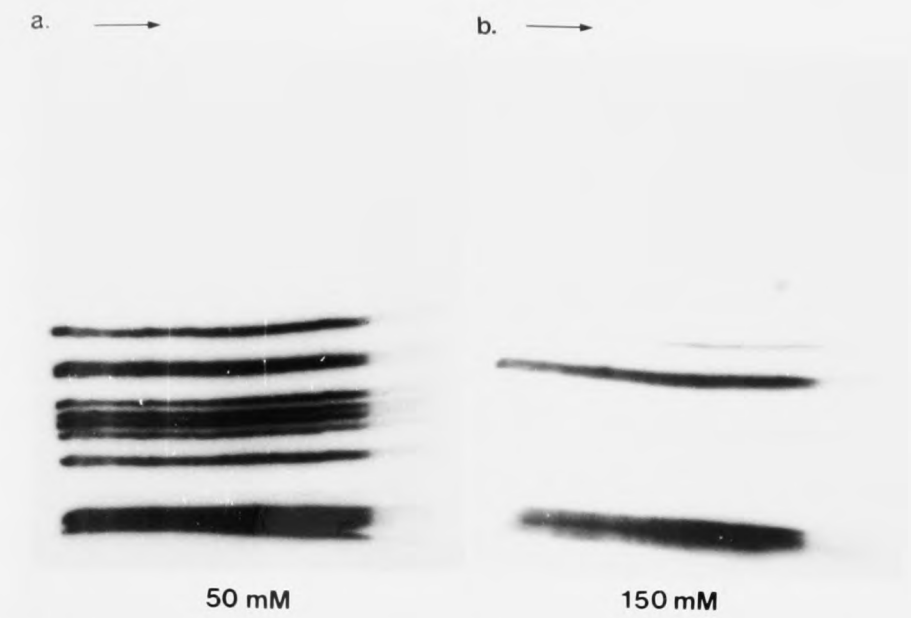


Fig. 39
Crossing lysogenic total cell protein with DNA from the lysogen 16μg of Eco R1-cut DNA from a lysogenic strain was crossed with total cell protein from the same strain (1.5mg/gel) at 50mM NaCl (a) or 150mM NaCl (b). No specific binding was detected [see text].

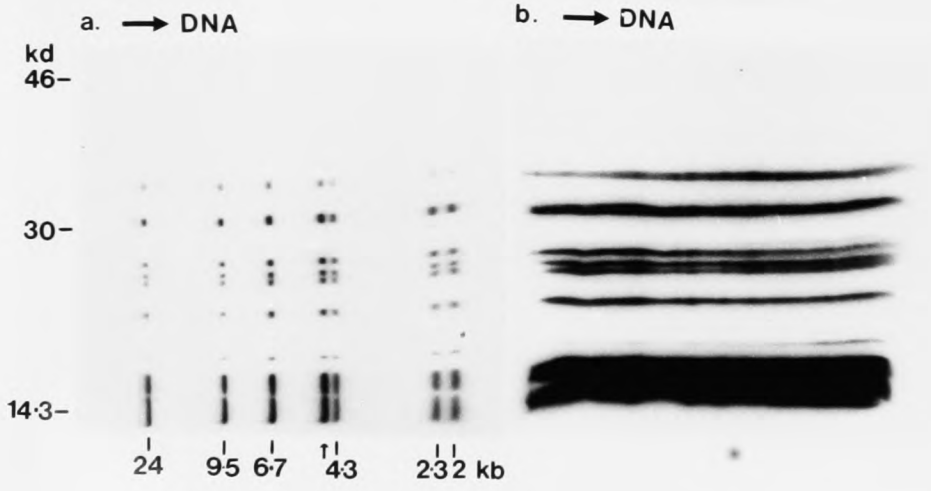


Fig. 40 Crossing Lex A protein with lac operator sequences Total cell extract from the Lex A over-producer (1mg/gel) was crossed with 0.5μg of λ DNA and a plasmid, pGH6, containing lac operator sequences [arrowed]. The crossing buffer was 10mM Tris, 10mM $MgCl_2$, 10mM KCl, 1x Denhardt's soln., 5% DMSO and 0.1mM EDTA, pH 7.5. The crossing time was 6h. For comparision, total cell extract from a λ lysogen crossed with total cell DNA is included (b). No specific binding was detected. Note, however, that the binding patterns of the Lex A over-producer and the λ lysogen are very similar.

loci, though only two or three have been identified to contain sequences specifically recognised by this protein[194]. High concentrations of lex A appear to be toxic to the cell, therefore the lex A gene was placed under the control of lac operator sequences. This enables the over-production of lex A protein , after induction with IPTG. Figure 40 shows the effect of crossing total cell extract from lex A overproducer with λ DNA and a plasmid containing the lac operator sequences, to detect the lac repressor. None is identified as it is only just above its natural abundance. As the cross was carried out at low salt, a variety of DNA-binding proteins are also identified. Comparison with the Lamda lysogenic strain shows the pattern of DNA binding to be similar.

Effect of pH

The effect of pH on crossing total DNA with total cell extract from the lex A over-producer was investigated [fig. 41]. The binding is considerably reduced at low pH, but unaffected at pH 8.5. so pH 7.5 was chosen as the optimal pH, after comparision with figure 40.

Specific binding by Lex A protein

Crossing total E.coli DNA with the lex A overproducer cell extract at 200mM NaCl and pH 7.5, a series of spots were retained, by a 21.5kd protein, that are suggestive of specific binding [figs. 42 and 43]. About 14 spots are seen when Bam H1 cut-DNA is used, with varying affinities. The Bam H1 result is reproduceable [fig. 43, compare a with b and fig. 44]. The pattern of spots is different when Eco R1 is used to digest the DNA [fig.42a], and a

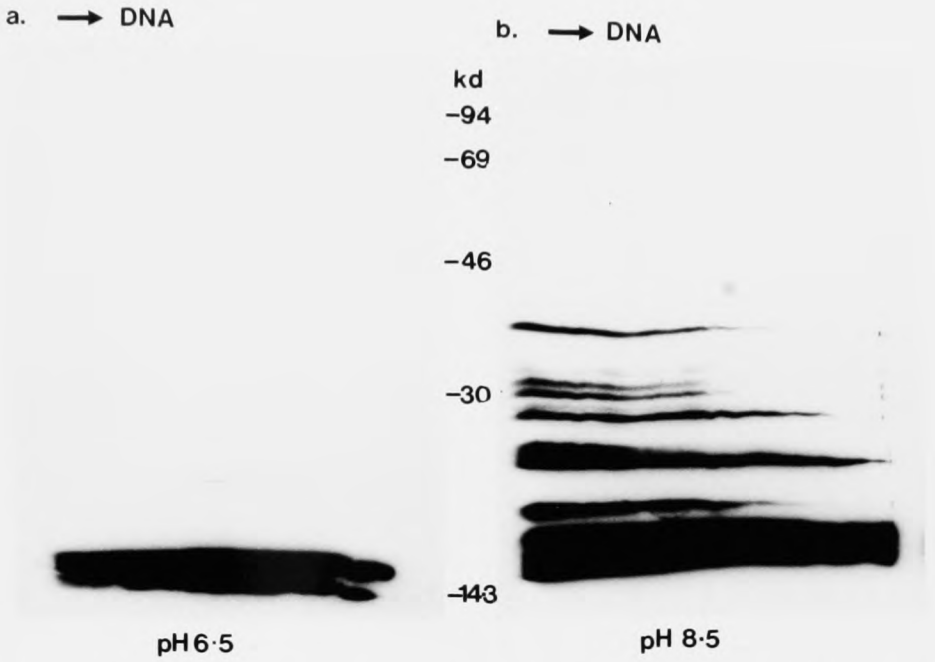


Fig. 41 Effect of changing pH on DNA-binding Total cell extract (1mg/gel) from the Lex A over-producer was crossed with 16µg of Eco R1-cut E.coli DNA in Hepes, pH 6.5 (a), or Tris, pH 8.5 (b). Total crossing time was 7h and autoradiography for 24h. More binding is seen at pH 8.5.

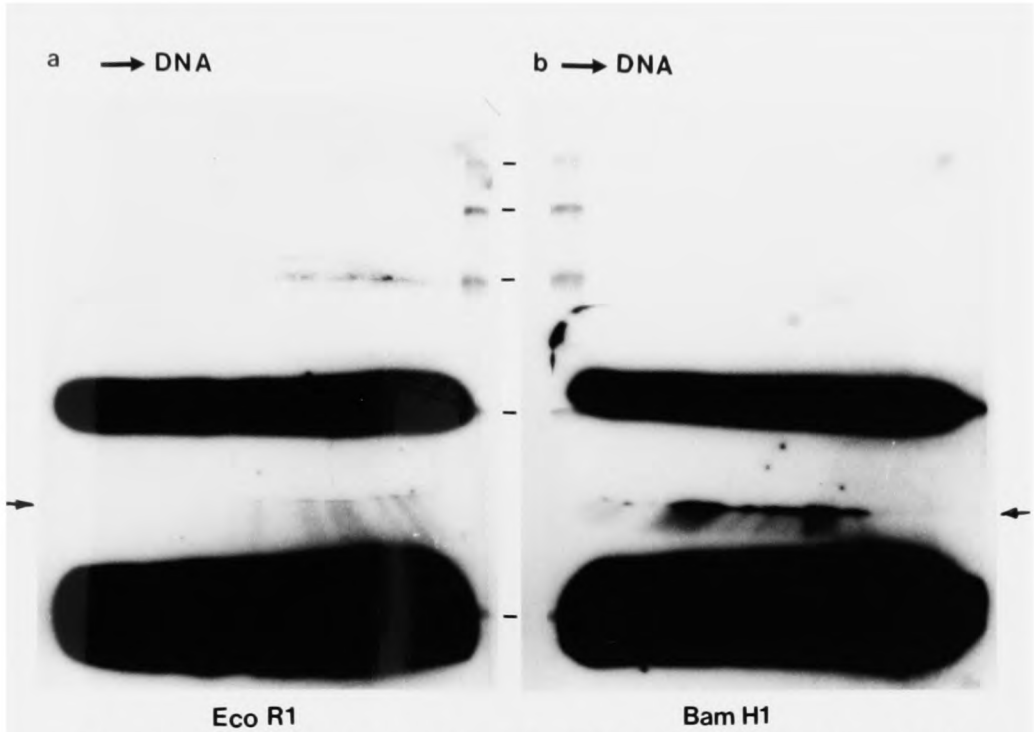


Fig. 42 Crossing Lex A protein with E.coli DNA Total cell protein from the Lex A over-producer (1mg/gel) was crossed with 16μg of (a) Eco R1-cut or (b) Bam H1-cut E.coli DNA in 10mM Tris, 1.5mM $CaCl_2$, 2.5mM $MgCl_2$, 200mM NaCl, 0.1mM EDTA, 5% DMSO, and 1x Denhardt's soln., pH 7.5 for 8h. Note the array of retained spots [arrowed] at 21.5kd. The bars indicate marker proteins of 94, 69, 46, 30 and 14.3 kd.

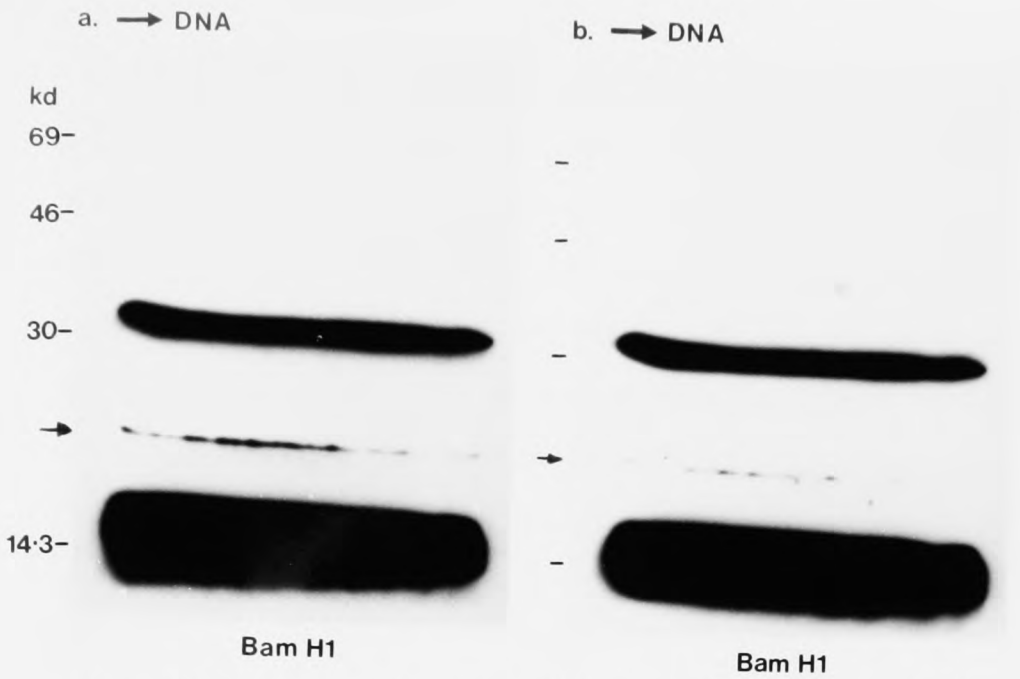


Fig. 43 Crossing Lex A protein with E.coli DNA Legend as for fig. 48, except 16µg of Bam H1-cut DNA was used on two different filters. Again, a similar array of spots is seen. Autoradiography time was 6h.

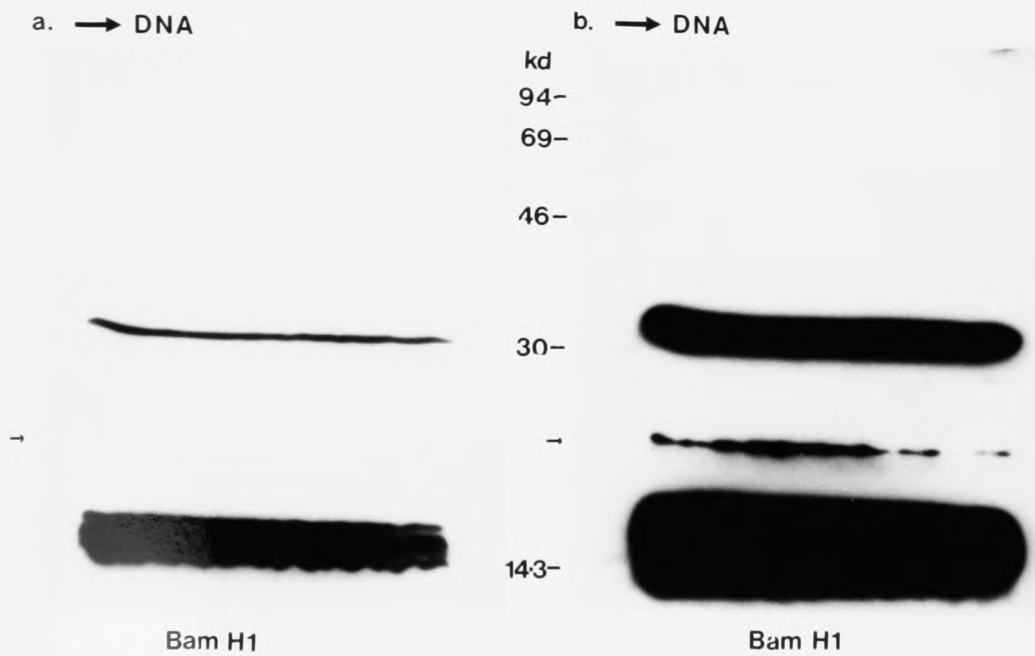


Fig. 44 Crossing Lex A protein with E.coli DNA The filter from fig 49a was exposed for either 1h (a), or 24h (b) to show the non-random retention of the spots by the Lex A protein. The DNA retained by the HU proteins does not reflect the pattern shown by the 21.5kd protein.

shorter autoradiograph [fig. 44a] shows that this pattern is not reflected by the HU proteins.

There is one objection to believing that this is true lex A binding to specific sequences. The size of the lex A protein in this study is 21.5kd, whereas others have reported sizes of around 24kd[207]. However, this matter has not been fully resolved, and this smaller lex A protein may possess functional characteristics of the true protein. This smaller protein has been observed in colonies, transformed for carriage of the lex A gene. It may be that this smaller protein is due an amber mutation, thus curtailing the transcript. Definitive proof that these spots are specifically retained by the lex A protein could be obtained by eluting the bound DNA, cloning it and re-crossing it to show specific retention.

T antigen studies

As a test for eukaryotic sequence-binding proteins, the T antigen of SV40 was chosen. A cell line, Cos 7, that constitutively produces T antigen, was obtained[195]. T antigen [T Ag] specifically binds to the origin site of SV40. Nuclear extract from Cos 7 cells [materials and methods] was crossed with a mixture of phage λ and a plasmid DNA, pSV01, containing the SV40 origin site [fig. 45]. Specific binding of the T antigen would be indicated by the retention of pSV01 DNA only. Because the T Ag is about 90kd, 7.5% acrylamide gels were used to enable efficient blotting. The abundance of T Ag in the nuclear extract was not known, though the Cos 7 cells allow the replication of plasmid molecules bearing the SV40 origin sequences[196], implying that there is functional T antigen present in these cells.

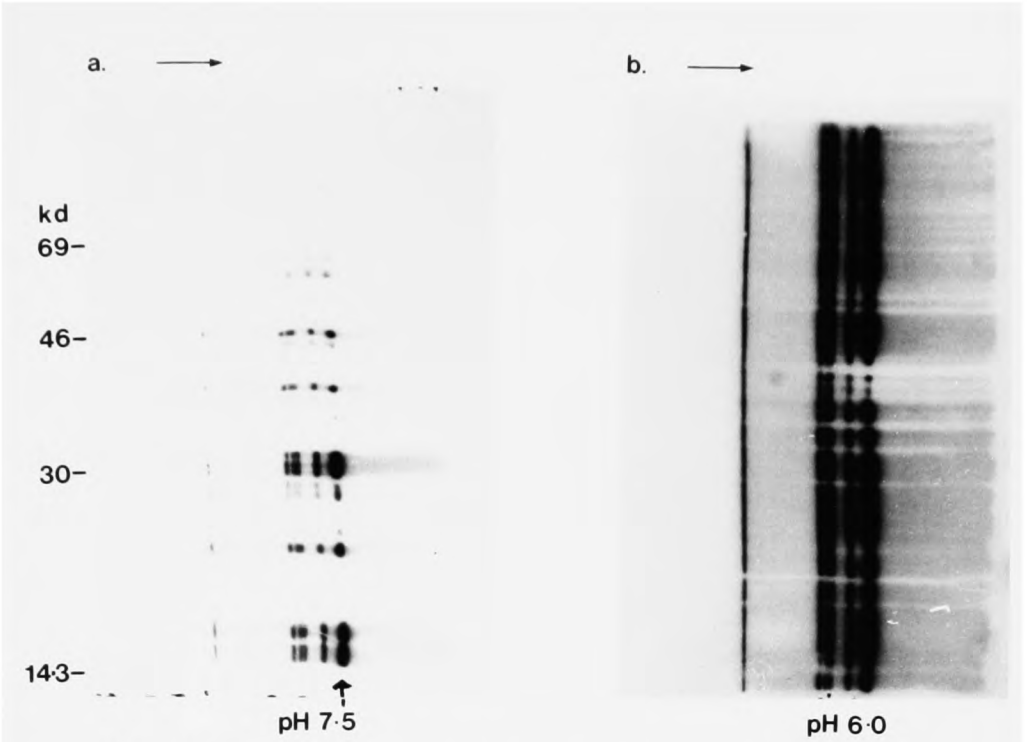


Fig. 45

Effect of pH on DNA-binding to nuclear extract from Cos-7 cells

Nuclear extract (1mg/gel) was crossed with 1.25μg of Bam H1-cut λ DNA and pSV01 DNA in 10mM Tris, pH 7.5 (a), or 20mM sodium phosphate buffer, pH 6 (b). The buffer, in addition, contained 50mM NaCl, 0.1mM EDTA, 5% DMSO, and 1x Denhardt's soln. Note the artefactual retention of the DNA at pH 6. Markers on the left indicate molecular weight standards. No specific retention of the SV40 origin sequence [arrowed] was noted.

Preliminary experiments failed to show any specific binding, even though various conditions were tried, such as changing pH, addition of ATP, $MgCl_2$, $CaCl_2$, or a combination of these [figs. 45, 46, 47]. One reason for the lack of identification may be that the T Ag is not in sufficient concentration in the Cos 7 nuclear extract. No enrichment steps were tried to investigate this. However, the above results show that ATP unmasks many DNA-binding proteins [fig. 46], as does 2mM $CaCl_2$, but not $MgCl_2$. One curious effect is shown in figure 45 where a phosphate buffer at pH 6.0 resulted in the retention of nearly all the DNA by the filter.

Results of binding DNA at higher salt concentrations are similar to those expected from previous findings [fig. 48]. Only the core histones bind DNA at 500mM, and no binding is obtained above this salt concentration.

Studies with yeast ARSs

Certain yeast DNA sequences promote independent replication and existence of circular plasmids in yeast cells. These sequences, called autonomous replicating sequences [ARSs], may contain yeast replication origins. Such sequences were therefore used to identify possible proteins in yeast cells that may specifically bind to these sites [Figs. 49 and 50]. The autonomous replicating sequences have been previously described[197]. Figure 49 shows the cross carried out using a mixture of λ DNA and pMA 9. The smaller pMA 9 fragment [cross, fig. 49] contains an ARS. No clear evidence of specific binding was found. However, since binding to such sites may be influenced by supercoiling, the cross was carried out using intact, unlabelled ARS DNA and the bound DNA detected by subsequent hybridisation with

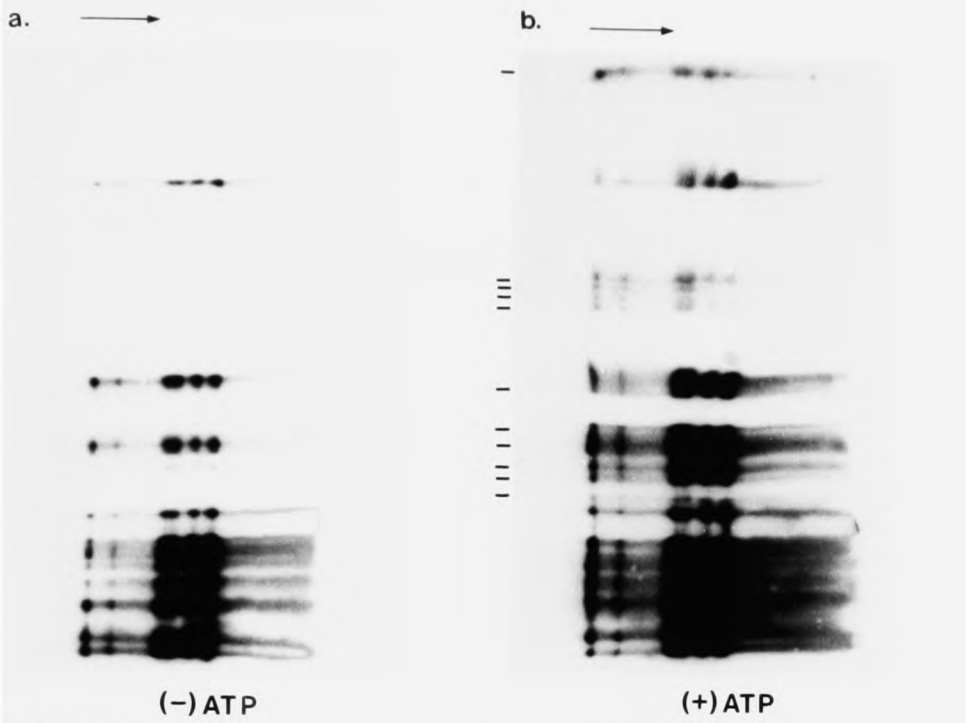


Fig. 46

Effect of ATP on DNA-binding to nuclear extract from Cos-7 cells

Nuclear extract from Cos-7 cells was crossed with λ and pSV01 DNA in 10mM Tris pH 7.5, 0.1mM EDTA, 50mM NaCl, 5% DMSO, 1x Denhardt's, 2mM $MgCl_2$, 2mM $CaCl_2$, and without (a), or with (b) 1mM ATP. Crossing time was 18h. The extra bands [bars] show DNA-binding enhanced by ATP.

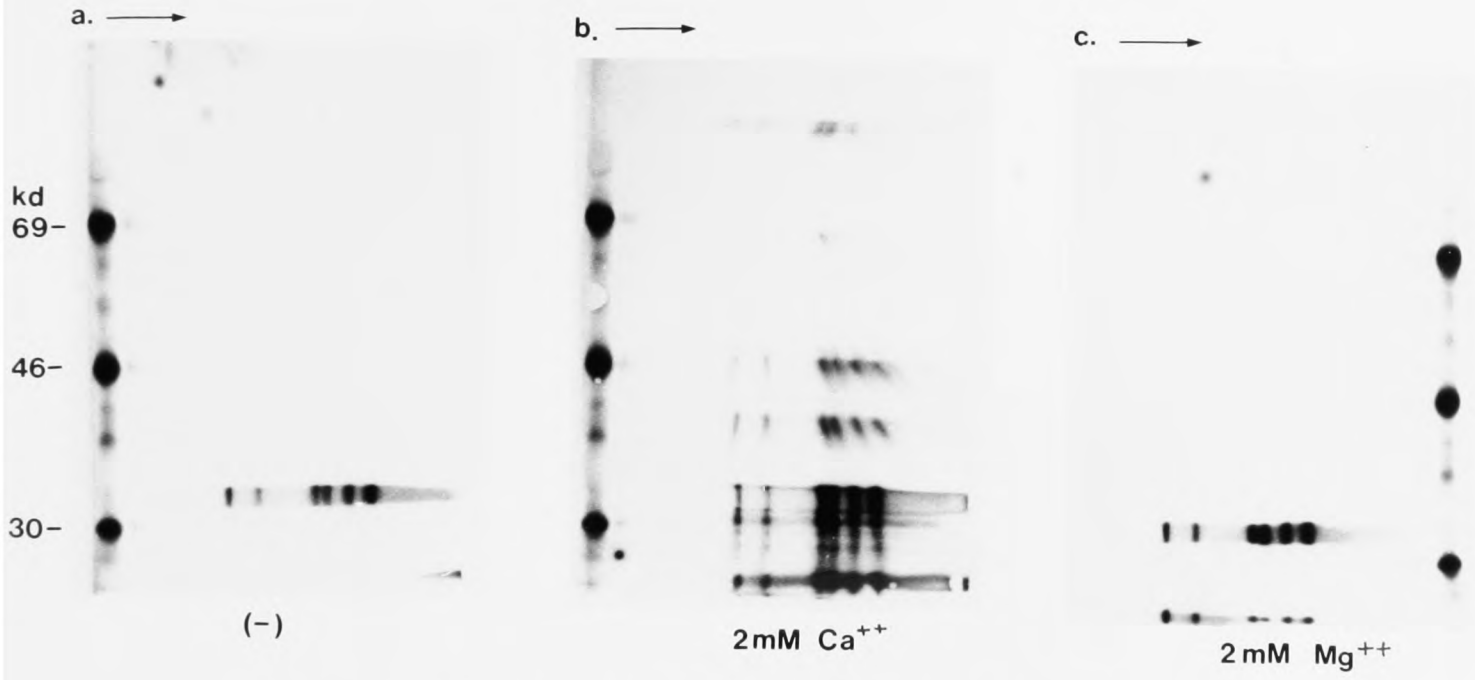


Fig. 47 Effect of Calcium, and Magnesium on DNA-binding Nuclear extract from Cos-7 cells was crossed as described for fig. 46. Additions were none (a), 2mM $CaCl_2$ (b), or 10mM $MgCl_2$ (c). Crossing time was 18h. The addition of calcium enhances DNA-binding.

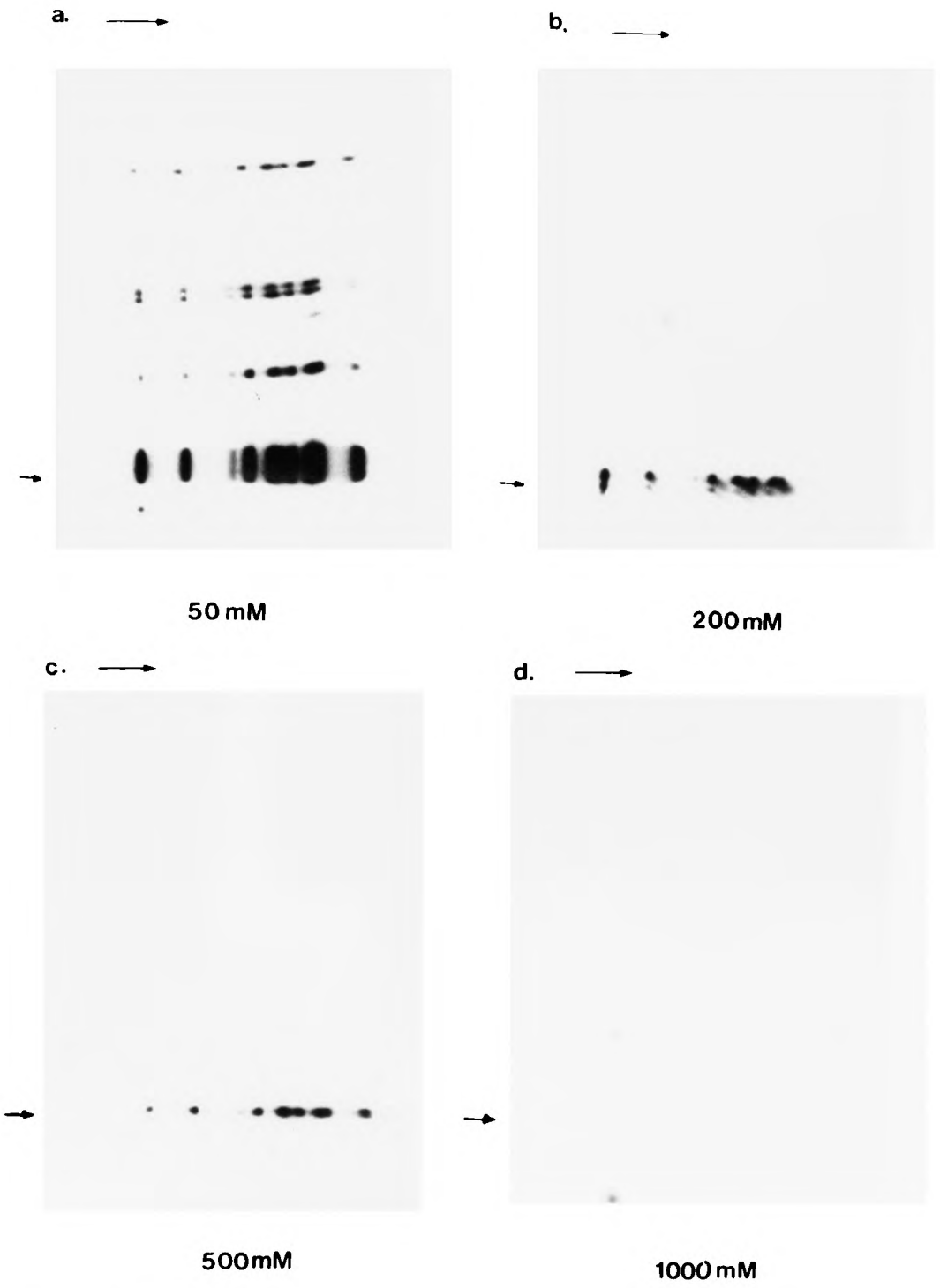


Fig. 48

Effect of salt concentration on DNA-binding by nuclear extract

Nuclear extract from Cos-7 cells (1mg/gel) was crossed with 1.25μg of λ DNA cut with Bam H1 and Eco R1. The binding buffer was 10mM Tris, 0.1mM EDTA, 5% DMSO, 1x Denhardt's soln., pH 7.5 and (a) 50mM NaCl, (b) 200mM NaCl, (c) 500mM NaCl, or (d) 1M NaCl. The arrow indicates core histones.

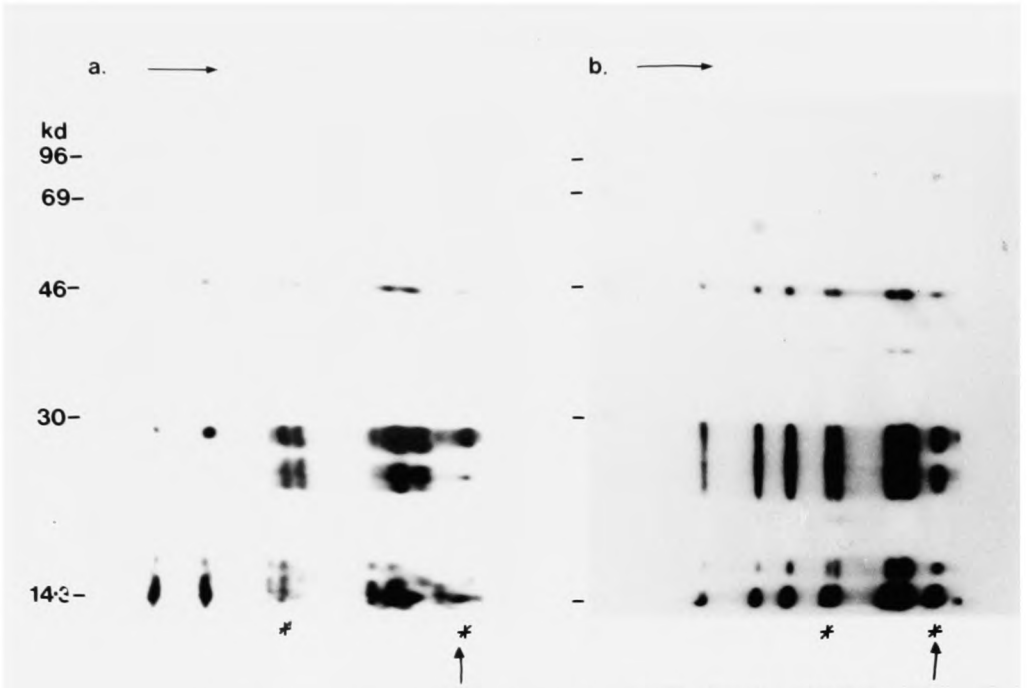


Fig. 49 Binding of yeast ARSs to yeast extract Yeast extract was crossed with Hind III-cut λ DNA, and Bam H1- and Eco R1-cut pMA 9 DNA [asterisked]. Binding buffer was 10mM Tris, 200mM NaCl, 2mM $CaCl_2$, 0.1mM EDTA, 5% DMSO, 1x Denhardt's soln., pH 7.5 and crossing time was 4h (a), 7h (b). No specific binding to pMA 9, carrying the ARS [arrowed], was detected.

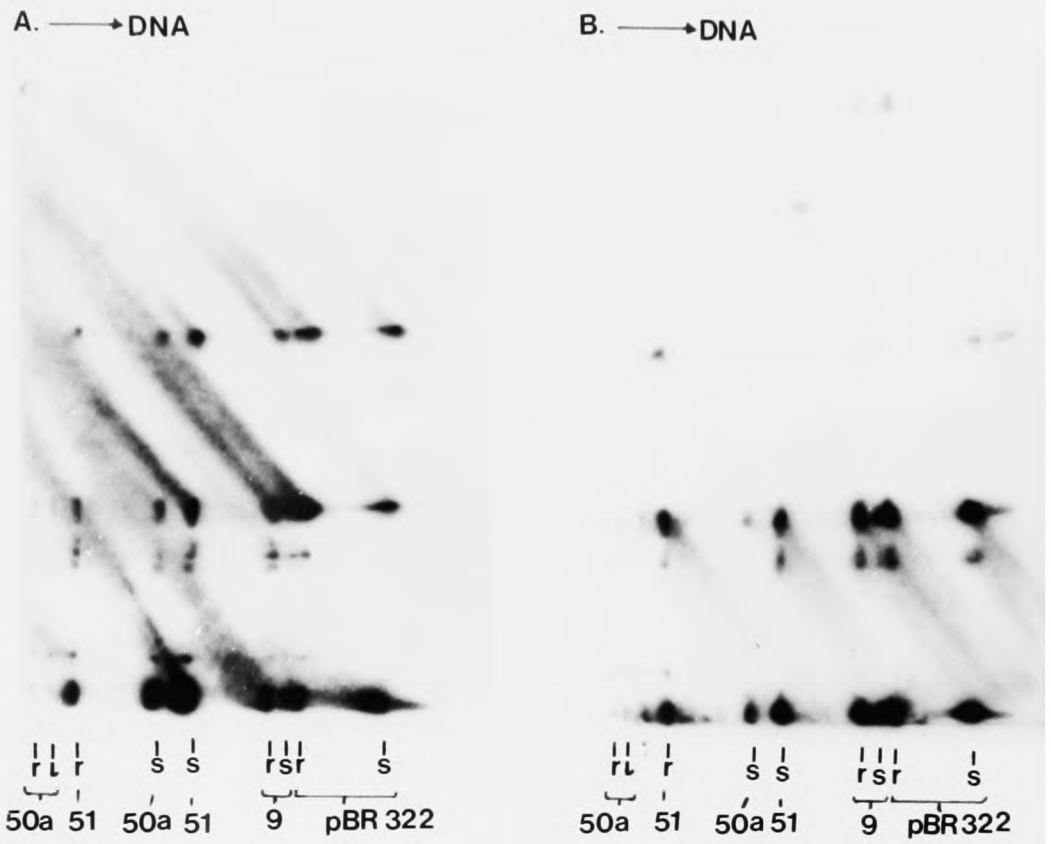


Fig. 50 Effect of crossing intact ARS DNA with yeast cell extract
Yeast cell extract was crossed as described for fig. 49, except that the DNA was intact. After crossing, the filter was treated with 0.5M NaOH for 20s, neutralised in 1.5M NaCl, 0.5M Tris for 20s, blotted dry, baked overnight, and hybridised with labelled pBR 322 DNA as described in materials and methods. This enables the retained DNA to be visualised. Fig. 50a and b are duplicates. The positions of the plasmids are as indicated ; s=supercoiled, r=relaxed, l=linear forms of DNA. Plasmids pMA 9, 50a, and 51 contain yeast ARSs. No specificity for intact DNA was noted.

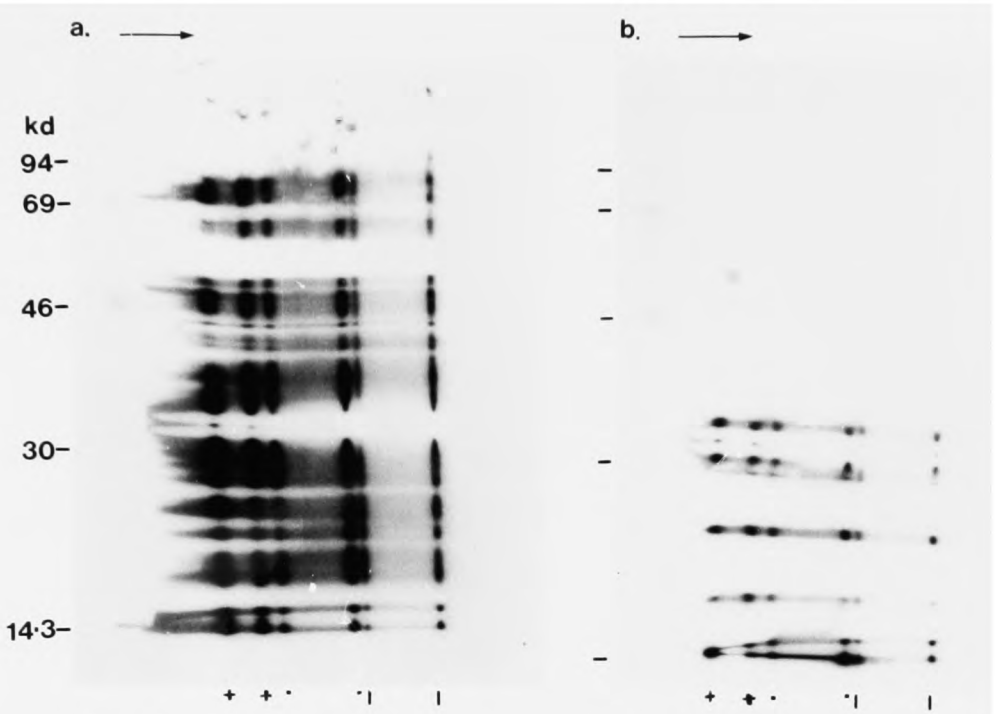


Fig. 51 Effect of crossing yeast ARS with HeLa nuclear extract HeLa nuclear extract (1.8mg/gel) was crossed with various ARSs; pMA9 cut with Bam H1 and Eco R1 (bars), pMA52 (dots) and pMA54 (+) cut with Eco R1. The cross was carried out as described for fig. 49, except 50mM NaCl (a), or 200mM NaCl (b) was used. No specificity was demonstrated.

a DNA probe [see materials and methods]. No consistent evidence of specific binding was detected [fig. 50]. Some of these ARSs were obtained from HeLa DNA, hence the crosses were repeated using HeLa nuclear extract [fig. 51]. Again no specific retention of ARS was noted.

Specific binding to 5S gene

Eukaryotic cells contain a protein that specifically recognises sequences in the 5S ribosomal gene[198]. HeLa nuclear extract was therefore crossed with a mixture of Lamda DNA and a plasmid containing Xenopus laevis 5S gene, pXLo 31 [fig. 52]. No specific binding was noted, even after using 200mM NaCl. The 5S binding protein was partially purified and crossed with the supercoiled form of the gene. As a control, a supercoiled plasmid containing 18 and 28S Xenopus ribosomal genes was used [fig. 53]. At 70mM $MgCl_2$, no specific binding was noted. However, at 140mM the supercoiled 5S plasmid was retained by a 54 kd protein [the asterisked band of DNA was due to a known contaminant in the DNA preparation]. This experiment, unlike the others, was not repeated.

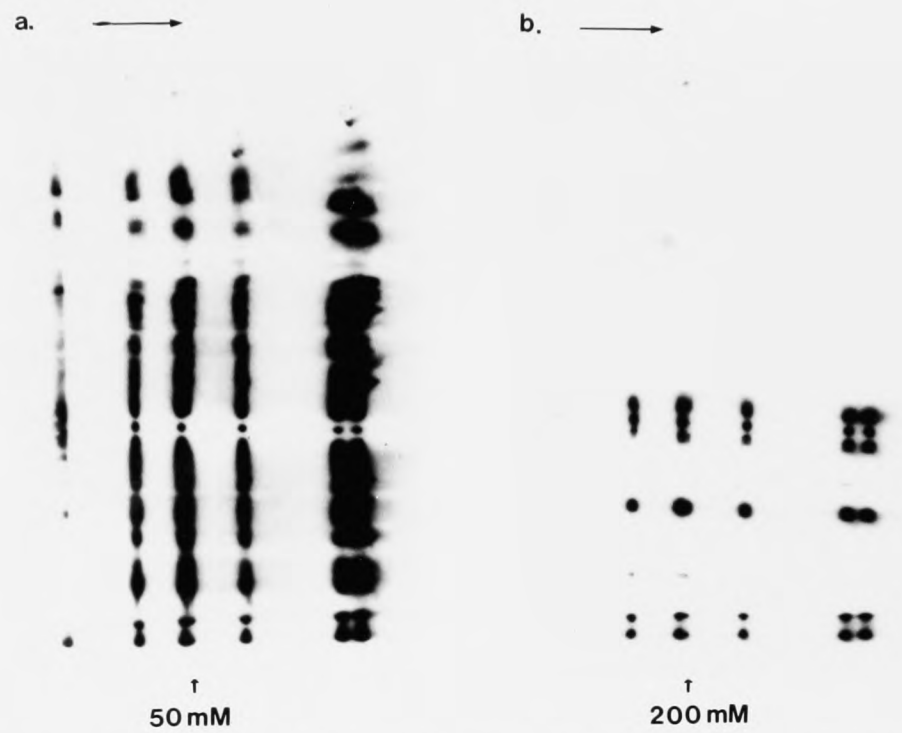


Fig. 52 Specific binding to 5S gene by HeLa nuclear extract HeLa nuclear extract (1.8mg/gel) was crossed with 2.3μg of λ DNA cut with Hind III and plasmid pXLo31 DNA, containing a 5S gene, linearised with Eco R1 [arrowed]. The binding buffer was 10mM Tris, 2mM $CaCl_2$, 0.1mM EDTA, 5% DMSO, 1x Denhardt's soln., pH 7.5 and NaCl at 50mM [a], or 200mM [b]. Crossing time was 6h. No specific retention of the 5S gene was noted.

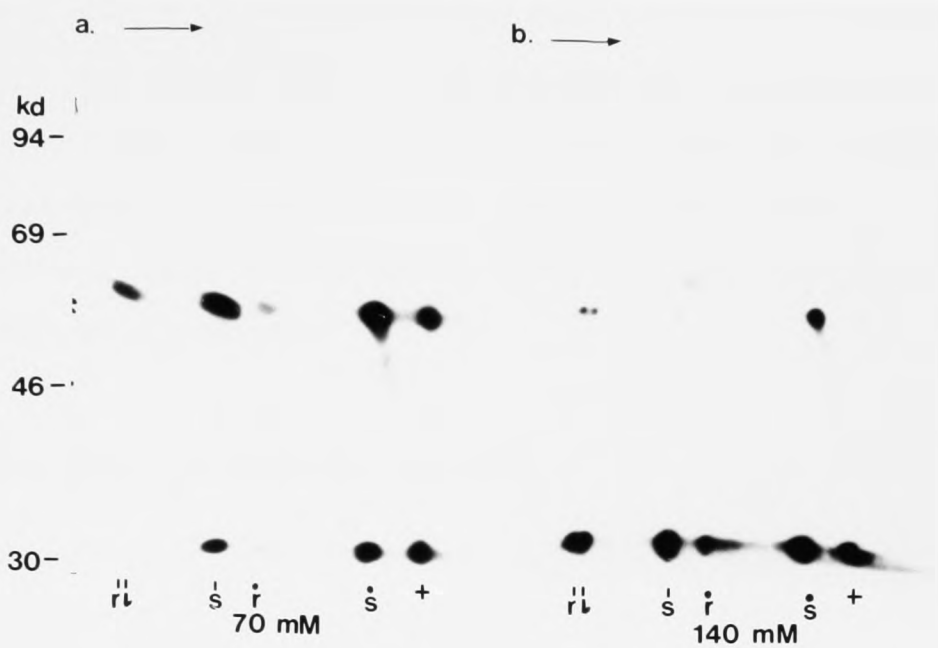


Fig. 53 Binding of supercoiled 5S genes to a HeLa cell sub-fraction
HeLa S100 ammonium sulphate fraction [see Materials and Methods] was crossed with X. laevis rDNA and 5S DNA in plasmids pXLr101 and pXLo31 respectively [s=supercoiled, r=relaxed, and l=linear]. The binding buffer was 20mM Tris, 5mM $MgCl_2$, 0.1mM EDTA, 1x Denhardt's soln., pH 7.5 and NaCl at 70mM (a), or 140mM (b). Crossing time was 8h. The filters were treated as described for fig. 56 to detect the bound DNA. The bars indicate pXLr101, and the dots pXLo31. Supercoiled pXLo31 is specifically retained by the 50 kd protein. The 'hot' spot (+) is due to a known plasmid contaminant.

Binding to u.v.-damaged DNA

HeLa cells contain a protein that shows considerable specifity towards u.v.-damaged DNA[199]. Intact circular DNA was irradiated with u.v. light [the intactness was checked before and after the procedure by gel electrophoresis and found to be similar], and crossed with HeLa nuclear extract [fig. 54]. Unfortunately, no specifity was demonstrable.

Nucleoid protein crossed with total DNA

HeLa nucleoid proteins were also crossed with Eco R1 split HeLa DNA in the hope that proteins that secure the DNA into loops and the sequences involved may be identified. A gel of nucleoid protein stained with Coomassie is shown in figure 55 and the results of the cross in figure 56. DNA-binding proteins described previously were detected [earlier results], but no specificity was evident. Although this cross shows proteins in the range 90-30kd; gels where the lower molecular weight species were adequately resolved were not significantly different.

Whilst the technique of 'Southern crossing' is useful in detecting sequence-binding proteins that are relatively abundant, the sensitivity of the technique does not permit detection of such proteins at their natural abundance. However, since a majority of such proteins can be made to be over-produced, using genetic engineering, this technique will allow detection of recognised sequences.

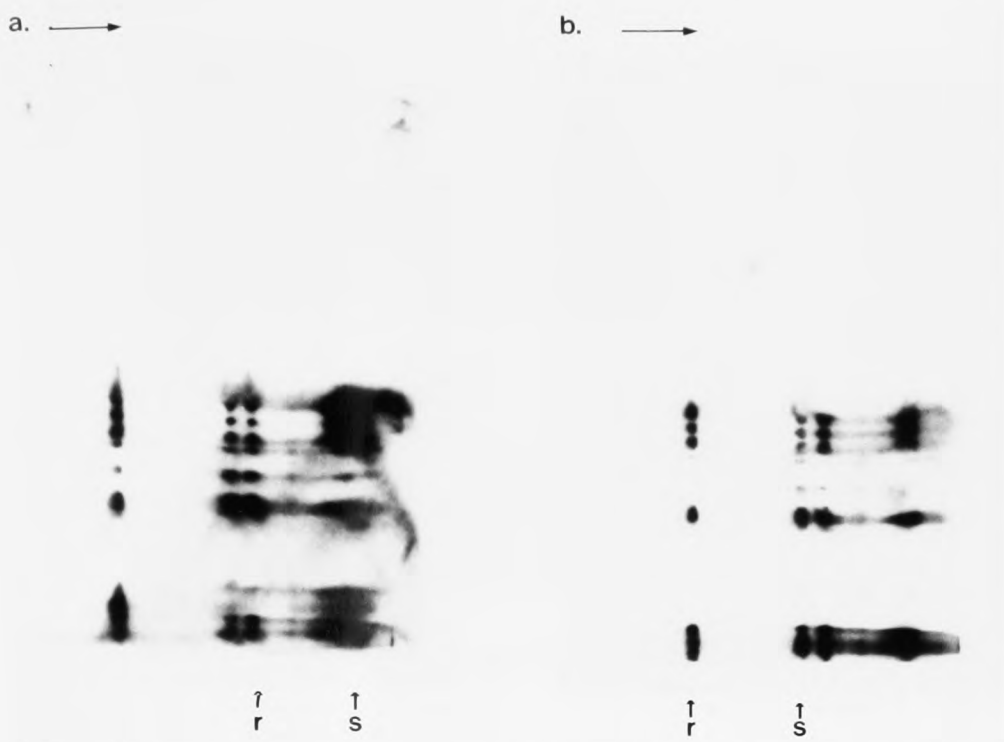


Fig. 54 Effect of crossing u.v.-damaged DNA with HeLa nuclear extract
HeLa nuclear extract (1.8mg/gel) was crossed with u.v.-damaged DNA [see Materials and Methods]. The crossing buffer was 10mM Tris, 200mM NaCl, 2mM $CaCl_2$, 0.1mM EDTA and 1x Denhardt's soln., pH 7.5. The intact plasmids were pBR 322 and pMCS, and the filters were hybridised with labelled pBR 322 [pMCS contains pBR 322 sequences]. The arrows indicate the u.v.-damaged species. The plasmid irradiated was reversed to negate any artefacts due to blotting [cf. a and b].

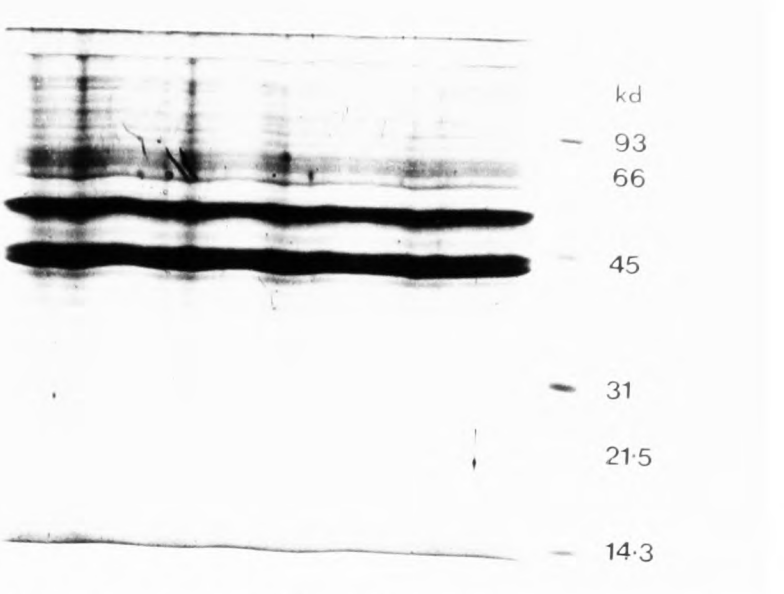


Fig. 55 Commassie stain of HeLa nucleoid proteins 1.8mg of HeLa nucleoid proteins were separated by SDS-PAGE and stained with Commassie [see Materials and Methods]. Molecular weight markers are indicated on the right hand side.

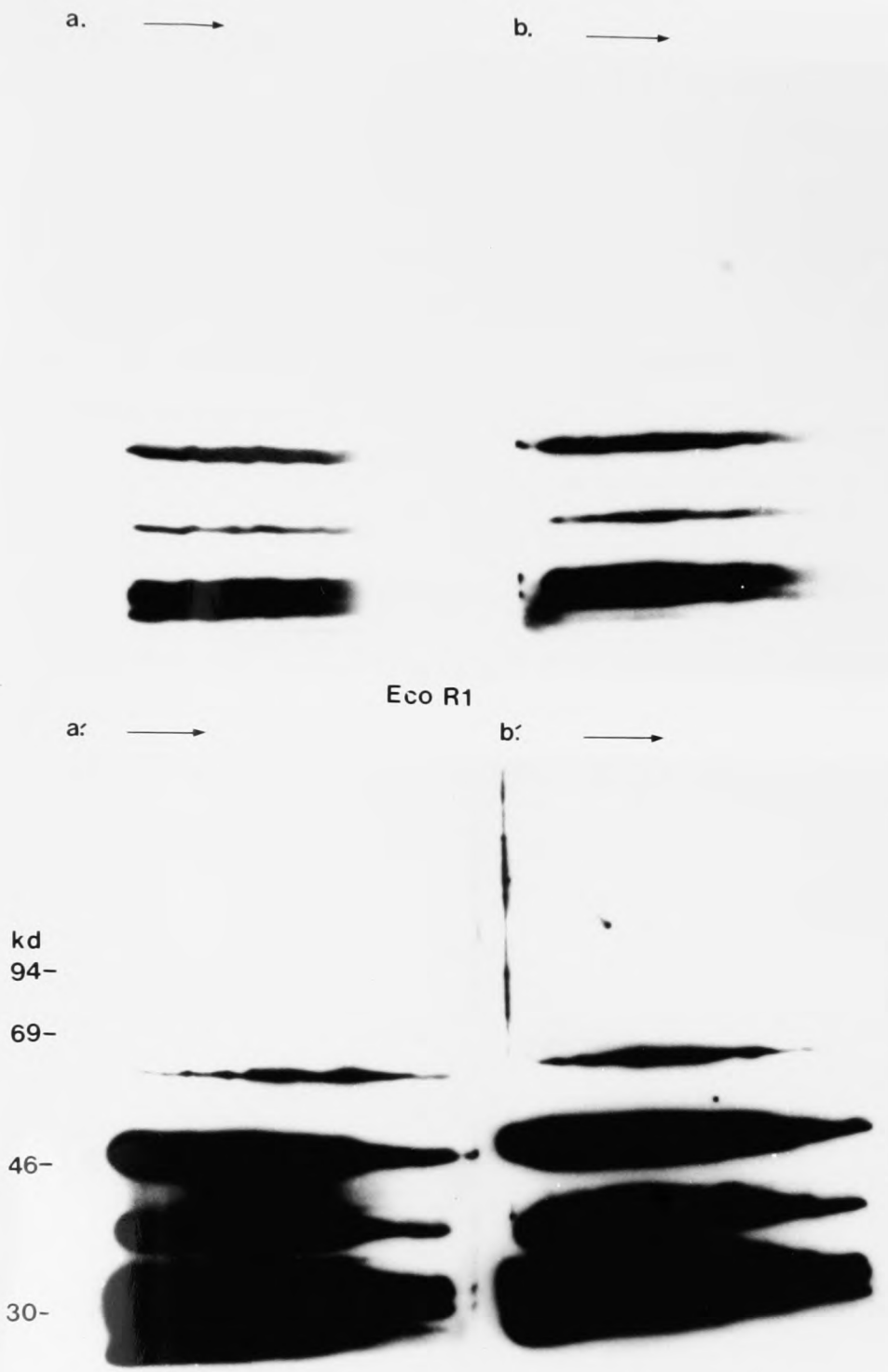


Fig. 56 Effect of crossing total HeLa DNA with HeLa nucleoid proteins HeLa nucleoid proteins (1.4mg/gel) were crossed with 12.5µg of total HeLa cell DNA cut with Hind III (a) or Eco R1 (b) for 18h in 10mM Tris, 0.1mM EDTA, 200mM NaCl, 2mM $CaCl_2$, 5% DMSO, and 1x Denhardt's soln., pH 7.5. Fig. 56a' and b' are longer autoradiographs. There is no evidence of specific DNA retention.

DETACHMENT MAPPING

Technique of mapping

The strategy used to insvestigate the nature of DNA attachment is shown in figure 57. The residual DNA is isolated, after *in situ* digestion with a restriction endonuclease, digested to completion with the same enzyme, and 'Southern blotted'. The filter is probed with radio-labelled DNA, and the abundance of a given sequence determined, relative to total DNA [see materials and methods]. If a sequence is specifically positioned close to the attachment point [fig. 57A], its abundance will be increased in the residual DNA, or remain unaltered if it is randomly positioned [fig. 57B]. The further the sequence is positioned away from the attachment site, the greater the chances of having restriction sites between it and the site, the greater its chances of being detached. Hence, the detachment mapping technique is a measure of the number of restriction sites between the attachment site and the sequence. Any consistent changes in abundance of DNA sequences therefore supports the theory that the DNA is non-randomly organized in the nucleus. This technique assumes that the palindromic restriction sites are uniformly distributed throughout the genome. Although this is not true, for practical purposes, they behave as if this were the case. The non-random distribution of such sites is also, in part, countered by using different enzymes.

Kinetics of digestion

Nucleoids, form HeLa cells labelled with ^{3}H-thymidine, were digested with a range of Eco R1 concentrations, and the level of digestion estimated by using a salt-wash assay, or a pellet assay.

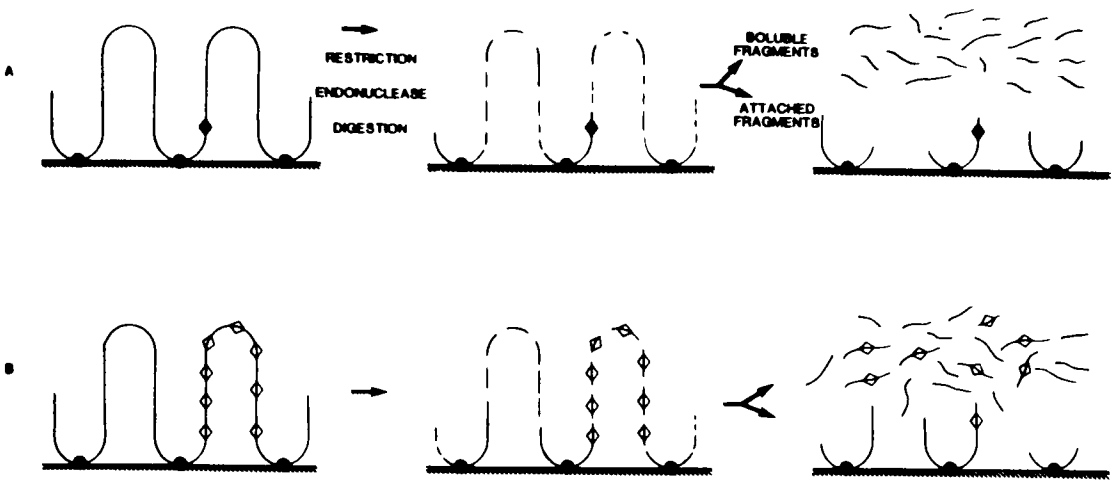


Fig. 57 The technique of detachment mapping The looped DNA in nucleoids is digested with a restriction endonuclease, the DNA remaining attached to the cages purified and the abundance of a particular gene determined by hybridisation. If this sequence is non-randomly positioned, its abundance will be a function of the number of cut-sites between the sequence and the attachment point. The closer the sequence is to the attachment site, the less the chances of having cut-sites in between, and therefore the greater its abundance in the residual DNA (A). If the sequence is randomly positioned, no difference in its abundance will be evident (B).

The salt-wash assay was performed by spotting a sample of the reaction mix onto a glass fibre disc, and washing this, under suction, with 2M NaCl, 10mM Tris, 0.1mM EDTA, pH 7.5, drying the filters and counting them for amount of DNA retained. DNA remaining attached to the cages is retained, and detached DNA washed away. The pellet assay was performed by spinning the reaction mix at 100,000g for 15 min, and counting the amount of DNA in the supernatant before and after spinning. The attached DNA pellets, and the detached DNA remains in solution. The results are shown in figures 58 and 59. HeLa cells, synchronised in various cell cycle phases were also used. The salt-wash assay consistently gave higher levels of DNA remaining attached to the cages [fig. 58], than the pellet assay [fig. 59] for a given enzyme level. No differences in the kinetics were observed for the different cell cycle stages. The DNA detachment is not linear, and the majority of the DNA is detached at low enzyme concentrations, and any further digestion needs much larger levels of enzyme. The shape of the digestion curves supports the idea that the digestion is directly proportional to the number of scission sites in a loop of DNA, and that two hits in a loop are needed to detach DNA. For loops that are large, few hits are required to detach the majority of the DNA, but once a segment of DNA has been detached, the attached DNA is now depleted in scission sites and hence requires more enzyme to detach further DNA.

Mapping ribosomal genes

Nucleoids from randomly growing HeLa cells were 'detachment mapped' [as described above, and see materials and methods], using Eco R1 and hybridised with *X. laevis* ribosomal genes [fig. 60]. The frog genes hybridise to the human sequences coding for 18s and

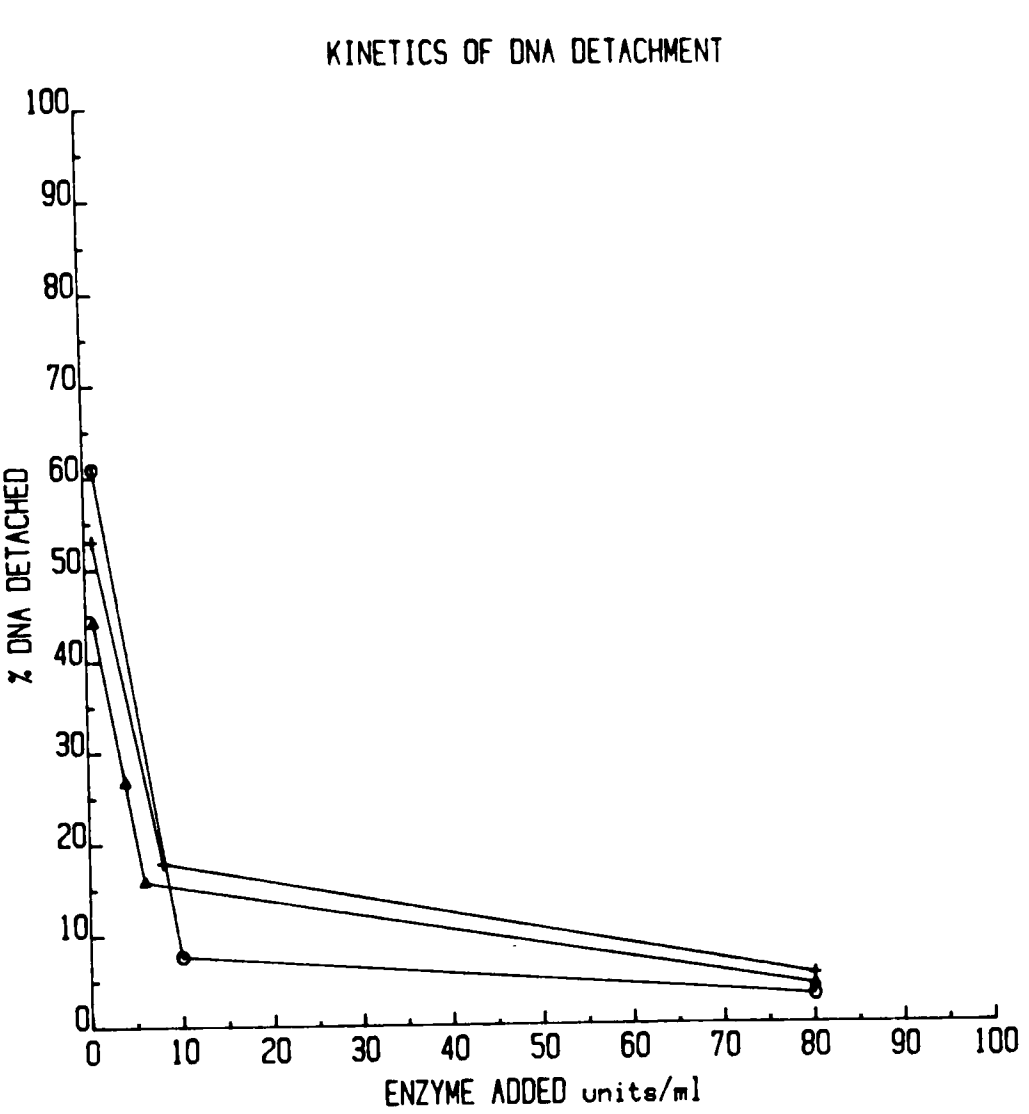


Fig. 58 Kinetics of DNA detachment HeLa nucleoids were digested to various levels with Eco R1, as described in Materials and Methods, and the amount of digestion obtained determined by the salt wash assay. After digestion, a sample was spotted onto glass fibre discs and washed by suction with 2M NaCl, 10mM Tris, 0.1mM EDTA, pH 7.5. The detached DNA is washed away, but the attached DNA is retained, by virtue of its bulk. Symbols; HeLa cells synchronised in G1/S (open cirles), M phase (open triangles), or S phase (cross). No significant cell cycle specific differences were noted.

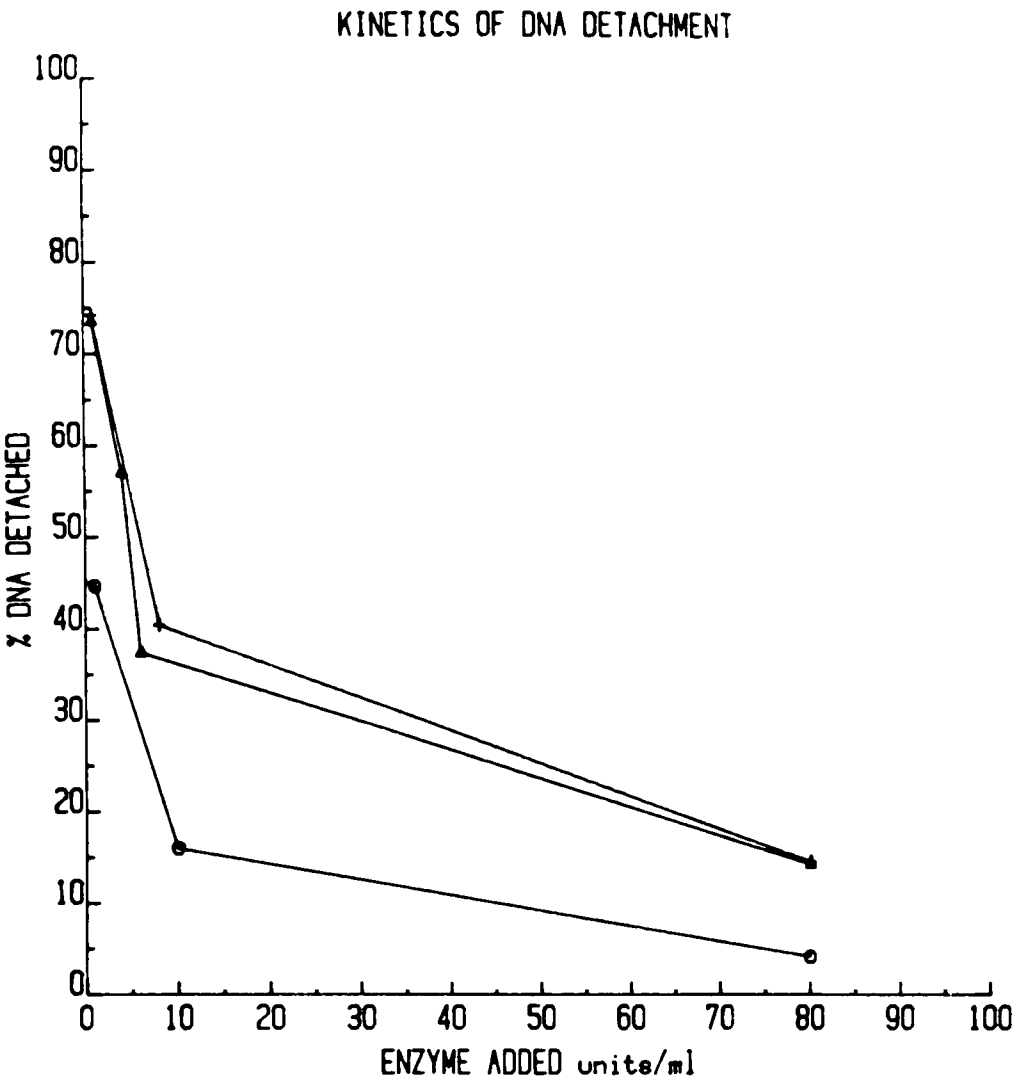


Fig. 59 Kinetics of DNA detachment The level of digestion was also determined by the pellet assay. The reaction mix was centrifuged to collect the digested cages, and associated DNA. The level of digestion was measured by sampling the supernatant before and after spinning. Compared with the salt wash assay [fig. 58], this gave lower digestion levels, and was therefore used as the estimation [resulting in an underestimation].



Fig. 60 Detachment mapping of 18S and 28S ribosomal genes HeLa nucleoids were detachment mapped, using Eco R1, and hybridised with X. laevis ribosomal genes [see Materials and Methods]. The DNA samples are as indicated. Track 9 contains end-labelled markers as indicated. Ribosomal sequences are enriched in the cage-associated DNA [cf. tracks 3 and 6]. Hybridisation to the ribosomal genes is also present in the DNA released from the cage [CR]; tracks 7 and 8. been detached away by Eco R1 [Symbols; + 4.1kb, o 3.2kb, 2.4kb].

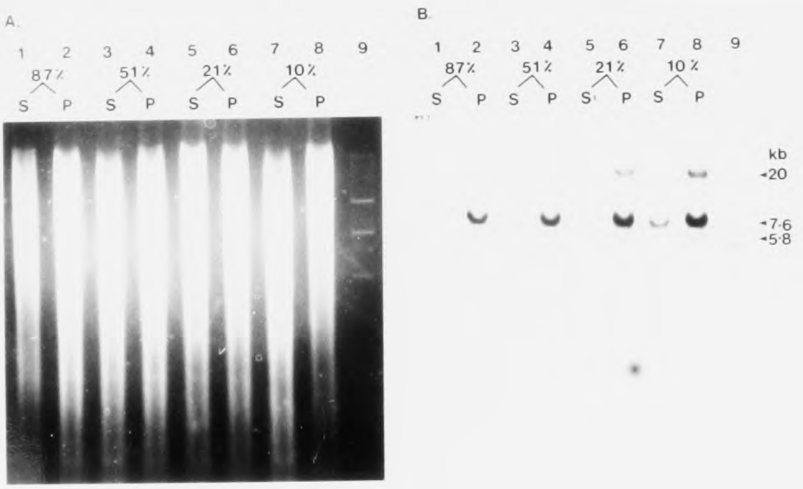


Fig. 61 Progressive detachment mapping of ribosomal genes HeLa nucleoids were digested with Eco R1 to various levels and both the detached (S), and the cage-associated DNA (P) were purified and hybrised to ribosomal genes, as described in Materials and Methods. Fig. 61A is the ethidium stain and B the autoradiograph, after blotting and hybridisation. Progressive enrichment of the ribosomal fragmnets [20, 7.6, and 5.8 kb] is seen in the cage-associated fractions with increasing levels of digestions [cf. tracks 2, 4, 6, 8]. Track 9 contains markers.

28S RNA. DNA associated with the nucleoids, after digestion, is relatively enriched in ribosomal sequences. With increasing levels of DNA detachment, progressive enrichment is seen in the residual fraction [p, fig. 61] and depletion in the solubilised fraction [s, fig. 61]. Joyce-Loebl densitometry indicates that the ribosomal fragments are not enriched to the same extent. This difference is more obvious if the DNA is digested by both Bam H1 and Eco R1, instead of only Eco R1, after the initial detachment with Eco R1 and hybridised for ribosomal genes [fig. 62]. The 2.3 kb fragment is relatively depleted, and the 1.4 kb fragment enriched [fig. 63]. This may reflect the proximity of the attachment site to one of the ends of the locus. Although an accurate map of the Bam H1 sites of the human gene were not available, comparision with a partial map of the 18S gene suggests that this site is closer to the 3' end of the 18S gene.

The results described here could be due to artefacts, such as selective protection against enzyme digestion. The DNA obtained after the initial detachment is only partially digested. To demonstrate that all Eco R1 sites 'in situ' are vulnerable to scission, the initial reaction was carried out for 48h and the cages isolated, DNA purified and 'Southern blotted' without a second enzyme digest. The filter was probed with ribosomal genes, and autoradiographed [fig. 64]. The results show that no partially digested bands were detected, i.e. given a long enough incubation, all the available Eco R1 sites in the ribosomal loci can be cut. Furthermore, if, after a brief initial digestion with Eco R1, the nucleoids are isolated and digested with Hae III enzyme, the ribosomal genes are now depleted in the residual DNA [fig. 65]. This also places any possible attachment sites outside the coding ribosomal genes, though not outside the transcriptional

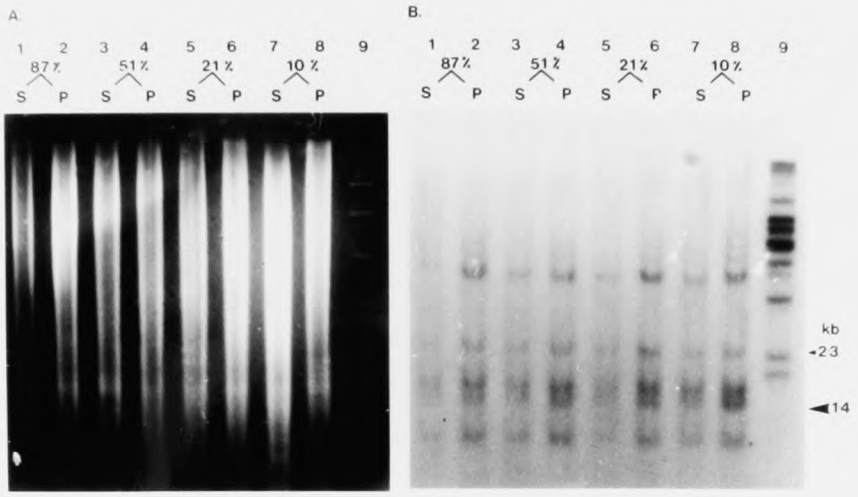


Fig. 62 Fine mapping of ribosomal genes The DNA, used in fig. 61, was cut with Bam H1 and Eco R1, instead of only Eco R1 as is normal, prior to gel separation and blotting. The autoradiograph, after hybridisation to ribosomal genes, was scanned with a Joyce-Loebl densitometer. Seven bands are evident [6.6, 5.5, 4.9, 2.3, 1.65, 1.4 and 0.9 kb]. The 2.3 kb fragment [small arrow] is relatively de-enriched, and a 1.4 kb fragment [large arrow] is relatively enriched, compared with the rest of the fragments [see fig. 63].

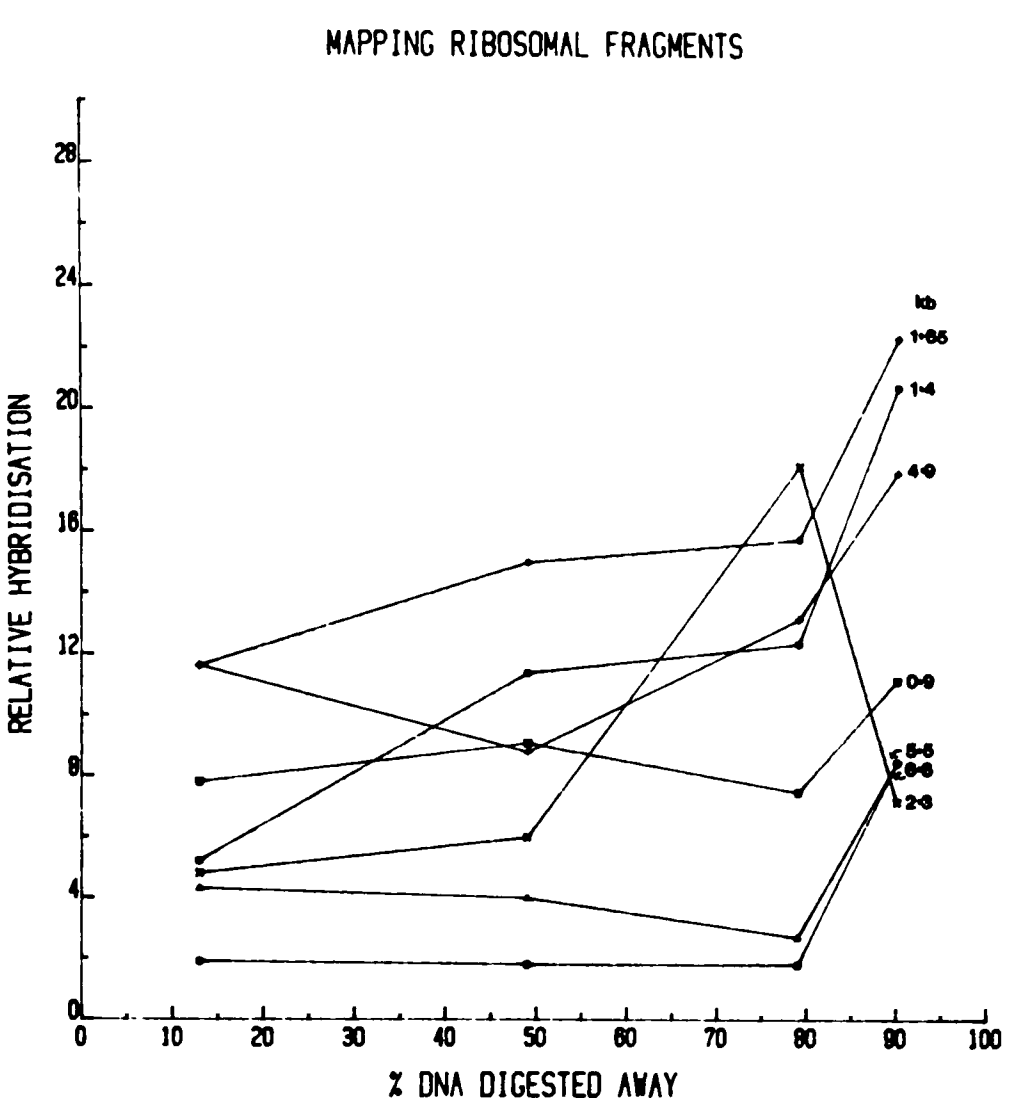


Fig. 63 Graph of the enrichments of the ribosomal fragments The densities obtained in fig. 62 were plotted against the level of digestion. The 2.3 kb fragment is relatively de-enriched, and the 1.4 kb fragment enriched [cf. the densities at 10% and 85% for each fragment].

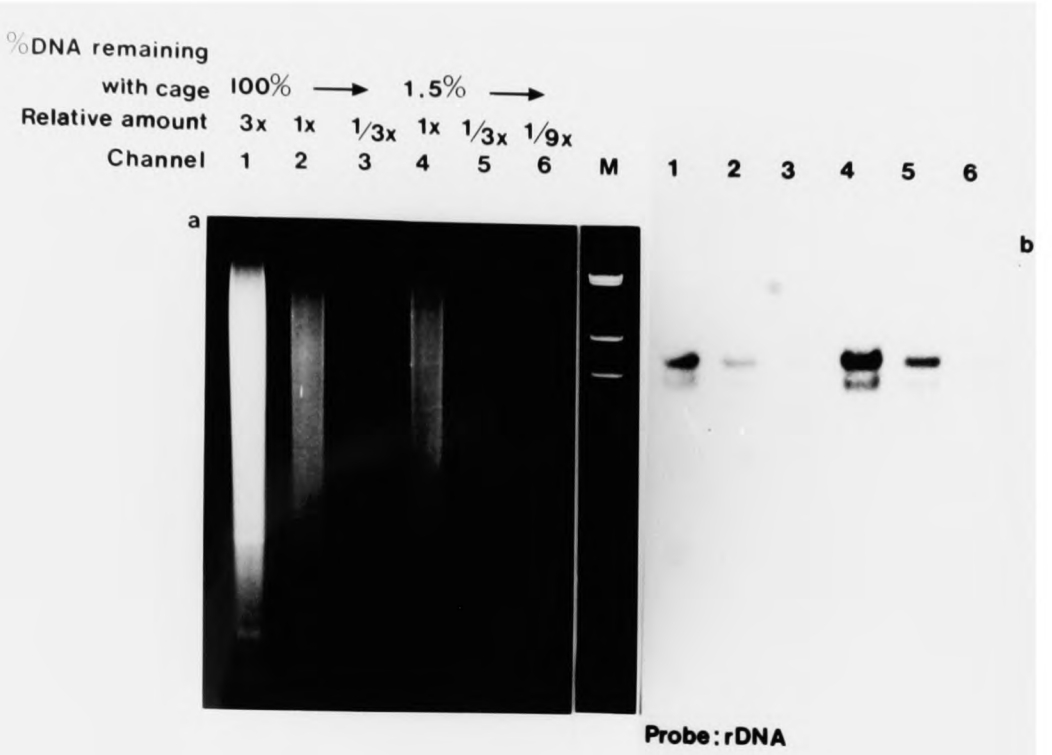


Fig. 64 Long incubation time and Detachment mapping HeLa nucleoids were incubated with Eco R1 [8u/ml] at 37 deg. C for 48h, the cage associated DNA purified, separated by gel electrophoresis, without the second enzyme digestion, blotted and hybridised for ribosomal DNA. About 1-2% DNA remains attached to the cages under these conditions. Total DNA, digested to completion is included for comparision. The amount of DNA loaded is as indicated [1 x is 1µg]. Fig. a is the ethidium stain, and b the autoradiograph [2h] after hybridisation to ribosomal sequences. No partially digested ribosomal bands were detected after longer exposures.

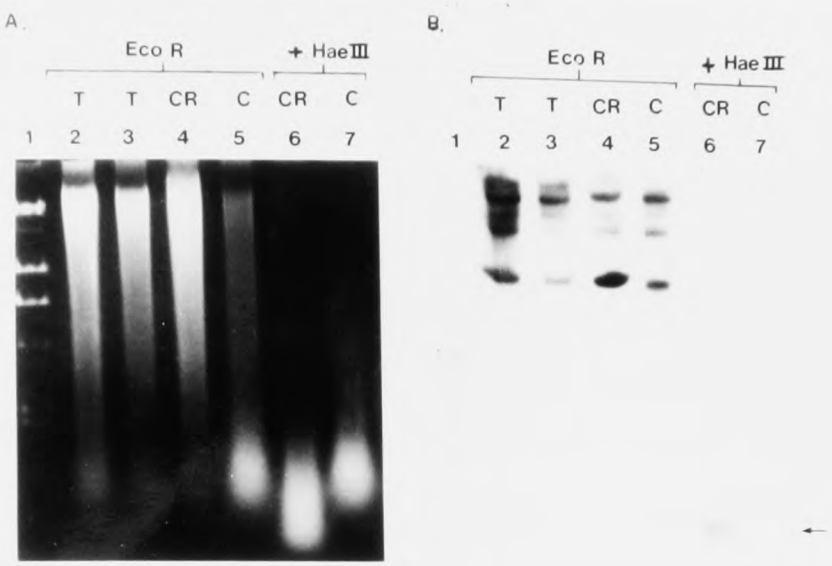


Fig. 65 Detachment mapping of ribosomal genes HeLa nucleoids were digested with Eco R1, the digested nucleoids purified and re-digested with Hae III, and a sample of solubilised and cage-associated DNA purified at each of these stages. The purified DNAs were loaded directly onto the gels, without the normal second enzyme digestion to completion. Fig. 65A is the ethidium stain and B the autoradiograph, after hybridisation to ribosomal genes. Track 1 is a marker track, track 2, 3 contain total [T] DNA at 2x and 1x loading respectively, track 4 is DNA solubilised by Eco R1 [CR, 1x], track 5 is DNA remaining associated with the cage after Eco R1 digestion [C, <1x]. Track 6 is DNA solubilised from the Eco R1-digested cages by Hae III [1x], and track 7 is DNA remaining attached to the Eco R1-digested cages after Hae III digestion. 1x loading is approximately 4μg of DNA. The ribosomal genes are enriched in the cage-associated DNA (C), after the Eco R1 digestion, but not after the subsequent Hae III digestion [cf. tracks 5 and 8]. The ribosomal genes appear to have been released, by Hae III, into the soluble fraction (CR). Note that the DNA is partially digested by these enzymes, as would be expected, under the conditions employed.

unit.

One criticsm levelled has been that of artefactual association via transcriptional units. Hence 'active' genes would be expected to be enriched in the residual DNA. Unfortunately, this cannot be differentiated from the model requiring such attachments as a pre-requisite for transcription. Whilst no control is adequate enough, the ribosomal genes remain enriched after the cells have been heat shocked [fig. 66], treated with actinomycin D, or during the cell cycle [data not shown]. Interestingly, γ-actin and histone H4A genes do not appear to be enriched in residual DNA. Both these genes are presumed to be active [see later].

Mapping proviral genes in nuclei

An interesting, though inconclusive, experiment was to detachment map the active proviral genes of Avian Sarcoma Virus [ASV] in nuclei. Active genes are known to be more susceptible to nucleases in nuclei[181]. The results show that after an initial enrichment, these genes are depleted [figs. 67 and 68]. When these genes are mapped in nucleoids, they are enriched. 'Active' conformation, and nuclease sensitivity, of these genes is mediated by the HMG 14 and 17 proteins, which are not present in nucleoids. The de-enrichment can be explained by the increased vulnerability of the Eco R1 sites in nuclei, in keeping with it's active conformation, or that the enrichment seen in nucleoids is via artefactual precipitation of the transcriptional complexes. It is not possible to distinguish between the two.

Mapping transcribed sequences

Solution hybridisation data has shown that the residual DNA is

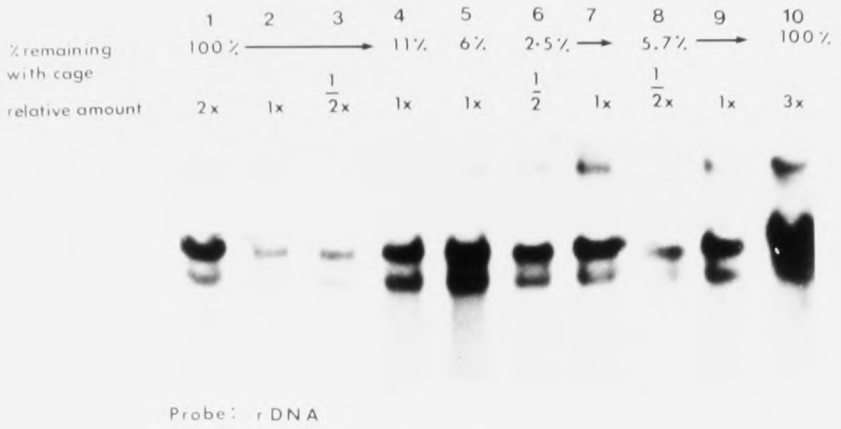


Fig. 66 Mapping ribosomal genes after heat shock treatment HeLa cells were heat shocked [see Materials and Methods], and then detachment mapped as described for fig. 60. The ribosomal genes remain enriched in the residual DNA. The greatest enrichment is when 94% of the DNA has been digested away [cf. tracks 4, 5 and 7].

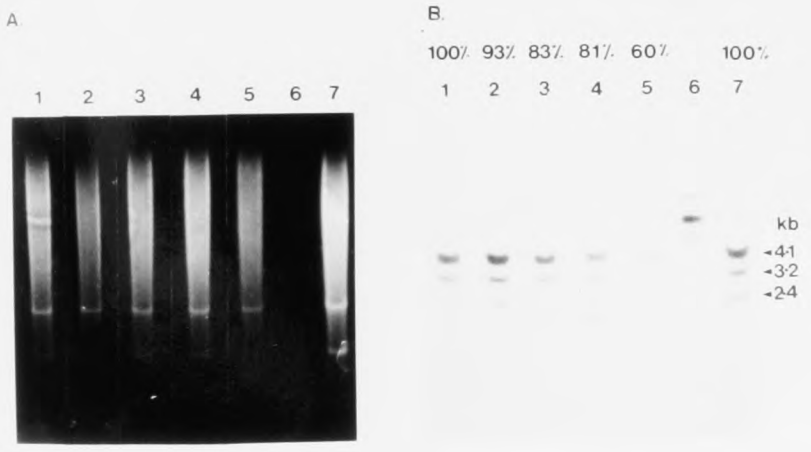


Fig. 67 Detachment mapping of ASV sequences in nuclei Nuclei, isolated from rat VIT cells, which have ASV provirus, were digested with Eco R1 to various levels, lysed in nucleoid lysis buffer [see Materials and Methods], and digested nuclear matrices isolated by centrifugation through 15% sucrose, 10mM Tris, 0.1mM EDTA, 2M NaCl, pH 7.5. The DNA was purified, digested to completion with Eco R1, gel separated, blotted, and hybridised with ASV genes. Track 6 contains end-labelled λ DNA, cut with Eco R1. The percentages above the tracks indicate the amount of DNA remaining associated with the matrices. Equal amounts of DNA were loaded onto the gel, except for track 7, where twice the amount was loaded. After an initial enrichment, a progressive de-enrichment is seen with increasing digestion.

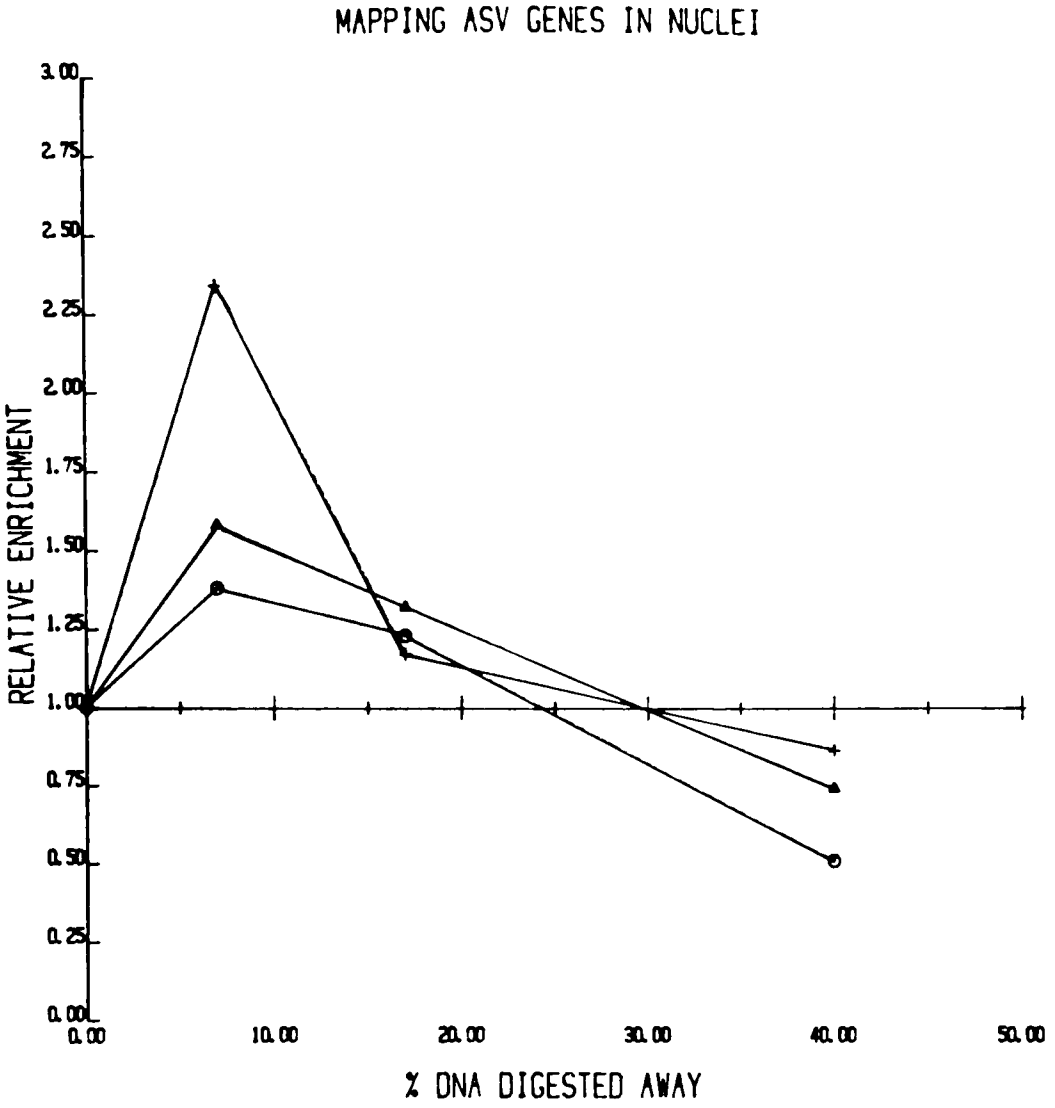


Fig. 68 Detachment mapping of ASV proviral genes The autoradiograph in fig. 67 was scanned with a Joyce-Loebl densitometer, and the densities of each band, relative to 100% DNA, plotted against the level of digestion. After an initial enrichment of the ASV genes, a progressive de-enrichment is seen that is absolute when 40% of the DNA has been detached away by Eco R1 [Symbols; + 4.1kb, o 3.2kb, ▲ 2.4kb].

enriched in transcribed sequences[94]. Poly-A selected and total RNA from HeLa cells were used as probes for detachment mapping [fig. 69]. The residual DNA is enriched in transcribed sequences. Whilst the low molecular weight bands are due to ribosomal genes [cf. fig. 69A and C], there are a variety of bands whose identities are not known. Due to the significant hybridisation, these may be repeated sequences that are known to be present within RNA molecules or are abundant RNA species[200,201]. These are not 'Alu' repeats though [see below].

Cell cycle studies on Histone H4A

If attachment is a pre-requisite for transcription, it may be possible to demonstrate the changing position of those genes that are cell-cycle specific fo expression. Whilst controversy exists about the cell-cycle specific expression of histone genes[204], the majority of data suggests that these are expressed in G_1/S and S phases of the cell[202,203]. HeLa cells were synchronised in G_1, G_1/S and S phases and the nucleoids detachment mapped for histone gene for H4A [figs. 70, and data not shown]. No significant differences in abundance were noted. However, several bands hybridise to this histone gene [arrowed, fig. 70], and varying conditions of stringency would have to be used to study these sequences.

Cell cycle studies on gamma-actin genes

Detachment mapping of γ-actin gene shows it to be considerably depleted in residual DNA, after Eco R1 digestion [fig. 71]. Again, mapping this gene at various levels of digestion shows a gradual depletion in the residual DNA and an enrichment in the

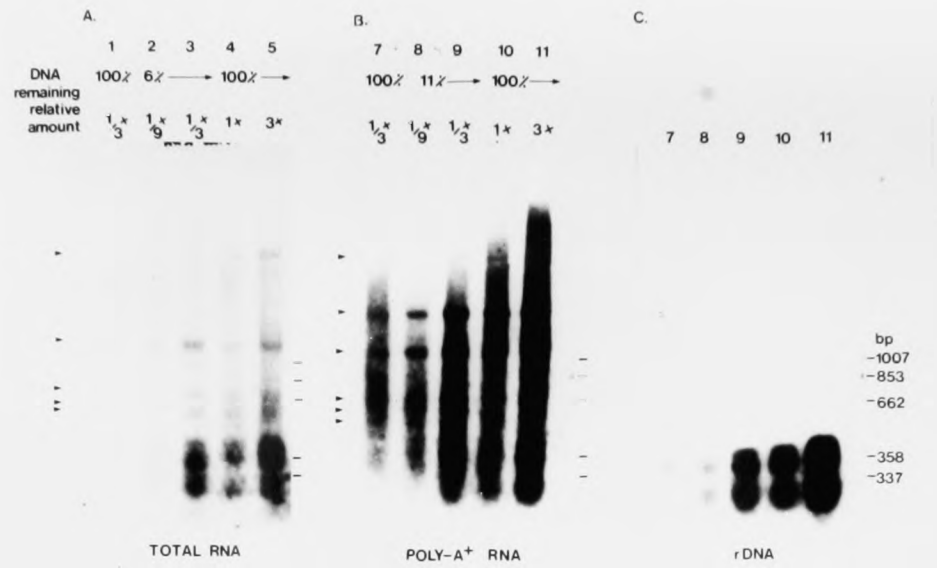


Fig. 69 Detachment mapping of transcribed sequences HeLa nucleoids were digested with Eco R1, the residual DNA purified, digested to completion with Hae III, gel separated and blotted [see Materials and Methods]. 94% or 89% of the DNA was digested away [tracks 2 and 3, and 8 and 9 respectively]. Tracks 1-5 were hybridised with total cell RNA, and tracks 7-11 with poly-A selected RNA [see Materials and Methods]. Track 6 contained end-labelled Hae III-cut PM2 DNA [not shown]. Transcribed sequences are enriched in the cage-associated DNA. Certain prominent bands are enriched after hybridisation to both the fractions of RNA [arrowed]. The low molecular weight bands are due to ribosomal sequences, as evidenced by re-hybridisation of tracks 7-11 with ribosomal genes. The identities of the others is unknown, though these are likely to be due to relatively abundant species.

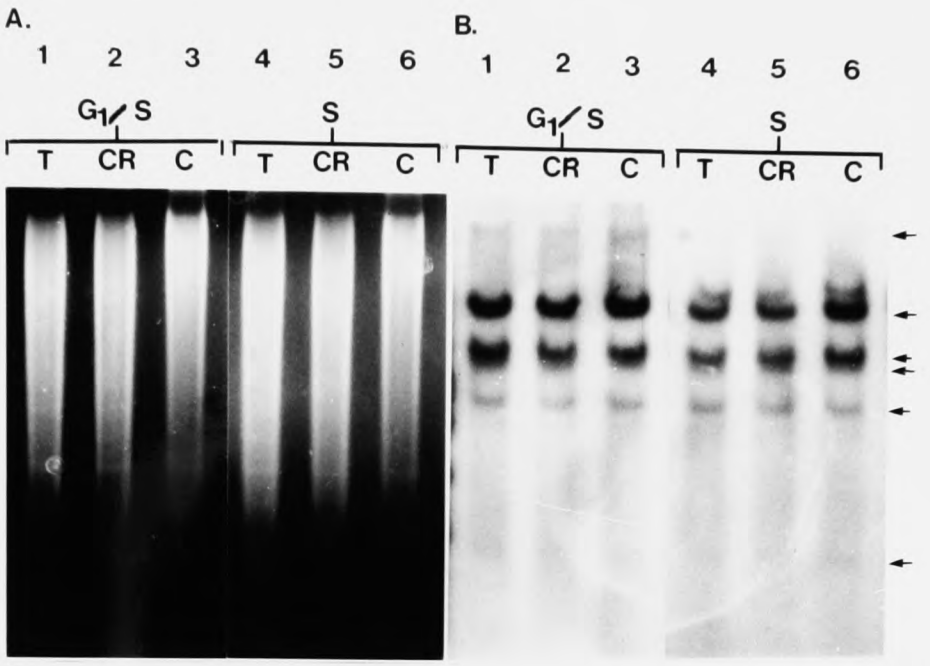


Fig. 70 Detachment mapping of Histone H4A genes [G1/S and S phase] Nucleoids from G1/S [tracks 1-3] or S phase [tracks 4-6] were detachment mapped using Eco R1. The ethidium gel (A), and the autoradiograph (B) are shown. Equal amounts of DNA were loaded. T=total DNA, CR= DNA released, and C= 2% DNA remaining associated with the cages. Arrows indicate hybridisation to specific bands.

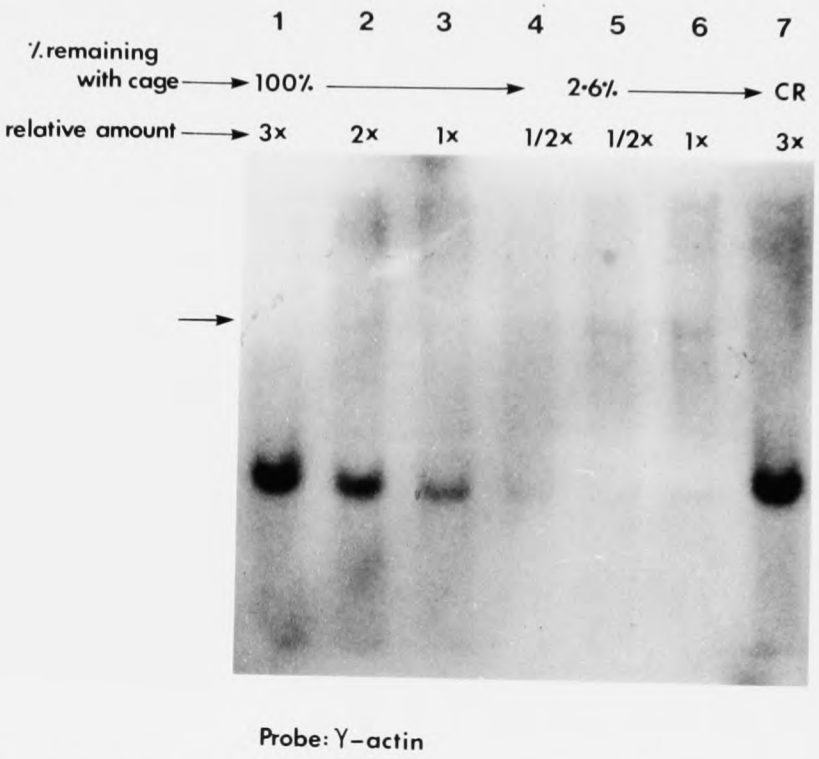


Fig. 71 Detachment mapping of actin sequences HeLa nucleoids, from randomly growing cells, were detachment mapped with Eco R1 and hybridised to γ-actin probe. Whilst the γ-actin band is visibly de-enriched, a faint cross-hybridising band [arrow] is enriched about 4-6 fold in the cage-associated DNA.

released DNA [fig. 72 and 73]. Unfortunately, very little is known of the expression of γ-actin gene in this strain of HeLa cells [cf. with the abnormal cytoskeletal protein patterns[186]]. If the gene is expressed, as has been found for HeLa cells elsewhere[206], then this would argue against artefactual association of active DNA with the cage. The actin gene family is known to be large, and this probe can hybridise to at least 20 Eco R1-cut DNA fragments[172]. Definitive proof of its expression in HeLa cells should be obtained by analysis of the RNA. Interestingly, there is a weak band that is enriched by the probe [arrow, fig. 71]. The actin gene does not appear to change its association with the cage in G_1, G_1/S phase, or 6h into S phase [fig. 74 and 75].

Direct examination of residual DNA

Direct staining of the residual DNA, after detachment mapping with Eco R1 or Bam H1 enzymes, does not reveal any significant differences. However, if this residual DNA, initially detached using Eco R1, is purified and further digested with Hae III, a number of differences become evident after ethidium staining [fig. 76]. The gel was photographed, the negative scanned and the relative enrichments of some of the prominent bands tabulated. There is no obvious repeat pattern, nor is there a single band that is considerably enriched. The maximal enrichment was about 4 x for a 960 bp fragment [fig. 76 and table 2].

DNA prepared thus was separated on 2% agarose gels [instead of the 4% acrylamide gels, as these are easier for blotting] and 'Southern' blotted. The filters were hybridised with total or 'Alu' DNA [fig. 77]. The results indicate that the residual DNA

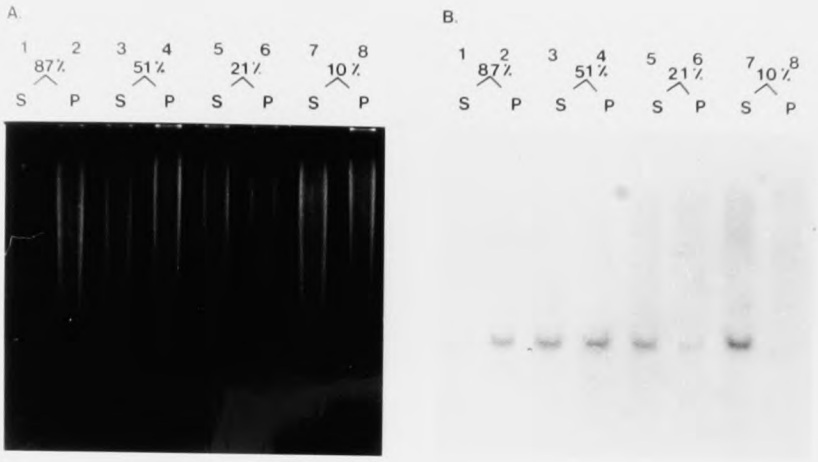


Fig. 72 Progressive detachment mapping of gamma-actin gene HeLa nucleoids were digested to various levels using Eco R1, and hybridised to γ-actin gene. Both the solubilised (S) and the cage-associated (P) DNAs were analysed. Fig. A shows the ethidium stain of the gel and B the autoradiograph of the filter. The percentages above the tracks indicate the amount of DNA remaining associated with the cages. A progressive depletion of the actin sequences is seen [cf. tracks 2, 4, 6, and 8] in the cage-associated DNA.

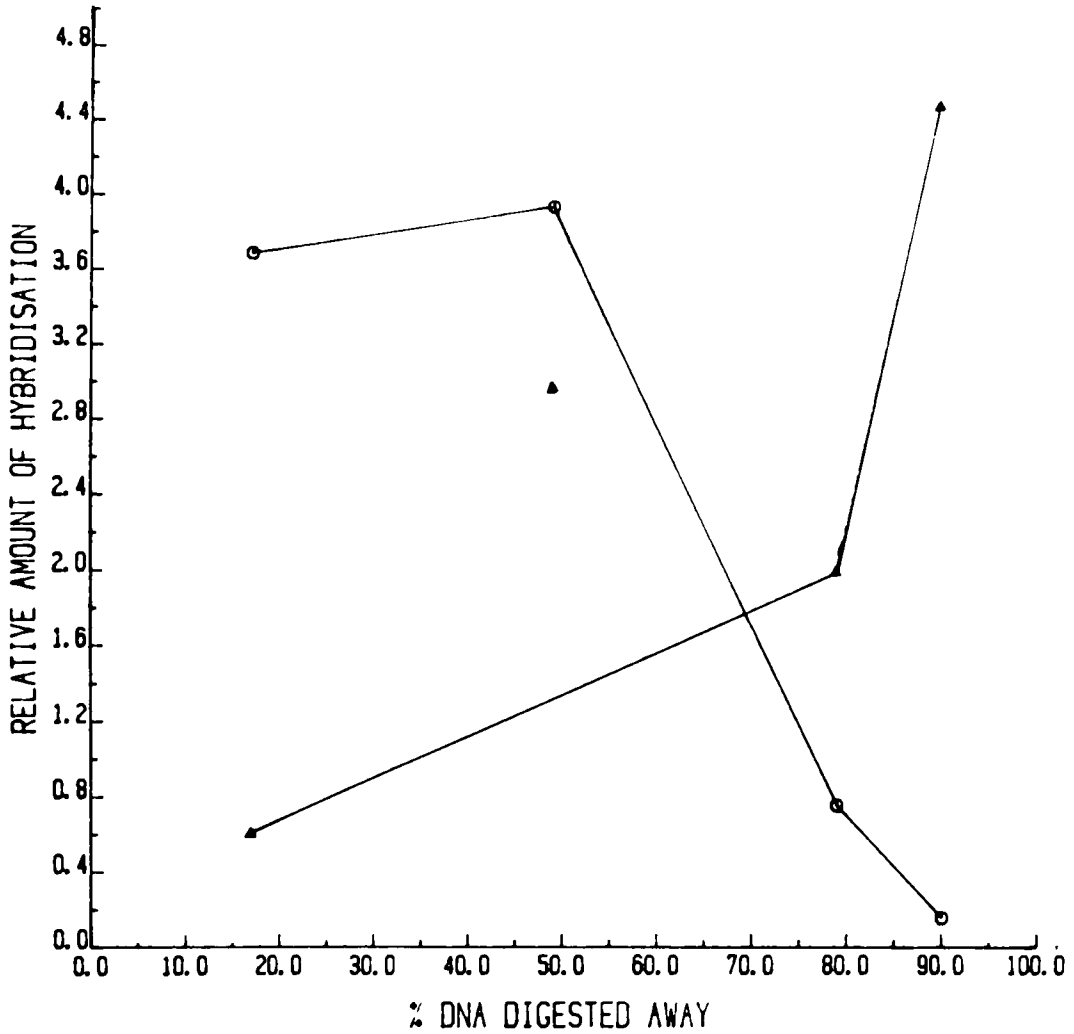


Fig. 73 Graph of relative hybridisation of actin sequences The autoradiograph in fig. 72 was scanned and the relative amount of hybridisation plotted against the level of DNA detachment. Except for the value at 49% digestion [triangle], there is good agreement between the depletion of the actin sequences in the cage-associated DNA [⊙], and the increase in the solubilised fraction [triangles].

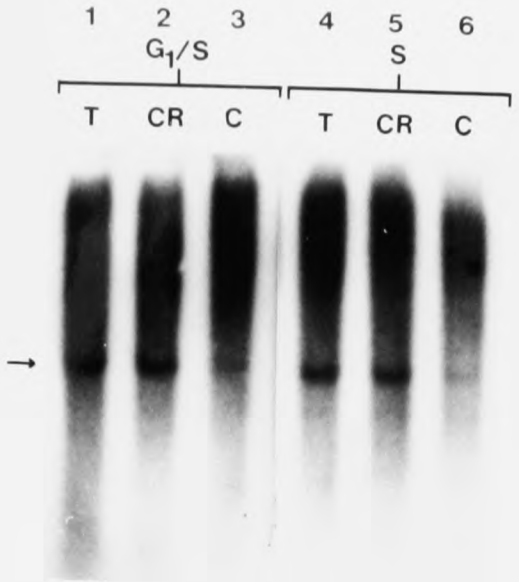


Fig. 74 Detachment mapping of actin genes [G1/S] and S phases] The filter, described in fig. 70, was re-hybridised with γ-actin probe [see Materials and Methods]. The γ-actin gene remains depleted in the residual DNA in both G1/S and S phases.

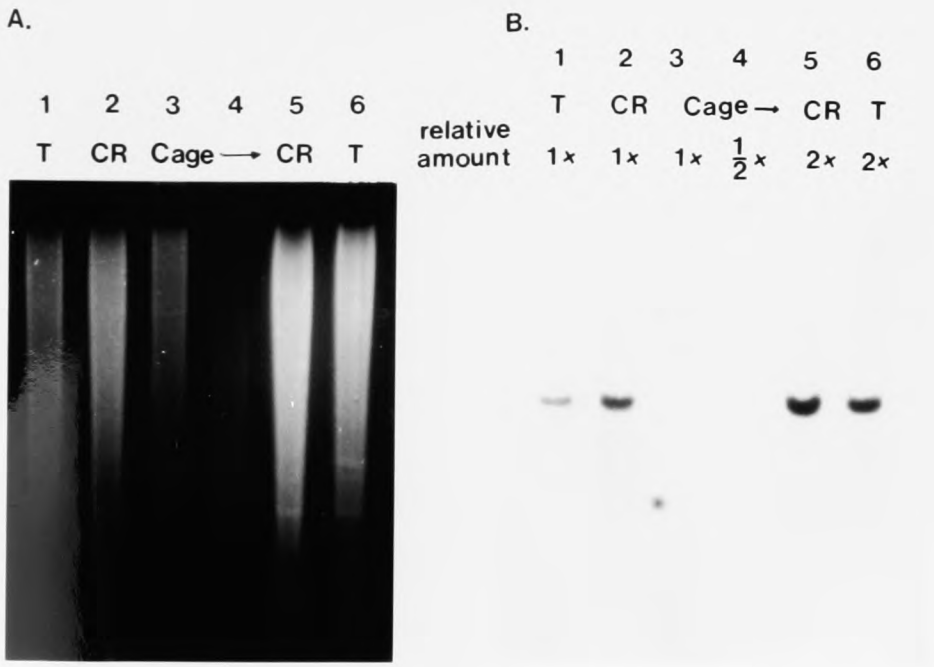


Fig. 75 Detachment of actin genes [G1 phase] Detachment mapping of the γ-actin gene was carried out on nucleoids isolated from G1 phase HeLa cells. Again, the γ-actin gene remains depleted in the cage-associated DNA.

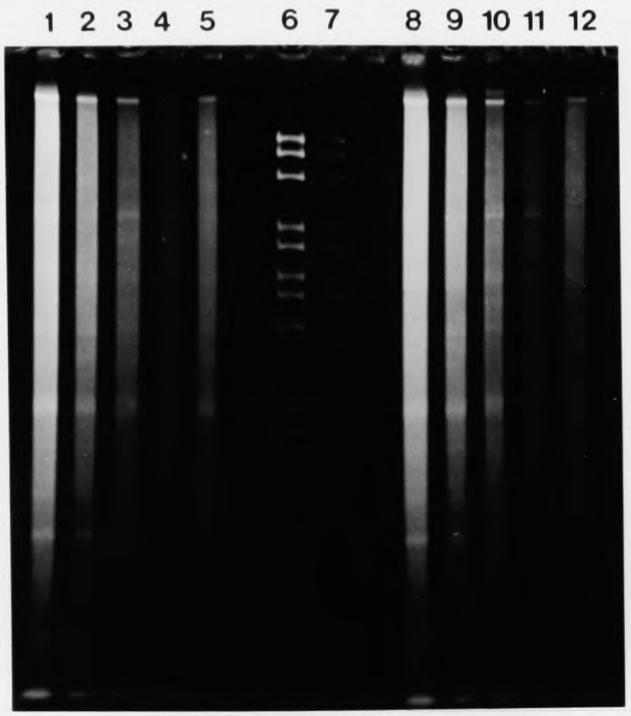


Fig. 76 Direct analysis of cage-associated DNA HeLa nucleoids were detachment mapped with Eco R1, and the purified DNA digested to completion with Hae III, rather than Eco R1, separated on a 4% acrylamide gel and stained with ethidium [see Materials and Methods]. Tracks 1, 2, 3 and 8, 9, 10 contain total cell DNA at a loading of 3x, 1x, and 0.33x respectively [1x loading =3.3μg]. The digestions resulted in 6% [tracks 4 and 5], and 11% DNA [tracks 11 and 12] remaining associated with the cages. The loadings were 0.33x [tracks 4 and 11] and 0.11x [tracks 5 and 12]. Tracks 6 and 7 contain Hae III-cut PM2 DNA. A variety of DNA bands are enriched in the residual DNA. The gel was photographed, and the negative scanned. Such gels were blotted and hybridised with repeated [fig. 77] and residual DNA [figs. 79-83]. The results are summarised in table 2.

is not enriched in 'Alu' repeats, and that the bands enriched after ethidium staining are not present in the same abundance in total DNA.

Table 2

Ethidium stain	Enrichment	Hybridisation	Enrichment	DNA probe stain
		1830	-	
		1470	-	
		1140	++	
960	++++			
840	++	880	+++	
660	++			
610	++	580	++	
525	++	500	++	
350	+/-	325	+++	225
330	+/-			215
165	-			160
100	+/-			130
				70
				15

Using residual DNA as probe

When nucleoids are extensively digested with DN'ases, a small amount of DNA remains resistant to digestion. Direct examination of this DNA, after purification, shows it to contain bands within a background of a smear [see fig. 78 and table 2]. Since nucleoids retain their supercoils in temperatures of 95 deg. C, the

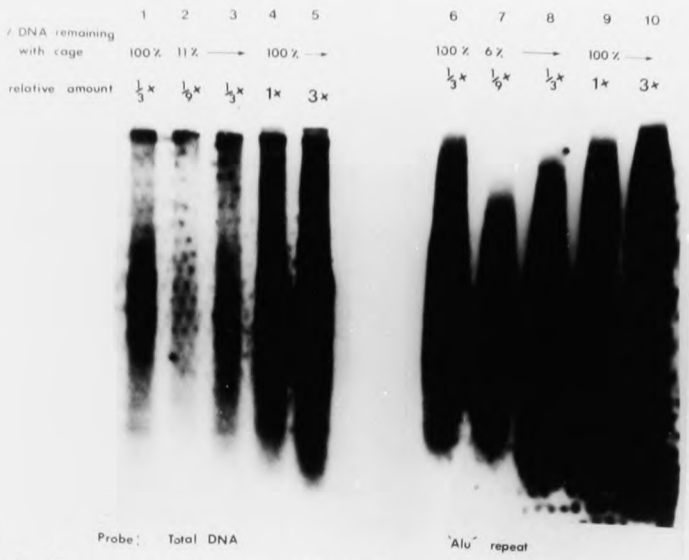


Fig. 77 Detachment mapping of repeated sequences DNA, prepared as described in fig. 76, was hybridised to total HeLa DNA (1-5), or 'Alu' repeat DNA (6-10). No obvious enrichment of the smear or any banding pattern was detected. The stippled backgroud was due to an artefact of drying the filter on a stippled blotting paper.

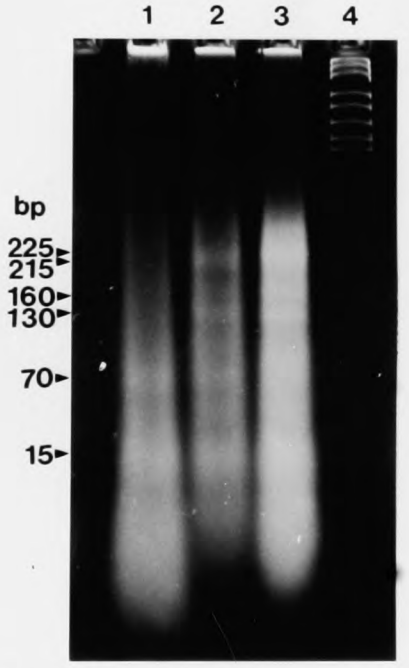


Fig. 78 Analysis of residual DNA HeLa nucleoids were extensively digested with DN'ase I, and the DNA purified (track 1), or the DN'ased nucleoids were treated with S_1 nuclease before (track 2), or after heating to 95 deg. C (track 3), and the residual DNAs analysed by gel eletrophoresis. Track 4 contains Hae III-cut PM2 DNA. A variety of 'bands' are visible [arrows], though these do not appear to have been affected by the S1 nuclease. There may be a slight difference in intensities, though this is not reproduceable.

attachment of the DNA is, by implication, also stable in these temperatures. One way of purifying the residual DNA of any contamination would therefore be to heat the DN'ase digested nucleoids to 95 deg. C, rapidly cool and re-digest with S_1 nuclease. Attached DNA will reanneal with zero order kinetics, but any contamination will remain susceptible. The residual DNA thus obtained is not signifinantly different from that after DN'ase treatment only [fig. 78].

Using this residual DNA as probe, a variety of DNA bands are enriched in the cage-associated DNA [fig. 79 and table 2]. The residual DNA probe was made from cells that had been synchronised in G_1 so that sequences that are attached, but not involved in replication are studied. There is little correlation between the Hae III-cut DNA fragments stained by ethidium and those detected by the probe. Obviously, the hybridisation bands are a sub-population of the 2% residual DNA associated with the cage. These bands may be those reported to be 'Hae III repeats'[61,62]. However, not all of these bands fall into this category. Their identities remain unknown.

Interestingly, hybridisation of the DN'ase-generated probe, to a filter produced after detachment mapping with Eco R1, results in the enrichment of two DNA bands of 8.3 and 7.5 kb [fig. 80]. If Bam H1 is used instead of Eco R1, a single band of about 15 kb is enriched in the cage associated DNA [fig. 81]. Since this band is visible even in total DNA, total DNA cut with Eco R1, Bam H1 or Hind III was hybridised to this probe [fig. 82]. The minimum length of this DNA is 25 kb. Using residual DNA probe, made after S_1 nuclease treatment, makes no difference to the result obtained [fig. 83]. Residual DNA, obtained after digestion of the

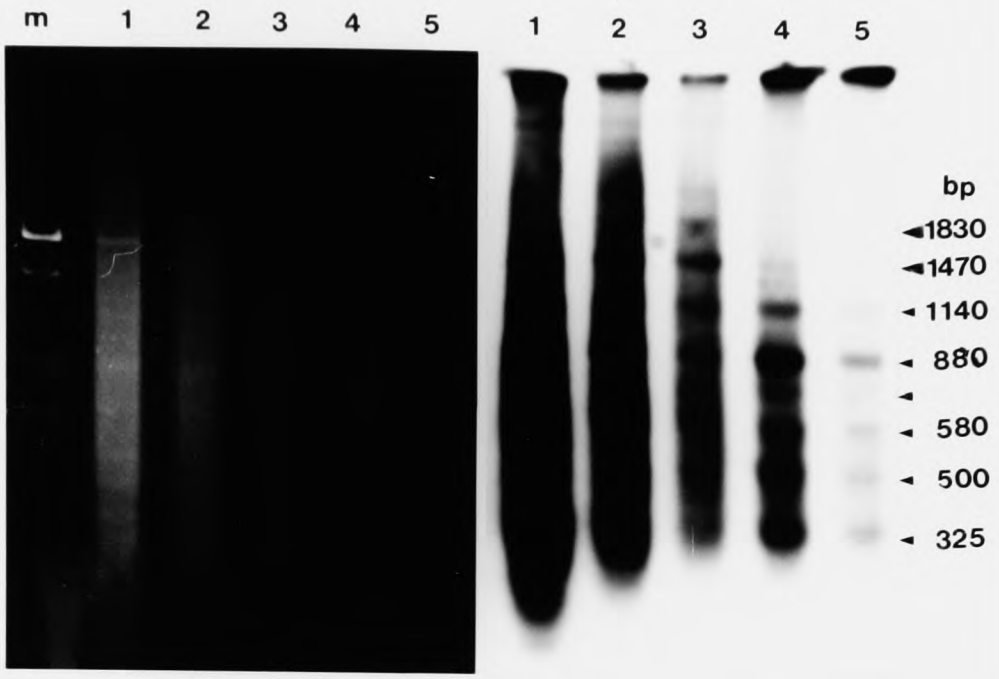


Fig. 79 Detachment mapping residual DNA DNA, prepared as described in fig. 76, was hybridised to nick-translated residual DNA, as shown in track 1, fig. 78. Fig. A is the ethidium stain of the gel, and B the autoradiograph of the filter. The relative amounts of DNA loaded are as indicated adove the gel. A variety of 'bands' are detected, of which some are enriched [small arrows], and some are depleted in the cage-associated DNA [large arrows, cf. tracks 3 and 4].

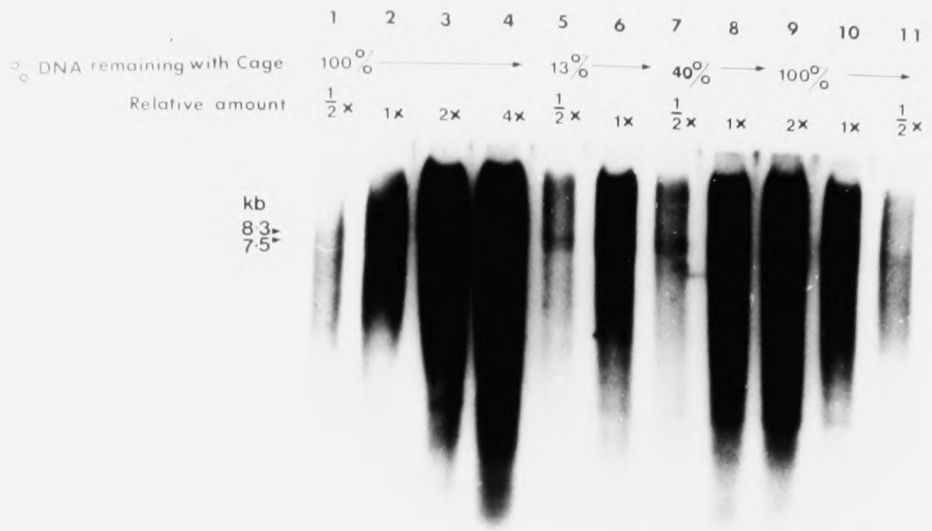


Fig. 80 Detachment mapping of residual DNA The DNA probe used in fig. 79 was also hybridised to a filter, produced after detachment mapping randomly growing cells with Eco R1 only. A slight increase in the 'smear' hybridisation is present, as well as two DNA bands of 8.3 and 7.5 kb that are enriched in the cage-associated fraction about 3-fold.

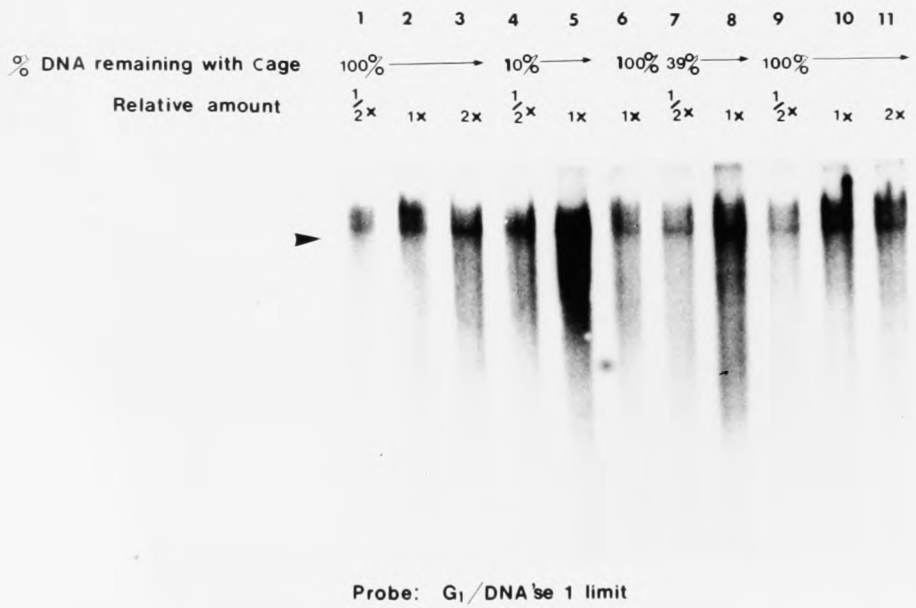


Fig. 81 Detachment mapping of residual DNA The probe used in fig. 80 was also used on a filter produced after detachment mapping using Bam H1 only. A single band of about 15 kb is now prominent.

nucleoids with micrococcal nuclease, hybridises to more bands than are recognised by the DN'ase I-generated residual DNA [fig. 84]. The former enzyme may not have digested the nucleoids to a same extent.

Table 3

Gene	% DNA remaining	Cell-cycle	Relative enrichment	Comments
ASV	60	random		Eco R1 digest of
4.1 kb			0.74x	nuclei
3.2 kb			0.51x	
2.4 kb			0.86x	
Histone	2	G1/S	1.1x	At least seven
H4A	2	S [6h]	1.1x	bands hybridise
	1	G1	0.8x	No significant difference
Actin	2.8	G1/S	0.1-0.2x	These are maximum
	4	S [6h]	0.1-0.2x	enrichments
	2.6	random	0.2-0.3x	
	1	G1	0.1x	
Ribosomal				
19.3kb	10	random	2.9x	
7.5kb	10		2.4x	
5.7kb	10		3.0x	

Conclusions

The above experiments do not prove specific attachments, but there is considerable evidence to suggest that this may be so. 'Active' genes are enriched in the residual DNA, though are all 'active' genes? There is no evidence to suggest any changes in the histone H4A genes, or for γ-actin genes with activity. Since the histone genes are so diverse[203,205], it may be argued that the 'active' genes were not detected by this cloned DNA. This could be resolved by isolating both the RNA, and detachment mapping using other cloned H4A genes. Similarly, the lack of enrichment of

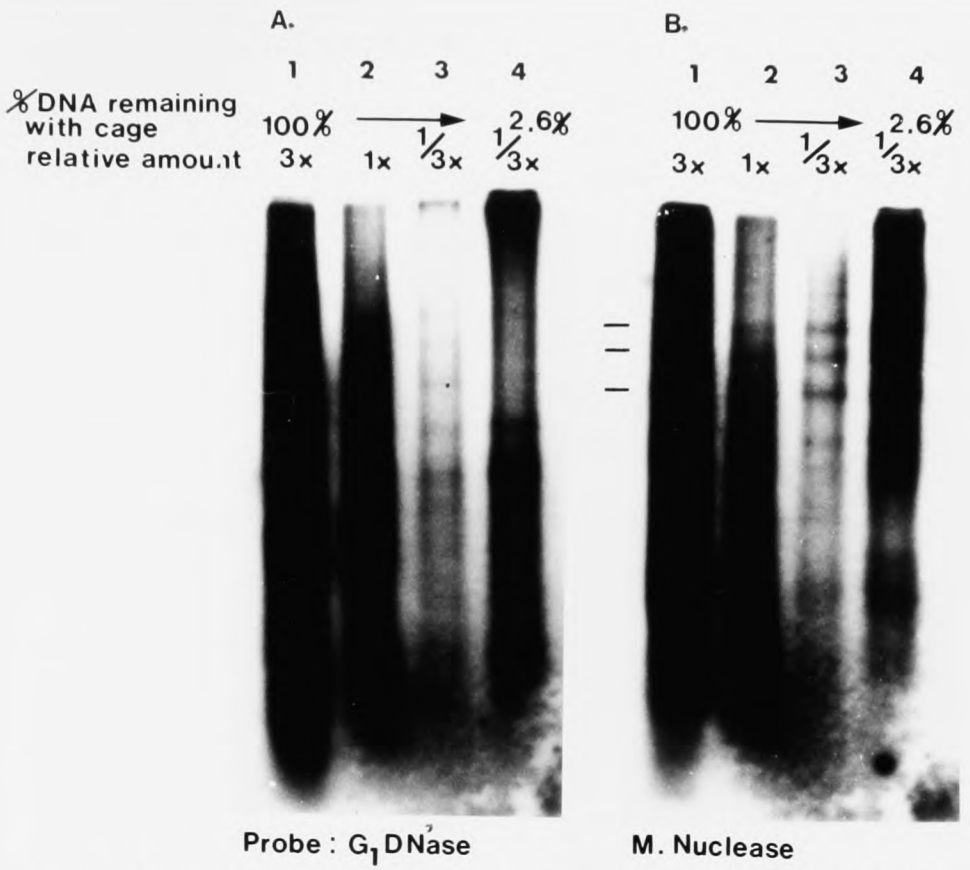


Fig. 84 Comparisions of nucleases to generate the residual DNA HeLa nucleoids were digested extensively with DN'ase I or micrococcal nuclease and the residual DNA labelled and hybridised to a filter, produced as described for fig. 76, and the autoradiographs are shown in (A) and (B) respectively. Note that the filter in B is the same as that used in A, after washing off the first probe [see Materials and Methods]. The main differences are that the DN'ase I treated probe recognises a sub-set of bands recognised by the micrococcal nuclease generated probe.

the γ-actin gene could also be similarly explained. Again, isolation of the mRNA for this gene from HeLa cells would increase our confidence in believing that this active gene was not closer to the cage. Interestingly, should the latter prove to be the case, this would remove the criticism that active genes were artefactually precipitated onto the cages.

For the artefactual association, one notices that the ASV genes are de-enriched after detachment mapping in nuclei, but enriched in nucleoids in transformed cells. Of course, it is well known that the active genes in nuclei are preferentially sensitive to nucleases[181]. However, we do not know whether these genes are lost because of scission away from the ASV genes, on the same loop of DNA, or whether the Eco R1 has preferentially cut these genes at their sensitive sites in nuclei. This could be resolved by detachment mapping with Eco R1, but digesting the isolated DNA with a different restriction endonuclease, prior to blotting and hybridisation. Cuts within the ASV genes will lead to smaller bands than expected.

CONCLUSIONS

When living cells are extracted with non-ionic detergents and high concentrations of salt, structures resembling nuclei are produced. These structures, termed nucleoids, contain intact supercoiled DNA and probably all the nuclear RNA. Since there is an underlying structure discernable, these structures have been investigated to ascertain whether this plays any role in the control of cellular function. Detailed characterisation of these structures is scant. However, since such structures have been implicated in DNA replication, RNA processing and perhaps transcription, I have studied these structures in order to ascertain whether the DNA is attached to these structures in a specific configuration, define the protein species that may be responsible for the tenacious attachment of DNA and partially characterise any of the proteins.

Nucleoid proteins are difficult to study, as they are not easily solubilised into conventional buffers, and denaturants such as SDS have to be employed to strip and solublise these proteins. Consequently, it has been difficult to identify DNA-binding proteins, or to investigate specific proteins, without attracting criticisms of artefactual extraction. Some of these proteins have been identified to be cytoskeletal proteins, though very little is known about the rest. Of the most studied, the lamins are probably the best characterised. No function for these have been found yet, though their localisation within cells is now known. These proteins are not entirely nuclear, and their intracellular distribution appears to change with the cell-cycle.

The presence of cytoskeletal elements suggests the possibility that some of the nucleoid proteins may be derived from parts other than the nucleus. To investigate this possibility, the presence of cell-surface proteins was looked for in the nucleoid proteins. Cell-surface proteins can be iodinated with considerable specificity, hence chance contamination seems less likely. Nucleoids contain a large proportion of proteins derived from the cell surface, though not all cell-surface proteins are found associated with nucleoids. Since the cytoskeleton is known to interact strongly with both the nucleus and the cell-surface, it is likely that the cell-surface proteins are associated with the nucleoids via it. Are these proteins cell receptors? It is likely that these proteins retain their biological activity in nucleoids, and this possibility may therefore be investigated. Since only a fraction of these cell-surface proteins are associated with nucleoids, this raises the possibility that these proteins may represent a biological sub-fraction, e.g. receptors that have been activated or inactivated. This is particularly intriguing, when one considers the role of steroid receptors, and the nuclear localisation of the receptor-steroid complex.

The majority of cellular glycoproteins are membrane proteins, and many of the receptor proteins are glycosylated. Investigation of nucleoid proteins using lectin staining shows that many of these proteins are also glycosylated. These studies also support the notion that some of the nucleoid proteins may be receptors. The lectin staining patterns also allow the detection of proteins that have disulphide linkages.

The only functional studies I carried out was to look for protein kinase and methylase activities. The former was studied

because DNA replication is known to take place at the nuclear attachment sites. This is also true for SV40 DNA in infected cells. Since the T antigen is phosphorylated, and it is the phosporylated form that mediates the viral DNA replication, I reasoned that there may be an equivalent nuclear protein that may mediate the attachment of the cellular DNA for replication. The methylase activity was studied at the suggestion of Professor Burdon.

There is kinase activity associated with nucleoids, but this is likely to be involved in mitosis, rather than replication. Specific cytoskeletal proteins are phosphorylated, both in vivo and in vitro. These changes have been well-documented in the literature as being associated with mitosis. This possibility should be tested by studying cell-cycle specific changes in nucleoid-associated kinase activity.

Professor Burdon, studying DNA methylase in HeLa cells, found that a significant amount of the methylase activity remained associated with the nucleus, even after a high salt wash [pers commun]. This lead to the intriguing suggestion that DNA methylases were tightly associated with the nuclear cage, and speculations of gene control using methylation. Whilst methlase activity was found to be present in the nucleoids, only 15-20% of the methylation was accounted for by the RNA and DNA. The rest was found in the protein fraction. The signification of this remains to be elucidated.

Because DNA is attached to the nucleoids, the nature of this attachment was studied by investigating DNA-binding proteins and the orientation of the genes with respect to the attachment sites. Since solubilisation of the nucleoid proteins requires

denaturation, study of these proteins using conventional column chromatography, or solution bio-assays has proved difficult. However, DNA-binding can be studied using protein blotting. The proteins renature spontaneously during blotting, and their biological activities appear to return. Nucleoid proteins contain a variety of DNA-binding proteins, most, if not all, are found tightly associated with the nuclear cage. DN'ase digestion of nucleoids does not lead to detectable solubilisation of such proteins. Of the DNA-binding proteins detected, none appear to be easily identifiable as DNA- or sequence-specific. The former is made difficult as it is not possible to perform quantitative studies easily due to the nature of the technique, and because saturation of binding is not easily obtained. However, sequence-specificity can be demonstrated using cloned probes. Unfortunately, there is no evidence to indicate that nucleoid proteins contain sequence-binding proteins, or whether HeLa DNA contains sequences that are specifically recognised for attachment.

I therefore devised a technique, 'Southern Cross', that does not depend upon the fore knowledge of such proteins or DNA sequences, but would allow their detection in a crude mixture. Using λ repressor as a test for the system, it is possible to detect specific DNA sequences at their natural abundance, and amongst a mixture of other DNA fragments. Detection of the protein is probably only possible if it is enriched about a 100-fold over its natural abundance, though a crude protein preparation can still be used. The main limitation of this technique is that it is dependent on renaturation of the proteins, after SDS-PAGE. This could be obviated by the use of native gel electrophoresis where possible. Applying this technique to study Lex A protein, I found that this protein appeared to bind to a specific set of DNA

fragments. Whilst it is possible to isolate the retained fragments, clone and re-test these to demonstrate sequence-dependent binding by the Lex A protein, this was not done.

Looking for sequence-specific proteins in eukaryotic cells was more difficult. Perhaps a 5S gene-binding protein was detected in a crude mixture. Nucleoid proteins do not appear to contain any such proteins, though lack of time prevented a complete search to be made.

Studying DNA arrangements within the nucleoids reiterates previous findings of specific orientation of the genes. However, artefacts caused by precipitation of transcriptionally active genes could not be ruled out. Of the most interesting findings, no change in the position of the actin, or histone H4A genes with the cell cycle were demonstrable. However, neither were found to be closer to the attachments sites. Should the γ-actin gene be found to be actively transcribed at any of the cell cycle phases, the criticism of artefact would be nullified.

Direct examination of the tightly bound residual DNA did not prove fruitful. A variety of repeated sequences were found to be present in this residual DNA. None of these behaved as if they were responsible for DNA attachment. This may suggest that there is no one specific sequence that attaches DNA to the cage, but that attachment may be mediated by any sequence. Specificity could be obtained by allowing attachments to take place at fixed times during the cell cycle. The role for attachments would then be purely from a structural necessity, rather than for transcriptional purposes. Replication, of course, needs these attachment sites, both for segregation of daughter chromosomes and for the inheritance of the differentiated trait.

Are we any further in understanding the nature of nucleoids as a result of the studies presented here? The answer is probably not. There are certain enzymatic activities found present in the nucleoids. Some of the nucleoid proteins can bind to nucleic acids. Yet, none of these observations allow definitive statements regarding the role of these elements in the functioning of the cell to be made. Potentially the most useful of the findings is the technique of 'Southern crossing', that can enable the detection of sequence-binding proteins and these sequences more easily. However, since the two years following publication, this technique does not appear to have been used by the scientific community.

Of the questions remaining unanswered, or raised by this study is the one about the underlying structure of the cell. There is enough evidence to suggest that there is such a structure. The DNA appears to be non-randomly positioned in the nucleoid, the proteins of the nucleoids are mainly the cytoskeletal elements, with a fraction of the cell-surface proteins that are preferentially represented. Yet none of these allows a firm conclusion to be drawn. Extension of these studies may lead to a greater understanding.

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