

Cbf1 Regulates Chromatin Remodelling
of the
Saccharomyces cerevisiae Genome
at
Multiple Binding Sites

Bertha Verónica Valverde Garduño

Thesis Submitted for the Degree of Doctor of Philosophy
at the
University of Oxford

**THE QUEEN'S COLLEGE
TRINITY 1999**



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ABSTRACT

The centromere binding factor 1, Cbf1, of *Saccharomyces cerevisiae* is a bHLH/ZIP protein which has been described as a determinant of specific chromatin structures and as a tethering factor for activators of transcription at the promoters of genes of the Methionine Biosynthesis Pathway. Deletion mutants show various phenotypes, among them methionine auxotrophy, an increased rate of chromosome loss, modifications in the growth rate and modification of the chromatin structure at *MET* genes. Meiosis competence also becomes greatly reduced in *cbf1* cells. The sequence motif (RTCACRTG) to which Cbf1p binds is found at multiple loci through the yeast genome. This thesis shows that the chromatin structure is reorganised at multiple Cbf1p binding sites *in vivo*, when yeast cells are starved to enter meiosis. Extensive remodelling occurs at the *MET16*, *MET17(25)*, *DRS2* and *GDH3* loci and at the YAL060W open reading frame, as detected by *in vivo* digestion of chromatin with micrococcal nuclease and indirect end-labelling. The same kind of analysis showed that the remodelling of chromatin at Cbf1p binding sites is not specific for meiosis, it occurs also in similarly starved haploid cells. The lack of methionine is a key trigger of these changes. This reorganisation of chromatin is dependent on Cbf1p, since starved *cbf1* cells do not display any modification in nuclease accessibility patterns at or around Cbf1p binding sites. Mutational analysis revealed that a negative charge at a putative phosphorylation site (serine residue 226) and the DNA-binding activity of Cbf1p are both required for the chromatin reorganisation to occur in response to starvation. *CBF1* mutants which do not reorganise chromatin were also shown to be unable to enter meiosis, suggesting that the remodelling of chromatin at multiple Cbf1p binding sites may be required to enter pre-meiotic DNA replication, since such cells arrest before the initiation of this process. In summary, the results presented in this thesis are compatible with a model in which Cbf1p plays an active role as part of a mechanism sensing the nutrient availability and regulates the reorganisation of chromatin, at multiple loci through the yeast genome, in response to starvation conditions.

To the beloved memory of my mother

Venancia Garduño González

Her never lost curiosity and her enjoyment of knowledge have been an
inspiration through these years.

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Chapter 1

General Introduction

1.1 Chromatin Organisation of the Eukaryotic Genome

Chromatin is the highly ordered supramolecular nucleoprotein complex in which genomes are organised (van Holde *et al.*, 1995; Widom, 1998; Wolffe, 1998). Although it was initially considered only as a highly efficient way of packing the long molecules of chromosomal DNA, the present view is that it is also, in fact, a very effective form of regulation of the fundamental informational transactions in the nucleus. Much progress in the understanding of such processes has been done with the use of isolated molecules of DNA for *in vitro* simplified studies, but despite the depiction of DNA on its own in various models, the real substrate is chromatin, rather than naked DNA. This organisation of the genetic information requires mechanisms to allow the proteins and protein complexes in charge of such transactions to gain access to the DNA template in a regulated manner. Therefore, the study of factors involved in determining chromatin structure and dynamics, is of particular interest in the understanding of the regulation of DNA transactions. Such is the case of a factor of the yeast *Saccharomyces cerevisiae*, best described as the centromere and promoter factor 1, Cpf1 (Mellor *et al.*, 1990), but at present referred to by the standard name of centromere binding factor 1, Cbf1, (Cai and Davis, 1990) which acknowledges only one of the functions of this protein.

There is currently much interest in the study of the relationship between the chromatin structure and the regulation of DNA replication (Itoh and Shimizu, 1998; Quintini *et al.*, 1996; reviewed by Gruss and Sogo, 1992), recombination (Fox and Smith, 1998; Nicolas, 1998; Singh *et al.*, 1998; Tsukamoto *et al.*, 1997) repair (Baxter and Smerdon, 1998; Surralles *et al.*, 1998) and transcription (Armstrong and Emerson, 1998; Dimitrov and Wolffe, 1996). It is now known that the meaning of a particular DNA segment, in terms of the correlation between its temporal expression and cell and life cycle, depends not only on its sequence, but it is highly dependent upon chromatin context and dynamics. For instance, genes otherwise active, become inactive when inserted into the more compact repressive heterochromatic regions such as telomeres (reviewed by Lustig, 1998). In the chromatin complex a great number of DNA binding and non-DNA binding proteins contribute to provide structural organisation and regulation through DNA-protein and protein-protein interactions. Among them, the histones, a group of highly

conserved basic proteins, are the most abundant and play a central role as part of the basic unit in the structure and regulation of chromatin.

The Nucleosome

The basic structural unit of chromatin in all eukaryotic chromosomes is the nucleosome. This unit was first detected when chromatin was partially digested with DNA endonucleases producing a regular ladder of DNA fragments which allowed researchers to associate a specific length of 200 base pairs (bp) of DNA with 2 sets of the four histones (Kornberg, 1974; Sahasrabudhe and Van Holde, 1974). In the first analysis of the crystal structure of the nucleosome core (Richmond *et al.*, 1984) it was found that it is a disk shaped histone octamer. It is constituted by two of each histone proteins H2A, H2B, H3 and H4, around which 145-147 bp of DNA are wrapped into ~ 1.8 left-handed superhelical turns. A simplified model of the nucleosome is presented in Figure 1. This complex structure is found every 200 ± 40 bp through all eukaryotic genomes. Stretches of linker DNA connect successive core nucleosomes in the linear beads-on-a-string relaxed structure (Olins and Olins, 1974). In the yeast, *Saccharomyces cerevisiae*, the nucleosomal length is 160-165 bp, the lower limit found so far in eukaryotic cells (Lohr and Van Holde, 1975). Histones are a group of small basic proteins highly conserved among eukaryotic cells. They have amino terminal regions (10-40 residues) rich in lysine, the histone tails, which can be reversibly modified by acetylation, phosphorylation, methylation, polyADP-ribosylation and in the carboxyl terminal tails of H2A and H2B ubiquitination. Wolffe and Hayes (Wolffe and Hayes, 1999) have recently pointed out that the histone tails constitute 25% of the total mass of the nucleosome, stressing the relevance of the multiple combinatorial possibilities for the chromatin structure. Figure 1 shows the residues which can be modified. It has been shown that these series of postranslational covalent modifications have a critical role in the regulation of the chromatin structure and activities, especially in transcription and replication. The crystal structure of the core particle has recently been described at the higher resolution of 2.8 angstroms; each histone heterodimer H2A-H2B and H3-H4 organises ~ 28 bp by making contact with the phosphate groups of the DNA backbone. Every time the minor groove faces the histone surface an arginine sidechain inserts into it. This structure also revealed one important feature: the histone tails of H4 can project outside the core particle, between the turns of DNA. It was then suggested that these tails can potentially interact with the lateral surface of the next nucleosome, contributing to the packaging of nucleosomes into higher levels of chromatin organisation (Luger *et al.*, 1997).

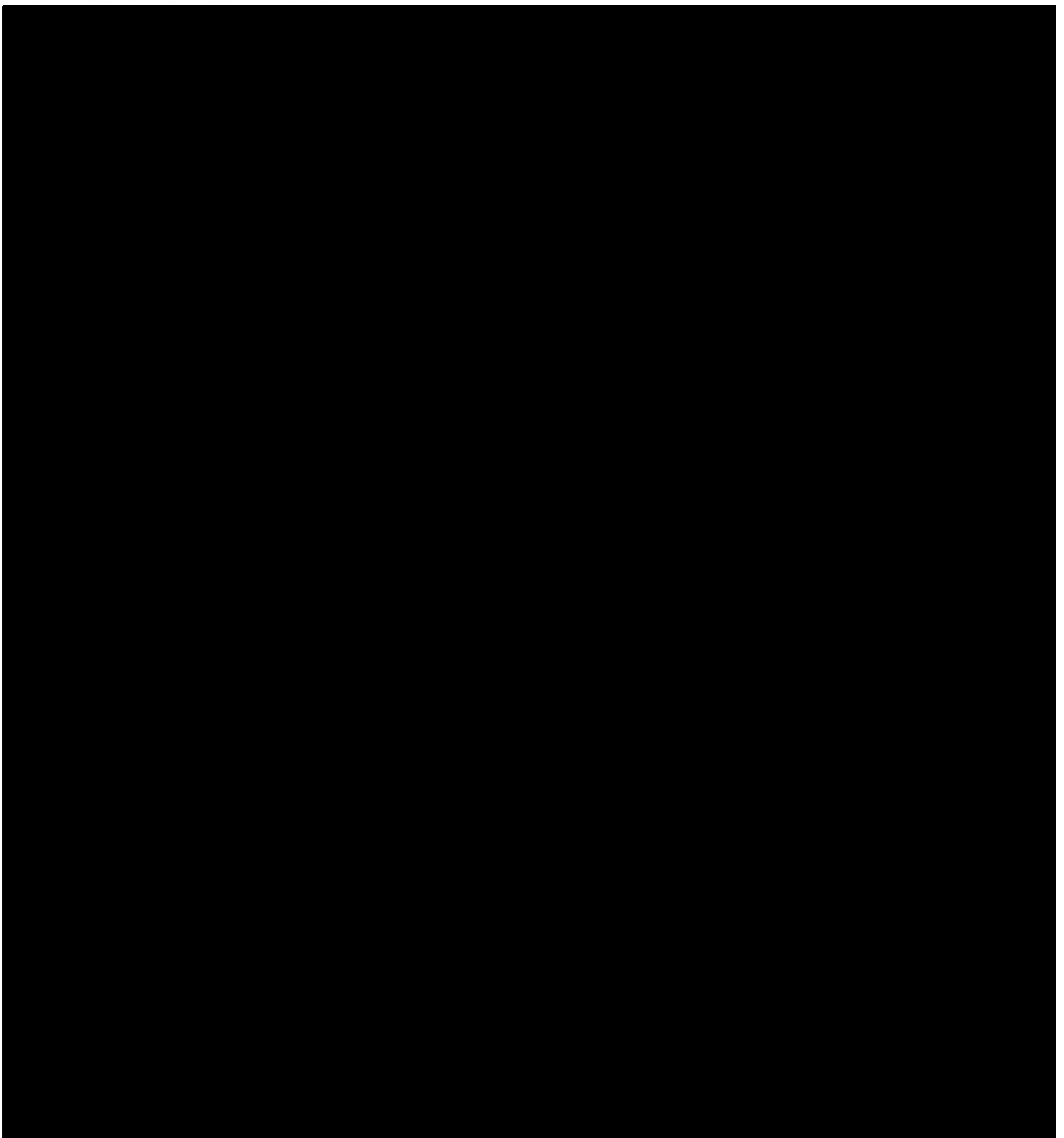


FIGURE 1. Nucleosome models. (A) A simplified representation of the core nucleosome indicating the dimensions (in nm) of the octamer and the two turns of DNA represented by a white cylinder around the central cylinder representing the octamer of core histones. (B) Diagram depicting the histone tails as lines projected from the central octamer (represented by a black circle) surrounded by DNA (gray zone). The residues which can present covalent modifications, with potential consequences for the chromatin structure, are indicated by the one character amino acid code along the tails. The left side of the diagram indicates the position of the residues and the right side their potential modifications: acetylation, Ac; methylation, M; phosphorylation, P; ubiquitination, Ub; ADP-ribosylation, ξ (Wolffe and Hayes, 1999). Residue K120 of H2B which can be ubiquitinated is not shown.

The fact that the histone tails can project outside the core and can be reversibly modified provides them with the potential to have a variety of interactions among them, with other proteins and with DNA. Nucleosomes do not lie at random positions with respect to a given DNA sequence, there is evidence of the sequence directed translational positioning of nucleosomes on DNA, for instance, the 5S ribosomal RNA gene can direct core histones of chicken, frog and yeast to the same position (Simpson, 1991).

It is possible to detect the presence and precise position of nucleosomes and other protein complexes on the DNA molecule, since its accessibility is modified by such interactions (Wu, 1980). For instance when chromatin is digested with micrococcal nuclease (MNase) it will first cut the linker DNA between nucleosomes, then it will continue digesting the linker DNA and finally the DNA within the nucleosome. Partial digestion of chromatin with MNase will preferentially cut linkers and non-nucleosomal DNA producing a pattern of accessibility that reveals the position of protein complexes along the DNA. Since MNase has specificity for A/T rich DNA sequences the chromatin patterns have to be compared with naked DNA digestions, also new accessible sites could appear in chromatin since the nuclease is forced to cut mainly linker DNA. Although the nuclease accessibility patterns reflect chromatin organisation, they do not provide information to determine to which extent nucleosome organisation could be affected, for instance the DNA-histone or histone-histone interactions. Some regions in chromatin have been shown to present two orders of magnitude more sensitivity to nuclease cleavage or chemical modification than the rest of the chromatin, they are devoid of nucleosomes and are termed hypersensitive sites (Scott and Wigmore, 1978; Varshavsky *et al.*, 1978; Wu *et al.*, 1979).

Higher order chromatin structure

Chromatin extracted under physiological salt concentration is organised in a condensed 30 nm fiber (Thoma *et al.*, 1979). However some studies have demonstrated that an extended fiber of 30 nm can be formed even at low ionic strength (Leuba *et al.*, 1994). Therefore it has been suggested that there are various degrees of compaction between the beads-on-a-string basic structure and the highly condensed chromatin fibre found at high ionic strength (reviewed by van Holde and Zlatanova, 1996; Zlatanova *et al.*, 1998). It is considered that additional non-core histones, the linker histones, are essential for this level of chromatin compaction (reviewed by Belikov and Karpov, 1998; Felsenfeld and McGhee, 1986). However there is biological evidence which demonstrates that the linker histone H1 is not required in the early development of some organisms. For instance

the protein similar to H1 expressed during early development of *Xenopus* was eliminated without significant consequences in the chromatin condensation or organism development (Dasso *et al.*, 1994; Ohsumi *et al.*, 1993). Due to the interaction of H3 with linker DNA (Belyavsky *et al.*, 1980) and the requirement for its N-terminal tail to reconstitute the fibre in the absence of the linker histone tail, it has been suggested that the tails of H3 may participate in the formation of the compact chromatin fibre structure (Leuba *et al.*, 1998a; Leuba *et al.*, 1998b). It has also been shown that the folding of the fibre in the absence of H1 requires the tail domains of the four core histones (Tse and Hansen, 1997). Yeast cells do not have an H1 linker histone and the only protein with significant similarity to the globular domain of H1, in the yeast genome, is not essential for viability (Patterson *et al.*, 1998; Ushinsky *et al.*, 1997). Nevertheless, a cation-dependent folding of yeast chromatin into a 30 nm fibre has been reported (Lowary and Widom, 1989). Some regions of yeast chromosomes are packed into a compact repressive heterochromatin at telomeres and mating type loci (reviewed by Lustig, 1998). Condensation of yeast chromosomes has been detected and some of the genes involved in this function isolated. In the case of a temperature sensitive mutant *smc2-1* the distance between two markers on one chromosome, as seen by microscopy, becomes increased when mitotically arrested cells are transferred to the restrictive temperature (Strunnikov *et al.*, 1995a).

Specific DNA binding proteins

In addition, there are also non-histone specific DNA binding proteins determinant for chromatin structure at specific locations. For instance, the interactions of these *trans*-acting factors at promoter regions may generate hypersensitive sites; at centromeres and telomeres these factors generate nuclease resistant regions. In some cases *trans*-acting factors may act passively, as boundaries to the ordering of nucleosomes, an example is the hypersensitive site generated by a factor at the intergenic region between *GAL1* and *GAL10* (Fedor *et al.*, 1988; Reagan and Majors, 1998). Other specific DNA binding proteins appear to play a more active role in determining chromatin structure through the interaction with the histones in nucleosomes. The formation of a stable positioned nucleosome, adjacent to the α -2-MCM repressor complex at the mating type loci of a cells, is prevented by deletion of the histone H4 amino terminal tail, indicating that interaction between the complex and H4 is required for the formation of the positioned nucleosome (Shimizu *et al.*, 1991). The budding yeast centromere and promoter factor Cbflp has been found to be required for the formation of specific chromatin structures associated to its

binding sites at *MET* promoters; in particular, it has been shown that at *MET25* (current standard name *MET17* will be used from here) two different arrangements of nucleosomes can be detected depending on the presence or absence of this factor in yeast cells (Kent *et al.*, 1994).

Despite of the apparent regularity and the presence of a basic universal subunit, chromatin is not uniform along the chromosomes. There are various specialised chromatin regions; among them and of prime biological relevance is the centromere, which at cytological level appears as a constriction of the condensed chromosomes, Cbflp also participates in the chromatin structure at centromeres.

1.2 Cbflp at the Chromatin of Yeast Centromeres

The viability of the offspring after cell division is dependent upon the faithful transmission of complete complements of genetic information after replication. The centromere is the chromosome region over which the kinetochore is assembled, through this centromere/kinetochore chromosomes bind and move along the spindle microtubules during cell division. This chromosome region also participates in the cohesion of newly replicated sister chromatids during prophase, metaphase and the first meiotic division. Various assays have been created to test centromere function in yeast cells to evaluate the effect of mutations on its components. All these methods are based on the loss vs faithful transmission of particular markers contained either in plasmids, chromosomal fragments or chromosomes. For instance, the stability conferred by a centromere to a plasmid can be estimated as the percentage of cells still expressing a particular marker on the plasmid after growth in non selective media (Panzeri and Philippsen, 1982). The first functional centromeric region (*CEN*) to be isolated was from yeast chromosome III and it was demonstrated that *CEN3* conferred proper mitotic segregation to circular plasmids bearing it, causing them to be inherited with high efficiency through mitosis (Clarke and Carbon, 1980; McGrew *et al.*, 1986). Budding yeast has the smallest centromeres described so far.

In the fission yeast *Schizosaccharomyces pombe* the centromere spans between 40 to 100 kb and is constituted by a series of elements around 5kb long centered on a core of 4-7 kb, but this core is not conserved among the three chromosomes (Takahashi *et al.*, 1992). The minichromosome Dp1187 of *Drosophila melanogaster* has a 420kb long centromere with 85% of it containing two different repetitive satellite sequences and 10% of transposons (reviewed by Wiens and Sorger, 1998). For humans it is less clear which specific segment of DNA in chromosomes constitutes the centromere, although the best

candidate is the alpha satellite found in all chromosomes and containing tandem arrays of 171bp monomers organised into higher order repeats (Heller *et al.*, 1985; reviewed by Murphy and Karpen, 1998).

In contrast, the first DNA segment found to be able to restore centromere function to a yeast chromosome was merely 289 bp long (Carbon and Clarke, 1984) and an even shorter region, whose length varies from 111 to 119 bp contains the three conserved elements (Hieter *et al.*, 1985). When analysed with nucleases a distinctive and unique chromatin structure is found at yeast centromeres, a nuclease resistant core of 160-220 bp flanked by hypersensitive sites on both sides (Bloom and Carbon, 1982) and these followed by positioned nucleosomes (Saunders *et al.*, 1988). Restriction enzyme analysis reveals an even more reduced central core of 150-160 base pairs (Funk *et al.*, 1989). Mutations which disrupt this core also alter centromere function, indicating that the components responsible for this structure are critical for centromere function. In fact a common feature among the centromeres of all eukaryotes, apart from some particular protein components, is the presence of a specialised chromatin structure (reviewed by Clarke, 1998).

Centromere DNA elements

A relatively short stretch of centromeric DNA is indispensable for proper chromosome segregation. In fact only 125 bp are sufficient for full centromere function during mitosis and meiosis (Cottarel *et al.*, 1989). Sequence analysis of this region over various chromosomes revealed a high sequence similarity and three clearly conserved sequences (Fitzgerald-Hayes *et al.*, 1982; Hieter *et al.*, 1985; Panzeri *et al.*, 1985) CDEI, CDEII and CDEIII (for centromere determining elements). As shown in Figure 2, these three CDE regions are present at the *CEN* loci of all *S. cerevisiae* chromosomes and confer proper mitotic segregation to plasmids bearing them (Cumberledge and Carbon, 1987; reviewed by Hegemann and Fleig, 1993). The function of each of these elements has been intensely studied by mutagenesis and deletion approaches, analysing the effect of mutations and deletions on the mitotic and meiotic stability of chromosomes and plasmids (Carbon and Clarke, 1984).

The most conserved of the three elements is CDEIII, a 26 bp sequence with bilateral symmetry and with 7 positions conserved among all 16 *CEN* loci. It is essential for centromere function (Clarke and Carbon, 1985), some point mutations on it completely destroy the centromere function (Hegemann *et al.*, 1986; Hegemann *et al.*, 1988; Jehn *et al.*, 1991; McGrew *et al.*, 1986). For instance substitution of the central C for any other

base results in total loss of centromere function. Other mutations have less detrimental effects, as in the case of positions 12-13 and 15-17 which increase the rate of chromosome loss, in fact mutations at any position of this element affect centromere function (Hegemann *et al.*, 1988; reviewed by Hegemann and Fleig, 1993). Inversion of this element also destroys centromere function, despite its partial symmetry (Murphy and Fitzgerald-Hayes, 1990).

CDEII is a region 78 to 86 base pairs long with a very high content of A/T (>90%). Short deletions or insertions within this element have only a mild effect on centromere function, but when long stretches of DNA are deleted or inserted the centromere function is strongly affected (Gaudet and Fitzgerald-Hayes, 1987; Panzeri *et al.*, 1985). The substitution of CDEII by a random AT sequence of DNA of the same length does not alter the centromere function. However, the substitution of the same stretch of DNA by GC rich sequences highly increases the mitotic loss rate with the increase in chromosome instability being directly proportional to the length of the deletions or insertions, indicating that it may function as a spacer. These facts indicate that the length and the composition of CDEII are essential for proper centromere function (Cumberledge and Carbon, 1987; reviewed by Clarke, 1990).

CDE I is an octamer sequence with partial bilateral symmetry: RTCACRTG. This element is not essential but it is required for optimal centromere function; a full deletion of it results in a 20 fold increase in chromosome loss (Cumberledge and Carbon, 1987). Individual bases are required for function as in the case of the last two bases of the palindrome, whose mutation at *CEN6* increase the mitotic loss rate by one order of magnitude (Hegemann *et al.*, 1988). The left side of the consensus (CAC) appears to be the most determinant for function, substitution of its third position increases chromosome loss by 25-30 fold (Niedenthal *et al.*, 1991). Deletion of CDEI from the centromere produces a nuclease resistant core reduced in size and intensity (Bloom and Carbon, 1982).

In general the most dramatic effect is seen when mutations which destroy centromere function are introduced, in this case the nuclease resistant core is completely lost (Saunders *et al.*, 1988). The CDEI consensus sequence octamer is of particular interest because it has been found at some other locations, particularly at some promoters (Bram and Kornberg, 1987).

The discovery of this nuclease resistant chromatin core at centromeres, and its clear difference from the nucleosomal subunit, prompted the isolation of the proteins involved in preventing nucleases to access this specialised chromatin region which spans the three

CDE regions. A number of factors that bind specifically to the CDE sequences have been described.

Centromere factors

A diagram showing the components of the yeast centromere identified to date is presented in Figure 3. A protein complex of 240Kd, Cbf3, which specifically binds to the essential CDEIII element has been isolated. The binding of this factor to CDEIII sequence is dependent on additional factors and on phosphorylation of one or more of its components (Lechner and Carbon, 1991). The four known components of this complex are Cbf3A (110KDa), Cbf3B (64KDa), Cbf3C (58Kda) and Skp1 (23KDa). Mutations at the essential genes encoding these factors, *NDC10/CBF2/CTF14*, *CEP3/CBF3*, *CTF13* and *SKP1* respectively, produce aberrant DNA segregation and mitotic delay. This suggests that they are functional components of the centromere *in vivo* (Goh and Kilmartin, 1993; Jiang *et al.*, 1993; Lechner, 1994; Lechner and Carbon, 1991; Stemmann and Lechner, 1996; Strunnikov *et al.*, 1995b). Ctf13 is activated for centromere assembly by phosphorylation mediated through Skp1 (Kaplan *et al.*, 1997).

Despite the fact that chemical footprinting and analysis of chromatin have shown protection of the CDEII motif *in vivo*, suggesting the presence of bound factors, no proteins have been isolated which bind to this element (Densmore *et al.*, 1991; Funk *et al.*, 1989). However, it has been suggested that Mif2, another putative component of the yeast kinetochore and homologous to the mammalian centromere protein CENP-C (Brown, 1995) could be binding to the CDEII element. This putative binding to bend DNA and facilitate interaction between Cbf1p and Cbf3, is based on the genetic interactions among them and on the presence in Mif2 of an A-T hook motif, common to chromatin proteins which bind A+T-rich DNA (Meluh and Koshland, 1995).

Using genetic approaches a number of additional factors have been identified that may be part of the centromere/kinetochore. These include Cbf5p, which interacts genetically with *CBF14* and is able to bind to microtubules, and Kar3p, a kinesin related protein, which appears to be involved in the *in vitro* movement of kinetochores along microtubules (reviewed by Pluta *et al.*, 1995). Some of them could be part of the centromere/kinetochore not by direct binding to the centromeric DNA, but through protein-protein interactions.

	CDEI	CDEII	CDEIII
	┌──────────┐	┌──────────┐	┌──────────────────────────────────┐
<i>CEN1</i>	GTCACATG	85bp/95%AT	TGTTTTTTGTTTTCCGAAGCAGTCAAA
<i>CEN2</i>	ATCATGTG	84bp/93%AT	TGTTTTTTGTTTTCCGAAAAAGAAAAA
<i>CEN3</i>	GTCACATG	84bp/93%AT	TGTATTTGATTTCCGAAAGTTAAAAA
<i>CEN4</i>	GTCACATG	78bp/92%AT	TGTTTATGATTACCGAAACATAAAAC
<i>CEN5</i>	ATCACGTG	85bp/91%AT	AGTATTAGATTTCCGAAAAGAAAAAA
<i>CEN6</i>	ATCACGTG	85bp/94%AT	AGTTTTTTGTTTTCCGAAGATGTAAAA
<i>CEN7</i>	ATCACGTG	86bp/93%AT	TGTTTTTGCCTTCCGAAAAGAAAATA
<i>CEN8</i>	ATCACATG	85bp/93%AT	GGGTTTTGTGTTCCGAACCTTAGAAAA
<i>CEN9</i>	TTCACGTG	84bp/98%AT	TGGTTTTGTTTTCCGAAATGTTTTTT
<i>CEN10</i>	ATCACGTG	86bp/94%AT	TGTTTATGATTTCCGAACCTAAATAT
<i>CEN11</i>	GTCACATG	85bp/95%AT	TGTTTCATGATTTCCGAACGTATAAAA
<i>CEN12</i>	ATCACGTG	84bp/94%AT	TGTATTTGTTATCCGAACAATAAAAA
<i>CEN13</i>	ATCACATG	84bp/87%AT	TGTGTATGCGTTCCGAACCTTTAAATA
<i>CEN14</i>	GTCACGTG	85bp/93%AT	TGTATTTGTCTTCCGAAAAGTAAAAT
<i>CEN15</i>	ATCACGTG	86bp/91%AT	TGTATATGACTTCCGAAAAATATATA
<i>CEN16</i>	ATCACGTG	83bp/95%AT	TGGTTAAGATTTCCGAAAATAGAAAT
	↓		↓
	→ ←		→ ← ←
	RTCACRTG		TGTTTTTG-TTTCCGAAA- - -AAAAA
	12345678		1 13 26

FIGURE 2. *CEN* DNA sequences from the 16 yeast chromosomes. The three conserved DNA elements CDEI, CDEII and CDEIII are indicated. Consensus sequences are shown below, the CDEI consensus octamer is RTCACRTG (R=purine). Vertical arrows mark the axis of a 2-fold symmetry, horizontal arrows indicate palindromic sequences (data taken from Hegemann and Fleig, 1993; Fleig *et al.*, 1995).

Another factor, possibly forming part of the centromere chromatin structure is Cse4. The chromatin structure of the centromere core becomes disrupted at the restrictive temperature in *cse4^{ts}* mutants. The carboxyl terminal region of this protein has a 64% identity with the histone H3; it also has similarity with the human centromere protein CENP-A and is essential for cell division (Stoler *et al.*, 1995). This finding has raised the proposition that at centromeres a specialised nucleosome exist as the base for a higher order chromatin structure (Basrai and Hieter, 1995; Meluh *et al.*, 1998).

A CDEI binding activity was first reported in partially purified extracts (Bram and Kornberg, 1987). Subsequent studies lead to the isolation and purification of the centromere and promoter factor 1 or Cpf1 (Mellor *et al.*, 1990), which was also named as

centromere binding factor, Cbfl (Cai and Davis, 1990), and centromere protein 1, Cpl (Baker and Masison, 1990). Although the apparent multifunctional character of this protein is best described by the name Cpl, the current standard name being used is Cbfl. Cbfl binds specifically to the CDEI element, it is not essential, but, as its cognate sequence, is required for optimal centromere function. It has been suggested that one of the functions of Cbfl at the centromere is to stabilise Mif2 and that the absence of Cbfl does not have a severe effect since Mif2 can still interact, though weakly, with the Cbf3 complex (Meluh and Koshland, 1995). Cbfl has also the ability to bend DNA *in vitro*, this has led to the suggestion that this property is relevant for its own interaction with the centromere factors which bind at CDEIII (Kent, 1994; Niedenthal *et al.*, 1993). This model has been further supported by recent evidence demonstrating that all 16 *CEN* DNA possess intrinsic DNA curvature indicating that the bending of centromeric DNA is important for its function *in vivo* (Bechert *et al.*, 1999). Although *CBF1* is not an essential gene, some other mutations require Cbfl for viability as in the case of the *mif2* deletion (Meluh and Koshland, 1995) or the deletion of the *cdpl* gene (Foreman and Davis, 1996). It is not clear whether Cbfl physically interacts with the product of *cdpl*. Another group of genes synthetically lethal with $\Delta cbf1$, the *CSL* genes, contains 3 genes coding for already known components of the kinetochore, one coding for a factor involved in transcription regulation and the new *CSL4*. The genetic interaction found between *csl4-1* and *cen3* Δ CDEI suggests that Csl4 is a component of the kinetochore and/or interacts with Cbfl at centromeres as a chromatin protein, since it has similarity to chromatin proteins with Glu-rich regions (Baker *et al.*, 1998). The Cbfl factor is of particular interest because its target site CDEI has been found at a number of promoters (Bram and Kornberg, 1987), and more recently at many other locations through the yeast genome (Kent, N.A., unpublished data). Disrupting the corresponding gene causes a number of cell defects: methionine auxotrophy, a higher chromosome loss rate and more sensitivity to microtubule destabilising drugs (Cai and Davis, 1990; Mellor *et al.*, 1990). The centromere chromatin structure is also altered, the nuclease resistant core becomes reduced in size (Mellor *et al.*, 1990). Sporulation competence dramatically drops, as well as spore viability; meiotic stability is also affected (Masison and Baker, 1992). The structural characteristics of this protein have been intensely studied in relation to its function at centromeres and other binding sites.

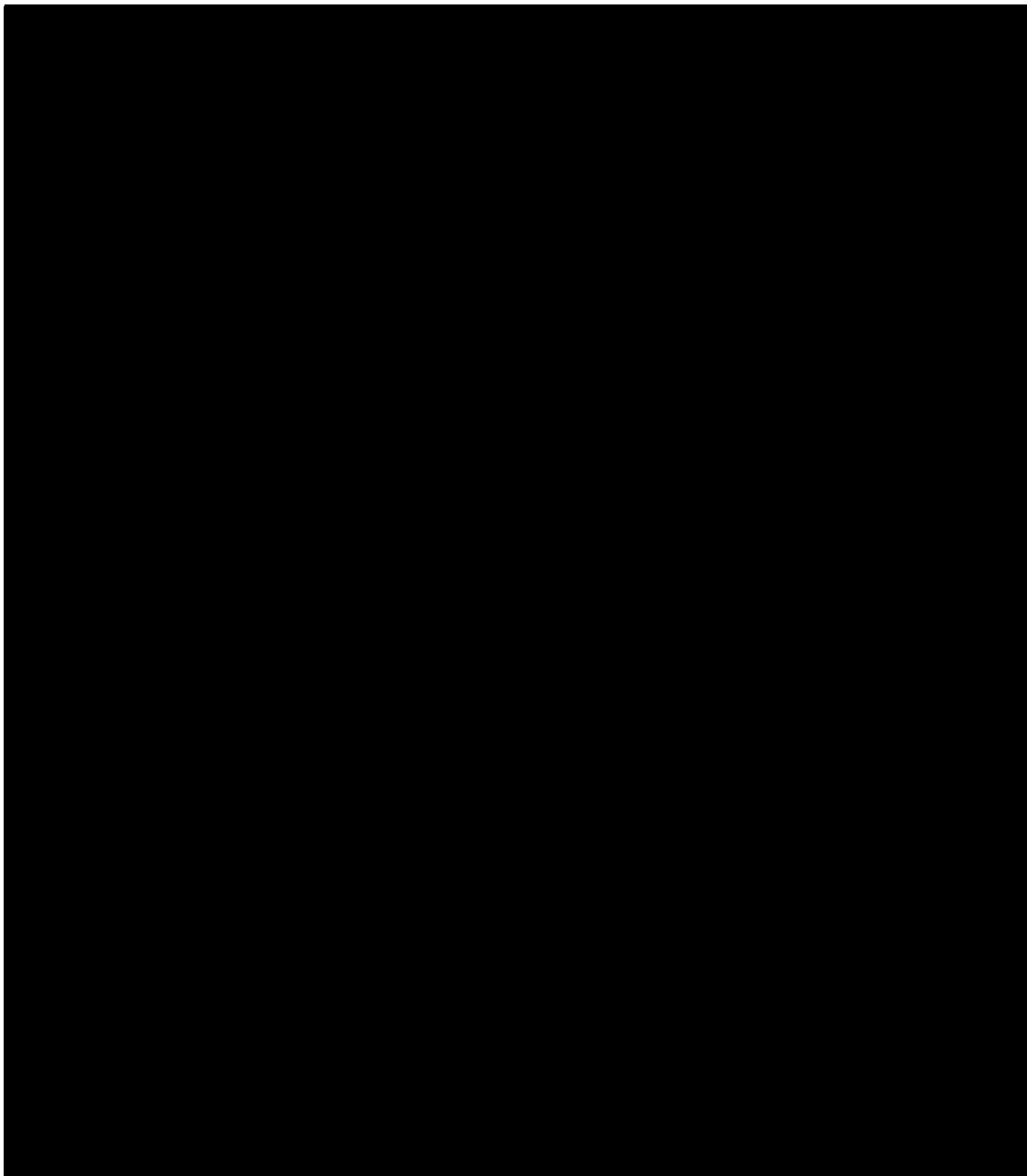


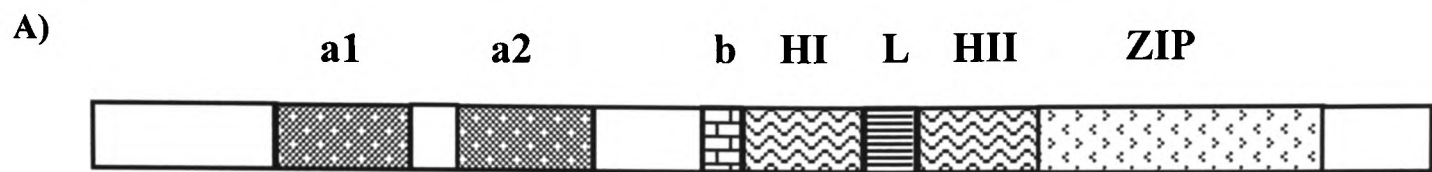
FIGURE 3. Models of the yeast centromere showing its components. **(A)** DNA elements CDEI and CDEIII are represented by boxes, CDEII is depicted as a double thin line between them. Thick line represents the DNA beyond the 125 bp of essential centromeric DNA. Cbf1p is represented as a dimer bound to its cognate sequence, it also interacts with Mif2 and Cbf3 according to genetic and biochemical evidences (Meluh and Koshland, 1995; see text for details). The protein factors of the Cbf3 complex are represented individually. The figures flanking the centromere core represent nucleosomes. **(B)** Model centered on a specialized nucleosome. The components of this hypothetical centromeric nucleosome (dark circle) are at least H4 and the H3 homolog Cse4, other histones may participate in this structure, the DNA bending and interactions among the factors are based on biochemical evidence (after Meluh *et al.*, 1998).

1.3 Structure and Functional domains of Cbflp

The centromere and promoter factor Cbflp of *Saccharomyces cerevisiae* was initially described as a protein with the ability to bind a DNA sequence motif at the upstream activation sequence of *GAL2*; this finding led to the suggestion that Cbflp was relevant for transcription regulation (Bram *et al.*, 1986). The Cbflp protein was later purified and it was estimated that there are at least 500-600 molecules per cell (Baker *et al.*, 1989; Cai and Davis, 1989; Jiang and Philippsen, 1989). The gene was localised on chromosome X and cloned by several groups which gave it various different names, *CEP1* (Baker and Masison, 1990) *CBF1* (Cai and Davis, 1990) and *CPF1* (Mellor *et al.*, 1990). The primary structure of Cbflp contains two regions rich in acidic amino acids at the amino terminal end. A distinctive structural characteristic of Cbflp, discovered when the gene was cloned, is the presence of a helix-loop-helix (HLH) domain in the deduced amino acid sequence (Mellor *et al.*, 1990). Additional domains have been detected in the primary structure of Cbflp, the basic and the ZIP domain; these three domains are described below. Figure 4 summarises the mutational analysis of such domains.

The HLH dimerization domain and the basic DNA binding domain

The HLH motif was discovered during an analysis of sequences in search for similarities to the E12 and E47 E2 box binding factors; these two factors were shown to share an homologous region with myc and MyoD proteins (Murre *et al.*, 1989a). More detailed analysis of the motif led to the finding that this region could potentially form two amphipathic α -helices with conserved hydrophobic residues on one side of each helix and these, Helix I and Helix II, separated by a flexible loop. The role of this domain has been confirmed by mutational analysis; dimerization was prevented when the conserved hydrophobic residues were mutated and when prolines were inserted into the putative helices. The crystal structures of various bHLH proteins, complexed with their cognate DNA fragments, have already been solved. The same topology of this domain is found among the bHLH factors MyoD (Ma *et al.*, 1994), E47 (Ellenberger *et al.*, 1994) and PHO4 (Shimizu *et al.*, 1997), and the bHLH/ZIP factors (see below) Max and USF (Ferre-D'Amare *et al.*, 1994; Ferre-D'Amare *et al.*, 1993). The HLH domain functions as a protein-protein interaction interface in such way that an HLH protein can interact with an identical partner to form homodimers or with a different protein bearing a compatible HLH domain to form heterodimers. A basic conserved region, required for DNA binding, is located contiguous to Helix I (Davis *et al.*, 1990).



B)

Mutation	Region	Phenotypes			
		In vivo		In vitro	
		Centromere	Methionine	DNA binding	Dim
Δ309(221-237)	Basic-HI	-	-	-	+
Δ324(243-260)	HI-L-HII	-	-	-	+
Δ317(302-343)	ZIP	-	-	-	-
K224A	Basic	+	+	+	/
S226A	Basic	+	+	+	+
S226E	Basic	+	+	+	+
H227N	Basic	+/-	+	+/-	/
E231A	Basic	-	+	-	/
E231G	Basic	-	-	-	/
E231V	Basic	-	-	-	/
R232A	Basic	+	+	+	/
R235H	HI	+	+	+	/
I238T	HI	+	+	+	/
I242T	HI	+	+	+	/
L245T	HI	+/-	-	+/-	/
L245S	HI	-	-	-	/
L249P	HI	-	-	-	/
P250F	HI	+	+	+	/
S255A	L	+	+	+	/
K256E	HII	-	-	-	/
259>PREG<260	HII	-	-	-	/
A263T	HII	+	+	+	/
L270T	HII	+	+	+	/
Q284P	ZIP	-	ts-	+/-	/
S293P	ZIP	+	-	+	/
N299A	ZIP	/	/	+	+/-
N299E	ZIP	/	/	+	+/-

FIGURE 4. Mutational Analysis of Cbflp. **A)** The diagram indicates the position of the structural and functional domains detected in the primary structure of Cbflp; a, acidic; b, basic. **B)** The table summarises the mutations introduced so far with their effects on function. For the point mutations the wild type residue, its position and the residue replacing it are given. For deletions the positions of residues excised are given. For insertions the residues inserted are indicated between >< signs. Dim column indicates the ability of mutant proteins to form dimers as measured by dimethylsuberimidate cross-linking assays. Data were taken from (Mellor *et al.*, 1991; McKenzie, *et al.*, 1993; Foreman and Davies 1993; Masison *et al.*, 1993; Nissom, 1998).

When two HLH proteins dimerize, the interaction between the hydrophobic faces of their helices is the basis for the formation of the dimer as a four helix bundle (see figure 5). The basic regions then lie in position to interact, each with half of the CANNTG palindrome DNA motifs on the major groove (Prendergast and Ziff, 1991). Thus the bHLH factors have the potential to form multiple kinds of dimers with a variety of DNA binding specificities, affinities and functional properties, providing the basis for fine tuned and complex regulatory networks (Jones, 1990).

There are various systems in which the bHLH factors play a key role in the differentiation process of cell lineages. For instance, in the fly neurogenesis the tissue specific bHLH factors *achaete* and *scute*, required for the formation of the large sensory bristles on the thorax, form DNA binding heterodimers with the ubiquitous bHLH *daughterless* (*da*) to function as transcription positive regulators. It has been suggested that these positive regulators can also form dimers with the *extramacrochaete* (*emc*) gene product which is a HLH factor lacking a basic domain and therefore acting as a negative regulator. The distribution of neural precursors is then refined by the expression pattern of *achaete*, *scute* and *emc* (Cubas and Modolell, 1992); additional factors in this system have been recently described, as the case of the hairy-related bHLH repressors (reviewed in (Fisher and Caudy, 1998). A similar system controls the differentiation of muscle cells; in this case some of the factors involved, MyoD, E12 and Id can combine to form various dimers. MyoD/E12 heterodimers, which bind to the creatine phosphokinase (MCK) enhancer with a ten fold *in vitro* DNA binding affinity and a different size preference compared to the MyoD homodimer (Murre *et al.*, 1989b; Voronova and Baltimore, 1990). Two types of heterodimers, E12/Id and MyoD/Id are unable to bind DNA due to the absence of a basic binding domain in Id (reviewed in Muscat *et al.*, 1995). The differentiation of muscle cells requires a decrease in the expression of Id to allow E12 and MyoD to interact with each other and activate transcription at the relevant genes (Weintraub *et al.*, 1991) Some other HLH proteins participate in the control of growth (reviewed by Murre *et al.*, 1994), among them the myc proteins and their interacting partners Mad and Max. An additional system controls the development of the vertebrate retina with another system of bHLH proteins (reviewed by Cepko, 1999). The N terminal domain and to some extent the bHLH domain of the Twist factor, have been implicated in the recruitment and regulation of the histone acetyl transferase activities of p300 and PCAF; the N terminal domain is also required for the inhibition of the p300 dependent transcription (Hamamori *et al.*, 1999a). A common feature of the bHLH proteins is their

ability to bind to the E box sequence CANNTG at enhancers and promoters (Baxeavanis and Vinson, 1993). Cbflp binds to this kind of sequence at centromeres and other locations through the yeast genome. An example of the bHLH fold is shown in Figure 5.

The bHLH domain of Cbflp has been subjected to mutagenesis and deletion analysis. The deleted molecules were tested *in vitro* for their ability to form homodimers in solution by dimethylsuberimidate crosslinking and to bind to a CDEI oligonucleotide. These molecules were also introduced and expressed in yeast by gene replacement to test the methionine and centromere related phenotypes. Deletion of the basic domain ($\Delta 309$) abolished *in vitro* DNA binding and *in vivo* centromere function, it retains the ability to form homodimers *in vitro* but produces a Met⁻ phenotype. Deletion of a region encompassing portion of Helix I, the loop and a portion of Helix II ($\Delta 324$) abolishes centromere function and methionine prototrophy; it retains the ability to dimerize in solution. The dimerization of molecule $\Delta 324$ involves a C-terminal region revealed as a potential leucine zipper or ZIP domain by sequence comparison and deletion analysis (Dowell *et al.*, 1992). The relevance of the basic region for Cbflp function has been demonstrated by domain swap experiments as well by point mutations. The replacement of the Cbflp basic region by the basic region of USF or c-Myc, which are able to bind the CACGTG sequence restore methionine prototrophy; substitution by the Ap4 basic region, which binds to the CAGCTG box, does not restore methionine prototrophy (Dang *et al.*, 1992).

The leucine zipper dimerization domain

As early as 1953 a structure for the interaction between two proteins was predicted to exist in the form of interdigitating "knobs into holes" at the hydrophobic interface of two proteins (Crick 1953; Pauling and Corey 1953). The first structure of this kind to be identified was that found in the CCAAT/Enhancer Binding protein C/EBP. It is also present in many other DNA binding proteins, among them c-, n- and l-Myc, Fos, Jun and Gcn4, a yeast transcription regulator (Landschulz *et al.*, 1988). Figure 6 shows the main features of this domain. A characteristic periodic heptad (a b c d e f g)_n along an α -helix in which positions a and d are predominantly occupied by hydrophobic and apolar residues, whereas charged and polar residues are predominant at the other positions (O'Shea *et al.*, 1991). Conserved leucine residues are found frequently, but not always, at d position (Alber 1992; Ellenberger *et al.*, 1994). Residues at positions a and d provide a hydrophobic interface for interaction between two proteins. The frequently charged residues at positions

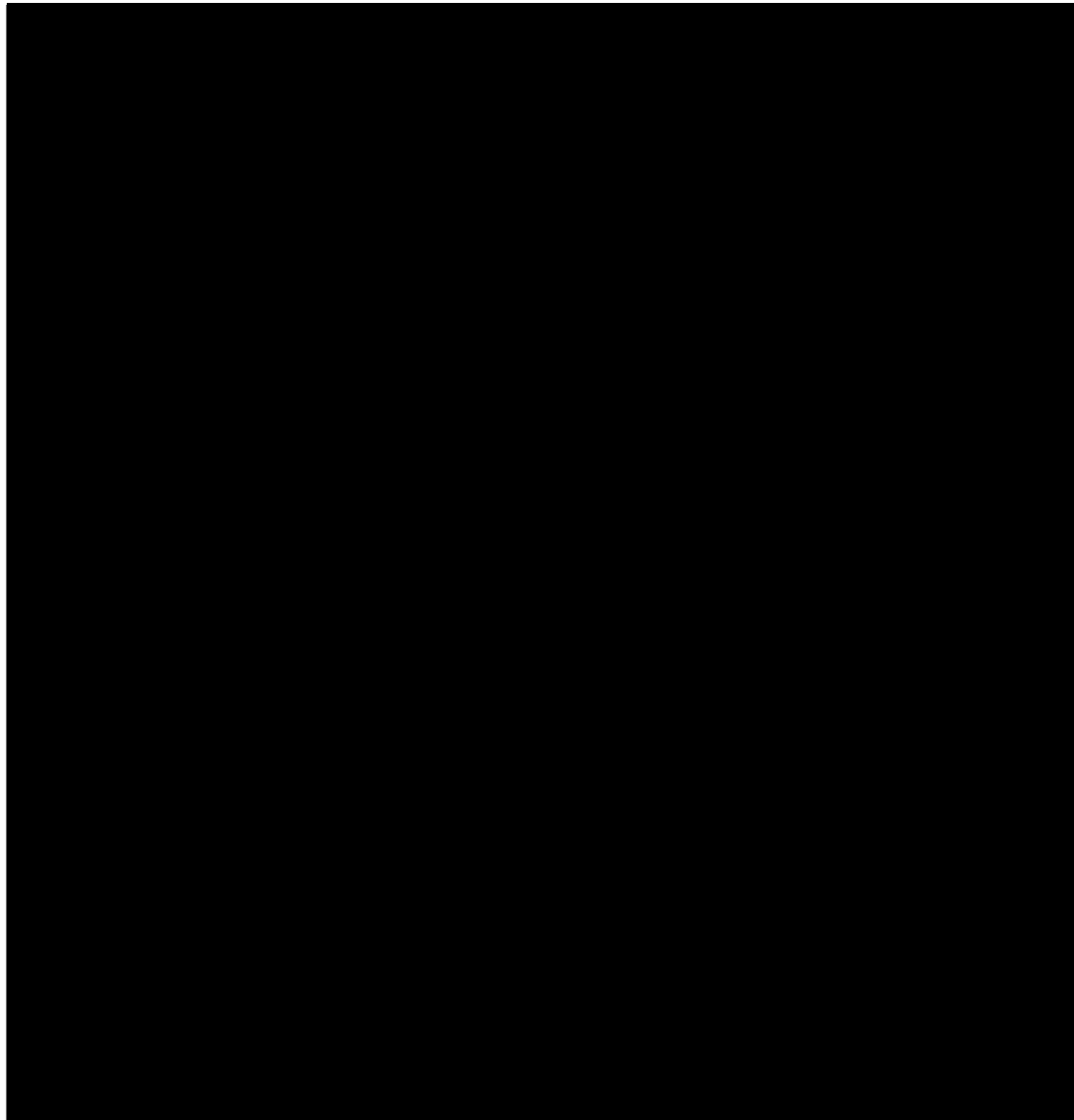


FIGURE 5. Diagram of the MyoD-DNA complex. The α -helices of the bHLH domain of MyoD (residues 105-166) are represented as cylinders, **b** is the basic DNA binding domain which interacts with the major groove of the double helix, **HI** and **HII** are helices I and II respectively. Two identical peptides interact to form the DNA binding homodimer which folds into a parallel, left-handed four helix bundle (diagram modified after Ma *et al.*, 1994).

e and g form intrahelical or interhelical salt bridges which protect such interface from solvent and add stability to the dimer (Cohen and Parry, 1990; Hu *et al.*, 1993). The structure of the ZIP domain (Vinson *et al.*, 1989) has been confirmed to be that of a short coiled coil, formed by two α -helices slightly curved over each other as revealed by the crystal structure of the Gcn4 ZIP domain (Ellenberger *et al.*, 1992). As in the case of the HLH domain the ZIP domain also allows proteins to form homo and heterodimers. The best known system is that of the oncoproteins Fos and Jun. The three dimensional structure of crystals containing the Fos-Jun heterodimer, the NFAT factor and cognate DNA has shown that the association of the three proteins on the DNA creates a continuous groove for the recognition of 15 base pairs (Chen *et al.*, 1998; reviewed by Chen, 1999). In some of these proteins an additional basic DNA binding motif participates in their function and they are known as the "bZIP" factors (Vinson *et al.*, 1989). In yeast a large family of bZIP proteins, which belong to the family of AP-1 (Fos-Jun) transcription factors, related to the YAP1, involved in the oxidative stress response has been revealed by the genome project (Toone and Jones, 1998; reviewed by Toone and Jones, 1999).

A putative ZIP domain is located at the carboxyl terminal end of Cbflp, contiguous to the bHLH domain. A mutant bearing a deletion of this region ($\Delta 317$) leads to the loss of the ability of Cbflp to dimerize and to bind to the CDEI DNA; the corresponding phenotype of this mutant is methionine auxotrophy and loss of the centromere function (Dowell *et al.*, 1992). Figure 6 shows this deleted region plotted as two hypothetical ZIP-like α -helices with the potential salt bridges. The putative ZIP domain of Cbflp can be replaced with that from the mammalian transcription factor USF (Gregor *et al.*, 1990). A fully functional Cbflp-USF fusion protein is then able to bind DNA and to restore the centromere function and methionine prototrophy; it has also been successfully used to produce homodimers in the two hybrid assay (Dowell *et al.*, 1992). More recent studies have provided additional evidence for a coiled coil ZIP domain at the C-terminal region of Cbflp. Circular dichroism spectroscopy reveals an α -helical secondary structure at this region (Nissom, 1998). It has also been found that this region satisfies many of the characteristics of a ZIP domain (according to Hu *et al.*, 1990), including at least two leucines at position d. Several ZIP domains have only two leucine residues, as in the case of CPC1 and TGA1 (O'Shea *et al.*, 1991). When these two residues are mutated to methionine in Cbflp, its ability to bind DNA decreases significantly (Dowell *et al.*, 1992). When this region is synthesised *in vitro* it has the ability to oligomerize on its own and it

has therefore been suggested that Cbflp could be forming oligomers with other ZIP proteins *in vivo* (Nissom, 1998). Interactions of Cbflp with other ZIP proteins have also been reported (See section 1.4).

The bHLH-ZIP protein family

A number of bHLH factors have leucine zippers carboxyl-terminal and contiguous to their helix-loop-helix domains; they constitute the family of the basic-helix-loop-helix-leucine zipper proteins (bHLH/ZIP). Although the actual three dimensional protein structure of Cbflp is unknown the evidence described above indicates that it belongs to the bHLH-ZIP family. This family of proteins includes Max (Blackwood and Eisenman, 1991), Mad (Ayer *et al.*, 1993), Mxi (Zervos *et al.*, 1993), AP4 (Hu *et al.*, 1990), TFE-3 (Beckmann *et al.*, 1990), TFEB (Carr and Sharp, 1990), USF (Gregor *et al.*, 1990), Rox (Meroni *et al.*, 1997) and the Myc proteins. Analysis of the role of the constituent parts of this class of proteins has revealed that the leucine zipper region of c-Myc can be replaced with that of GCN4, resulting in a chimera able to dimerize and bind DNA. This suggested that the ZIP region in this class of proteins is compatible with a coiled-coil structure, as in bZIP proteins (Davis and Halazonetis, 1993). The three dimensional structure of Max and USF is known, in these structures the ZIP domain prolongates from the Helix II of the bHLH domain to form a continuous α -helix (Ferre-D'Amare *et al.*, 1993; Ferre-D'Amare *et al.*, 1994).

A fusion protein containing the HLH region of TF3E and the ZIP region of USF forms dimers. Since the native TF3E is unable to form heterodimers, the specificity of the protein-protein interactions during the formation of dimers appears to be regulated by the leucine zipper and its position relative to the helix-loop-helix bundle (Beckmann and Kadesch, 1991; Baxeavanis and Vinson, 1993). As in the case of bHLH and bZIP proteins, the members of the bHLH-ZIP also participate in the combinatorial regulation of transcriptional activity. For instance the Max factor is able to form heterodimers with the Myc proteins which can then strongly bind DNA (Prendergast and Ziff, 1991), Max can form additional heterodimers with Mad and with Mxi to repress transcription (Ayer *et al.*, 1995). These heterodimers are also formed *in vivo* and participate in the regulation of cell proliferation (Facchini and Penn, 1998).

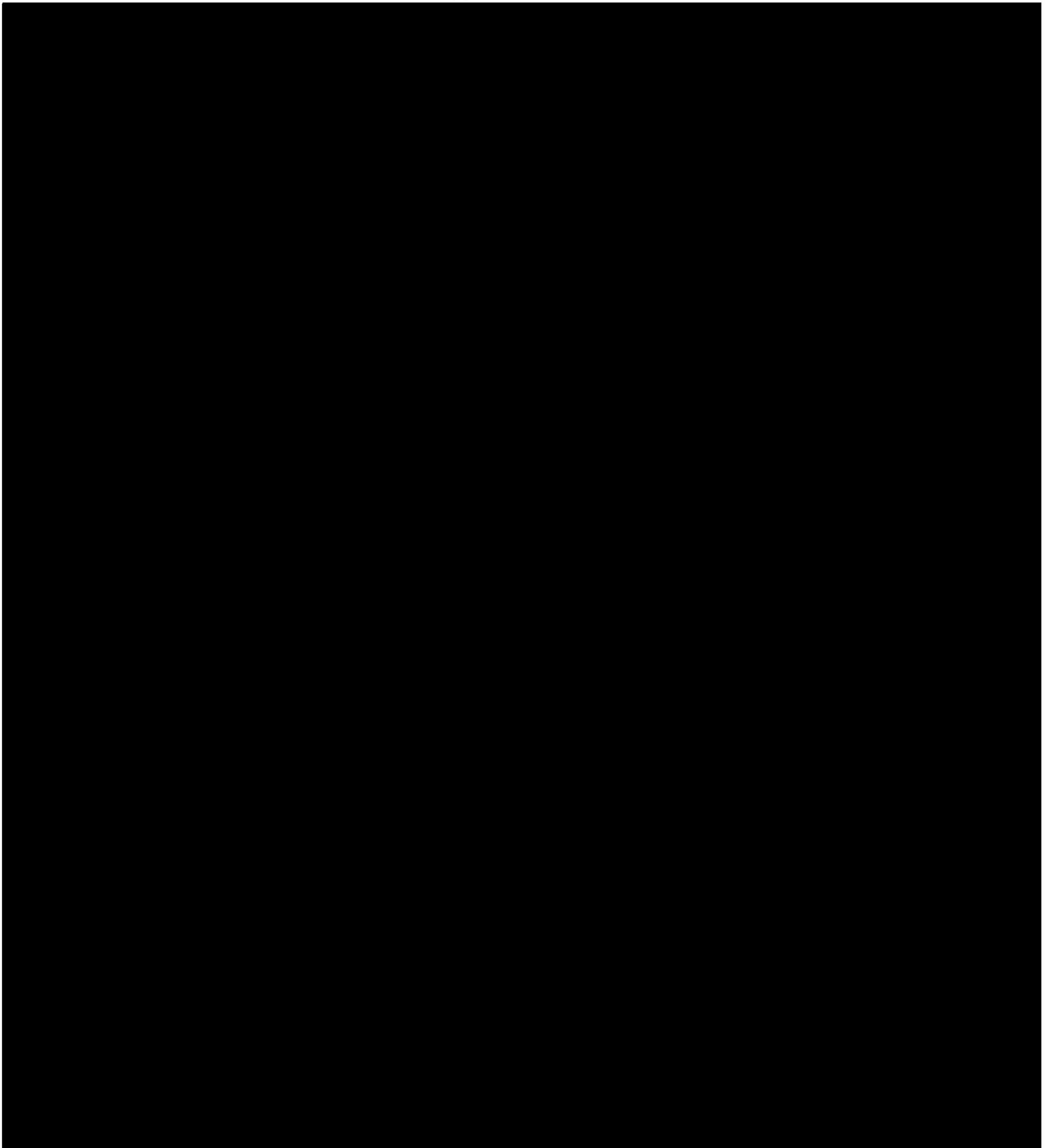


FIGURE 6. The ZIP domain. **A.** Residues 292-320 of the putative ZIP domain of Cbflp depicted as a pair of 3-4 helical plots. The helical wheel diagrams show the juxtaposition of residues **a** and **d** from one helix against the residues **a'** and **d'** of the other in the coiled coil structure. The potential stabilizing salt bridges between residues at positions **e** and **g'** and **e'** and **g** are indicated by dashed lines (Dowell *et al.*, 1992). **B.** Structure of the bHLH/ZIP protein Max (Ferre-D'Amare *et al.*, 1993).

1.4 Cbflp and Regulation of the Methionine Biosynthesis Pathway

The target site to which Cbflp binds, the CDEI conserved octanucleotide, is also found at the promoter regions of genes involved in the methionine biosynthetic pathway: *MET17* (non-standard name *MET25*), *MET16*, *MET2*, *MET14*, *MET8*, *MET3* and *SAM2* (Thomas *et al.*, 1989), *MET5*, *MET6*, *MET10* and various other genes such as *TRP1* (Bram and Kornberg, 1987), *QCR8* (Mulder *et al.*, 1994) and *CYT1* (Oechsner and Bandlow, 1996). Figure 7 shows that the proteins encoded by these *MET* genes belong to the biosynthesis pathway for cysteine and methionine (reviewed by Thomas and Surdin-Kerjan, 1997).

Biosynthesis of methionine is essential for cells since it is the first amino acid incorporated during the synthesis of proteins. Another essential product of this pathway is S-adenosyl-methionine (AdoMet), which is the methyl group donor in most of the transmethylation reactions in the cell. Although addition of methionine or AdoMet to the growth medium has long been known to repress the synthesis of enzymes required for methionine biosynthesis (Cherest *et al.*, 1969), the intracellular concentration of AdoMet is in fact the signal for repression (Thomas *et al.*, 1988).

Some of the genes in this pathway are regulated by the general control of amino acid biosynthesis; in this mechanism the absence of one of various amino acids in the growth medium results in the increased expression of many genes encoding proteins for different amino acid biosynthesis pathways. This mechanism functions through the presence, at the upstream activation sequence (UAS) of all those genes, of the binding site for Gcn4. Among *MET* genes only *MET4*, *MET5*, *MET6* and *MET16* contain such sequence and can potentially be regulated by this system (Mountain *et al.*, 1991; Mountain *et al.*, 1993; (O'Connell *et al.*, 1995). In the case of *MET16* the absence of regulation by the GCN4 mechanism has also been reported (Thomas *et al.*, 1990). In contrast, as shown in Figure 8, most *MET* genes present at least one copy of the CDEI sequence in their UAS's, with the exception of *SAM1*. The ubiquity of this sequence among this group of genes supported its relevance in the regulatory function.

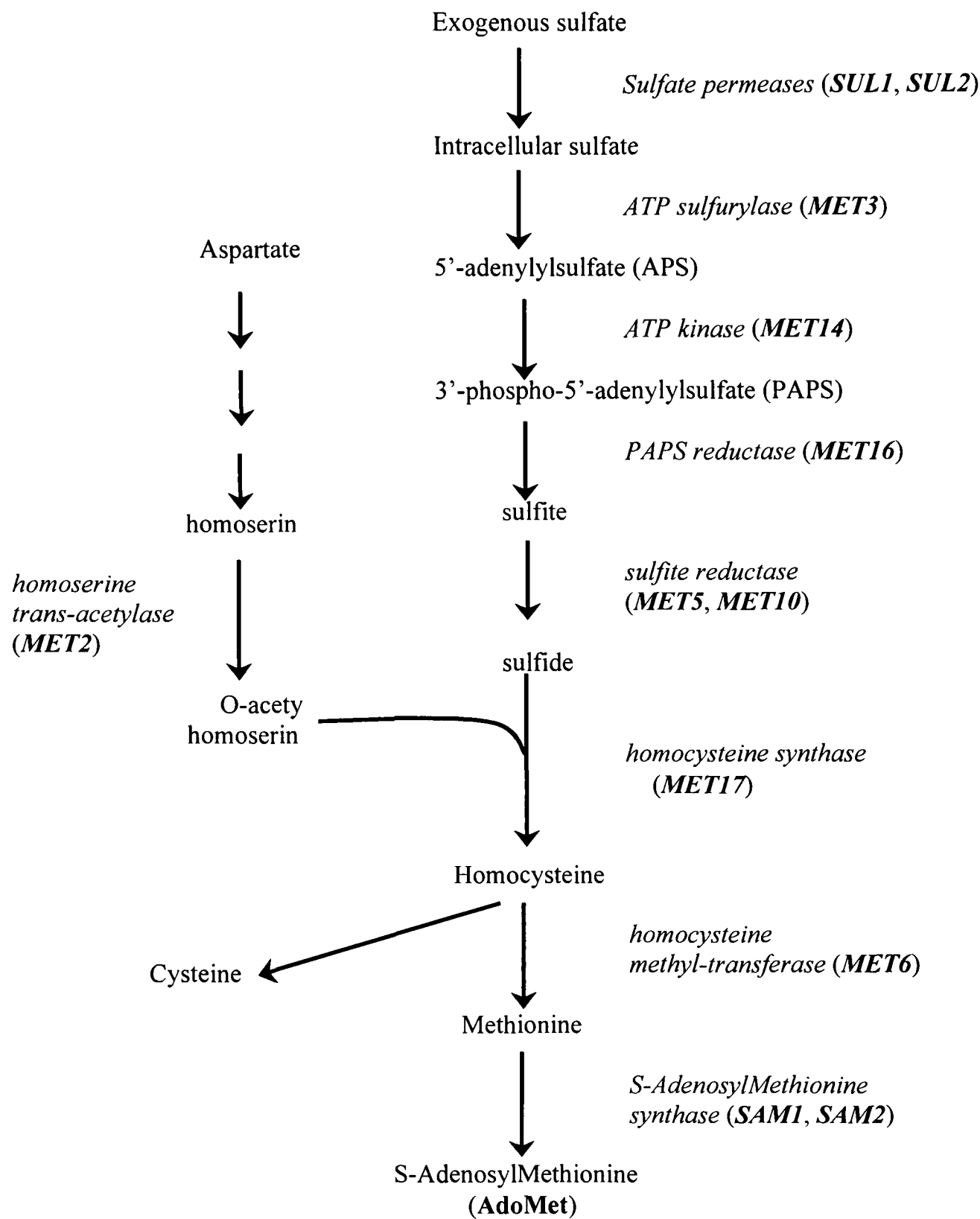


FIGURE 7. The Methionine Biosynthesis Pathway in *S. cerevisiae*. Genes encoding the various enzymes are indicated in parenthesis. This pathway produces two essential molecules; methionine, the first residue in protein synthesis and AdoMet, the principal donor of methyl groups in trans-acetylase reactions (reviewed by Thomas and Surdin-Kerjan, 1997).

CDEI at the regulatory regions of *MET* loci

Deletion analysis of the upstream region of *MET17*, the gene encoding O-acetylhomoserine sulfhydrylase, lead to the identification of DNA sequences which specifically regulate *MET* genes (Thomas *et al.*, 1989). One of the functional sequences is centered on two CACGTG (CDEI) palindromes and is required for derepression of *MET* genes under low intracellular AdoMet concentrations. Additional support for the involvement of the CDEI sequences in regulation was provided by the proven ability of CDEI oligonucleotides, cloned into mutants with deletions at the promoter region, to restore *MET17* transcription. The TCACGTG sequence however is not sufficient to activate transcription; for instance A CDEI motif inserted in a UAS does not activate transcription in *CBF1* cells (Buchman and Kornberg, 1990). Only the addition of the 9 5' nucleotides adjacent to it at *MET17* (oligonucleotide from positions -310 to -294) enabled transcription of a reporter gene and made it responsive to AdoMet concentration (Thomas *et al.*, 1992). Although an 18 bp sequence of *MET17*, containing the CDEI site, is able to activate transcription of the reporter gene *lacZ*, this activation seems to be Cbflp independent, since the reporter gene is expressed in *cbfl* cells (Kent *et al.*, 1994).

Interacting partners of Cbflp at *MET* promoters

Various lines of evidence suggested the possible involvement of Cbflp in the transcription activation of *MET* genes. Some of the structural features of Cbflp are common to activators of transcription: the DNA binding motif, the dimerisation domain (bHLH/ZIP, see section 1.3) and, as in some activation domains, the presence of two acidic regions on the N-terminal portion of the protein. This Cbflp structure together with the identification of its cognate sequence CDEI as an element capable to restore transcription of *MET17* from deleted *MET17* (Thomas *et al.*, 1992), all supported the hypothesis of its role as an activator of transcription. A genetic interaction recently found between *CBF1* and the *RTF1* gene, which codes for a factor acting at promoters during the transcription site selection by RNA polymerase II (Stolinski *et al.*, 1997), has also suggested a direct involvement in transcriptional activation (Baker *et al.*, 1998). However, direct evidence does not support this view, Cbflp seems to interact with activators of transcription, but it is not a transcription factor.

<i>MET2</i>	-550	aatCACGTGat	a	-539
	-356	tttCACGTGat	g	-345
<i>MET3</i>	-380	ggtCACGTGtaa		-369
	-363	agtCACGTGtaa		-352
<i>MET8</i>	-212	tttCACGTGtta		-201
<i>MET10</i>	-254	aagCACGTGagc		-243
	-328	ggtCACGTGatc		-317
<i>MET14</i>	-231	tttCACGTGatc		-220
<i>MET16</i>	-181	tttCACGTGgct		-170
<i>MET17</i>	-328	ggtCACATGatc		-317
	-303	tggCACGTGaag		-292
<i>SAM2</i>	-380	cttCACATGtga		-369

FIGURE 8. The CDEI motif among methionine biosynthesis pathway genes. The octanucleotide forming the consensus TCACGTGA is enclosed in a box. The core palindrome is indicated in uppercase letters (data compiled from Kent, 1994; Kuras and Thomas, 1995).

Although it has been reported that in *cbfl* strains the levels of *MET17* mRNA are reduced by one third and those for *MET16* are undetectable (Kuras and Thomas, 1995; Thomas *et al.*, 1992), Cbflp has failed to specifically activate transcription in a number of experiments. In fact it has not been possible to reproduce elsewhere the effect of Cbflp on the transcriptional activation of these two genes. For instance it has been shown that the *MET17* mRNA levels are not affected under repressing or derepressing conditions in cells lacking Cbflp and under some conditions the levels actually increase in those cells (Mellor *et al.*, 1991). The analysis using RNase protection assays also demonstrates that there are no differences in the expression levels of those two genes between *cbfl* and *CBF1* cells, either under repressing or derepressing conditions (Kent *et al.*, 1994). Moreover, a LexA-Cbflp fusion protein expressed in yeast was unable to activate the expression of the reporter gene *lacZ*, downstream of a *GALI* promoter with LexA binding sites (Thomas *et al.*, 1992). These evidence indicated that Cbflp is not a transcription factor and that other proteins are required for activation of transcription at CDEI containing *MET* genes. In fact, the only factor known to be absolutely required to activate transcription at all *MET* genes is Met4. This protein is a bZIP factor which, in contrast to Cbflp, possesses intrinsic transcription activator properties (Kuras *et al.*, 1996). It has been reported to be part of a

complex together with Cbflp and an additional bZIP factor, Met28, formed on the promoter region of *MET16*. Using the two hybrid system (Fields and Song, 1989), physical interactions between Cbflp and Met4 were detected, suggesting that the bHLH/ZIP motif of Cbflp interacts with the bZIP motif of Met4, but the formation of heterodimers has not been demonstrated (Kuras *et al.*, 1996). *In vitro* studies with protein fragments had shown that the Cbflp DNA binding activity over the *MET16* CDEI site is stimulated in the presence of Met28, but failed to demonstrate the presence of this protein in the Cbflp-DNA complexes formed during the band shift assays (Kuras *et al.*, 1997).

It has also been suggested that Cbflp participates in regulation of transcription at other locations. The promoter of *QCR8* gene (which codes for the subunit VIII of the mitochondrial ubiquinol-cytochrome c oxidoreductase) has a binding site for the Abfl factor, within this site the CDEI sequence is contained. It was considered that Cbflp and Abfl compete for this site in order to couple mitochondrial biogenesis with cell growth, since Cbflp had a weak repressive effect (de Winde and Grivell, 1992). However the activation of transcription from the *TRP1* promoter is not affected in *cbfl* cells (Mellor *et al.*, 1991). Recent studies indicate that Cbflp may be interacting with other factors of the transcription machinery. A *rtfl* allele, coding for the Rtf1 factor which appears to act at gene promoters during the process of transcription initiation site selection by RNA polymerase II (Stolinski *et al.*, 1997), is synthetically lethal with *cbfl* (Baker *et al.*, 1998). Although Cbflp participates in the assembly of protein complexes formed on CDEI sites, the direct involvement of Cbflp during transcriptional activation *in vivo*, remains controversial. The lack of evidence to demonstrate a direct involvement of Cbflp in transcriptional activation lead to the hypothesis that this factor could be acting at the level of chromatin organisation (Masison *et al.*, 1993; O'Connell and Baker, 1992; Thomas *et al.*, 1992). It has been shown that Cbflp is indeed a chromatin modulator (Kent, 1994; Kent *et al.*, 1994).

1.5 Cbflp and Regulation of Chromatin Structure

The tight association of DNA with the histone octamer and its further compaction into the higher order chromatin fibers are known to constitute repressive states of transcriptional activity (reviewed by Armstrong and Emerson, 1998). Multiple protein factors are required for transcription activation: RNA polymerase II and the general transcription factors which constitute the holoenzyme, sequence specific DNA binding proteins and non-DNA binding adaptors. This complicated apparatus has to gain access to

the promoters and enhancers deeply entangled in nucleosomes to initiate transcription and produce proper amounts of messenger RNAs. The direct involvement of nucleosomal proteins and structure in regulating transcription began to be appreciated when the possibility to manipulate the expression and to produce mutant versions of the core histones became available. When the dosage of H4 was altered in yeast, disrupting nucleosomal chromatin, it caused transcription from a number of promoters (*CUP1*, *GAL1*, *HIS3*, *PHO5*) under repressing conditions for them (Durrin *et al.*, 1992; Han and Grunstein, 1988; Kim *et al.*, 1988); reviewed by Gregory and Horz, 1998). Nucleosomes, however, do not always prevent activation; a nucleosome mediated cooperativity between the nuclear factor 1 and the glucocorticoid receptor has been suggested for the mammary tumor virus promoter due to the lack of synergism among them when H4 is depleted (Chavez and Beato, 1997). Repression and activation become disrupted by mutations in H4 residues relevant for its interaction with H2A-H2B heterodimers (Santisteban *et al.*, 1997). Recent evidence shows that the N-terminal tails of H3 and H4 are required for gene repression mediated by $\alpha 1$ - $\alpha 2$ in yeast (Huang *et al.*, 1997). This evidence indicates that the nucleosomal structure is relevant for both repression and derepression. Various types of modifications affecting histone-histone and histone-DNA interactions have been found *in vitro* and *in vivo*. From the loss of H2A/H2B dimers (Baer and Rhodes, 1983; Hansen and Wolffe, 1994), to the accessibility of the H3 thiol, thought to be due to unfolding of nucleosomes (Bazett-Jones *et al.*, 1996), these alterations are considered to be required for factors, involved in transcription and other DNA transactions, to access DNA sequences (reviewed by Workman and Kingston, 1998). There are various mechanisms to regulate the repression caused by the nucleosomal organisation; among them are two highly conserved events, the postranslational reversible modification of chromatin components, particularly acetylation of histones, and the remodelling of chromatin.

Chromatin modulation at specific sites

One of the best characterised systems involving chromatin transitions upon gene expression is the yeast *PHO5* gene, which codes for an acid phosphatase. The expression of this gene is controlled by phosphate content, it remains repressed in the presence of high concentrations of phosphate; under low phosphate conditions it becomes highly expressed. The bHLH transcription factor Pho4 can bind to two E-box sites in the UAS region of *PHO5*. Under repressing conditions there are 4 nucleosomes positioned over the promoter region of *PHO5* and although one of the E-box binding sites is not occluded by the nucleosomes, Pho4 does not bind to these sites (Almer and Horz, 1986). When cells are

transferred to low phosphate conditions the activation domain of Pho4 becomes liberated from its interaction with the Pho80-Pho85 cyclin-cdk complex and interacts with the homeobox protein Pho2. The Pho4-Pho2 factor then binds cooperatively to the *PHO5* promoter, activates transcription and triggers a chromatin transition of the nucleosomes in which the entire promoter becomes nuclease sensitive and Pho4 binds to its two cognate sites (Barbaric *et al.*, 1996). This chromatin transition is dependent upon the activation domain of Pho4 and does not require either transcription from the *PHO5* TATA box or DNA replication (Fascher *et al.*, 1993; Schmid *et al.*, 1992). A recent mutagenesis study of the activation domain of Pho4 failed to identify mutations which could show chromatin modulation and transcriptional activation as separable events, suggesting the possibility that the activation domain interacts with a single factor capable of both, transcriptional activation and chromatin modulation (McAndrew *et al.*, 1998). The activity of Pho4 is regulated by phosphorylation of one of its serine residues by the Pho80-Pho85 complex (Kaffman *et al.*, 1994).

Acetylation and Deacetylation

The first histone modification known to be relevant for transcription was suggested early in the study of chromatin, Vincent Allfrey and collaborators demonstrated a correlation between the synthesis of RNA and acetylation of chromatin (Allfrey *et al.*, 1964). Later a direct link between acetylation of core histones and transcriptionally active chromatin was established (Hebbes *et al.*, 1988). Although hypoacetylated states of core histones have also been associated with transcriptional activity, it is clear that the extent of histone acetylation is central to the structure and regulation of DNA accessibility in chromatin (reviewed by Davie, 1997; Davie, 1998). There are two types of histone acetyl transferases, HAT As acetylate nuclear histones and HAT Bs acetylate newly synthesized H4 in the cytoplasm (Brownell and Allis, 1996). The first histone acetyl transferase (HAT) was isolated from *Tetrahymena* and this HAT A is homologous to the yeast transcriptional adaptor Gcn5, which also possess HAT activity (Brownell *et al.*, 1996). The assay to test acetyl transferase activity soon lead to the identification of various other transcriptional coactivators as HAT's, most of them able to acetylate nucleosomal core histones and in some cases also other protein components of the transcriptional machinery (Table 1).

Protein	Activity	Substrates	Organism
Gcn5	HATA	H3, H4, H2B*	Yeast, Human
SAGA	HATA	H3, H2B	Yeast
Gcn5p/Ada3	HATA	H4, Sin1p	Yeast
p55	HATA	H3, H4, H2B*	<i>Tetrahymena</i>
yTAF _{II} 250	HATA	Unknown	Yeast
dTAF _{II} 250	HATA	H3, H4	<i>Drosophila</i>
hTAF _{II} 250	HATA	H3, H4*, TFIIE β	Human
CBP/p300	HATA	4 core, TFIIE β ,	Human
P/CAF	HATA	TFIIF, p53	Human, <i>Drosophila</i>
ACTR	HATA	H3	Human
SRC-1	HATA	H3, H4	Human
Es1p	HAT	H3, H4	Yeast
Tip60	HATA	H2A*, H3*, H4*	Human
Hat1p	HATB	H2A, H3, H4	Yeast
		H4*	
Rpd3p	HDAC	4 core histones	Yeast
mRpd3	HDAC	Unknown	Human
Hda1p	HDAC	4 core histones	Yeast
HDAC1	HDAC	Unknown	Human

TABLE 1. Proteins which modify the acetylation state of histones and transcription factors. These can have histone acetyl transferase activity (HAT) or histone deacetylase activity (HDAC). Free histone substrates are indicated by an asterisk.

Histone deacetylation has also been linked with transcriptional regulation, the mammalian histone deacetylase (HDAC) is related to the yeast transcriptional regulator Rpd3 (Taunton *et al.*, 1996). Several other HDAC complexes have also been described (Table 1). The current model for the role of HDAC's in the regulation of transcription is that they deacetylate nucleosomal core histones leading to a compaction of chromatin which prevents the access of transcriptional activators to their cognate sequences (Wolffe, 1997). The role of HAT's and HDAC's can be summarised as components of a system of various complexes which regulate the accessibility of other factors to the DNA template. A dynamic process of acetylation/deacetylation of histones and other chromatin components leads to cycles of local condensation/decondensation of chromatin. Although most of the studies on acetylation-deacetylation, and their effects on chromatin structure and transcription have been done *in vitro*, the overexpression of Gcn5 leads to histone

hyperacetylation. The expression of mutants with very low efficiency as transcriptional activators fail to produce such histone hyperacetylation (Kuo *et al.*, 1998; Wang *et al.*, 1998), confirming the relevance of these histone modifications for transcriptional regulation *in vivo*. Twist, a bHLH protein factor, involved in the inhibition of differentiation during embryogenesis, has been found to interact with the HATs p300 and PCAF through its N-terminal acidic regions; this interaction results in the inhibition of their acetyltransferase activities (Hamamori *et al.*, 1999b).

Chromatin Remodelling

Recently a number of complexes with the ability to modify chromatin structure have been isolated (reviewed by Cairns, 1998). The first of these chromatin remodelling machines (CRM's) to be reported was the SWI/SNF complex. Mutations on some components of this complex (*SWI1*, *SWI2(SNF2)*, *SWI3*, *SNF5* and *SNF6*) were shown to interfere with the transcription of various genes, among them *HO*, required for switching mating type in yeast (Stern and Hotta, 1984) and *SUC2*, required for sucrose fermenting (hence sucrose non-fermenting, SNF; (Neugeborn and Carlson, 1984). Suppressors of some of these mutations turned out to encode mutant versions of H3, H4 (Kruger *et al.*, 1995) or, in the case of Sin1, a protein homologous to HMG-1 (high mobility group), a structural component of chromatin (Kruger and Herskowitz, 1991). The involvement of the SWI/SNF complex in remodelling chromatin was further supported by the finding that the sensitivity of the *SUC2* promoter to micrococcal nuclease is lost and transcription from it is not fully induced under derepressing conditions in *snf5* or *swi2/snf2* mutants (Hirschhorn *et al.*, 1992; Matallana *et al.*, 1992; reviewed in Kingston *et al.*, 1996). Four additional CRM's have been isolated with a variety of specific chromatin remodelling activities (reviewed in Armstrong and Emerson, 1998). The nucleosome remodelling factor, NURF, has the ability to mediate the binding of transcription factors to the DNA template in chromatin in a ATP-dependent manner (Tsukiyama and Wu, 1996). The chromatin accessibility complex, CHRAC, strongly increases the accessibility of restriction endonucleases to chromatin DNA templates and has a nucleosome spacing activity (Varga-Weisz *et al.*, 1995; Varga-Weisz *et al.*, 1997). The ATP-dependent chromatin assembly and remodelling factor, ACF, facilitates the binding of activators to chromatin by assembling properly spaced arrays of nucleosomes at the expense of ATP (Ito *et al.*, 1997). These three complexes were isolated from *Drosophila*; the complex which remodels the structure of chromatin, RSC, was isolated from yeast and it makes nucleosomal DNA susceptible to cleavage by DNase I, almost to the same extent as naked DNA (Cairns *et al.*,

1996). Using a ³²P labelled DNA fragment in a reaction mixture containing RSC, unlabeled nucleosome core particles and ATP, it was demonstrated that RSC is able to transfer a histone octamer from a nucleosome core particle to naked DNA *in vitro* (Lorch *et al.*, 1999; reviewed by Travers, 1999). All these CRM's are high molecular weight (up to 2 MDa) multisubunit complexes, their features are summarised in Table 2.

Complex	Size	Subunits	ATPase	Organism
SWI/SNF	2 MDa	11	SWI2/SNF2	Yeast
Brahma	2 MDa	?	BRM	<i>Drosophila</i>
SWI/SNF	~2MDa	9-12	HBRM	Human
RSC	~1MDa	15	STH1	Yeast
NURF	500KDa	4	ISWI	<i>Drosophila</i>
CHRAC	670KDa	5	ISWI	<i>Drosophila</i>
ACTF	220KDa	4	ISWI	<i>Drosophila</i>
NRD	?	6	CHD3/4	Human
NURD	1.5MDa	18	?	Human

TABLE 2. A number of multiprotein complexes remodel chromatin. A common feature is the ability to modify chromatin structure at the expense of ATP.

More recent reports begin to link the various levels of regulation with the series of factors and complexes which modify the chromatin structure, from histone tail covalent modification to nucleosome rearrangement. For instance, it has been reported that the retinoblastoma protein, a transcriptional repressor, recruits the histone deacetylase HDAC1 to repress transcription of the cyclin E gene (Brehm *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). At the same time transcriptional activators have been revealed as the targeting factors of HAT complexes, it is the case of the yeast Gcn4 and the VP16 factor both of which recruit HAT complexes to direct nucleosomal acetylation to specific locations in order to stimulate activated transcription (Utley *et al.*, 1998). The viral transactivator Tat of HIV-1 has also been reported as a targeting factor for the CBP and p300 histone acetyl transferases. It brings them to the long terminal repeat (LTR) where a remodelling of the nucleosome arrangement, downstream of the transcriptional initiation site, occurs as a result of these interactions (Marzio *et al.*, 1998). The acetylation will facilitate the interaction between transacting factors and cis elements whereas deacetylation will prevent or inhibit such interactions (reviewed by Kuo and Allis, 1998). More important, a direct physical and functional link has been found between deacetylation activity and chromatin

remodelling activity. A new complex with the ability of nucleosome remodelling and deacetylating histones, NRD, has been reported to contain HDAC1, HDAC2 and two proteins, CHD3 and CHD4, which have an ATPase/helicase domain similar to that found in Swi2/Snf2 superfamily of proteins (Tong *et al.*, 1998). An additional complex containing, among other factors, the histone deacetylases HDAC1/2, histone binding proteins and the dermatomyocytis-specific autoantigen MI2B is also capable of both nucleosome remodelling and histone deacetylation (NuRD; Xue *et al.*, 1998; Zhang *et al.*, 1998). In summary these studies indicate that some regions of the genome are maintained in a repressed state in which histones are deacetylated and the chromatin is compacted, whereas other regions present an activated state in which histones are acetylated and chromatin is in a relaxed state. To reverse each of these states, in response to environmental and/or cell cycle conditions, various protein complexes are present in the cell which are targeted to specific sites to modify histones and other factors and simultaneously (or perhaps as a consequence) remodel the chromatin structure. It is interesting that a number of DNA binding factors with transcriptional activation or repression activities are being revealed as the targeting factors for chromatin modification. It is then clear that protein factors involved in maintaining and/or changing the chromatin structure play a central role in the regulation of DNA transactions by providing or preventing accessibility to this template, especially those with the potential to bind to many sites through the genome. Cbflp is known to modulate chromatin and there are many potential binding sites for it through the yeast genome.

Chromatin modulation by Cbflp

The lack of evidence to consider Cbflp as a transcription factor has lead to the suggestion of its function as a chromatin modulator. A model was proposed in which it performs the same function of establishing a chromatin structure to allow other factors to access DNA at centromeres and promoters. Various lines of evidence pointed to an involvement of Cbflp in chromatin structure.

Additional interacting partners of Cbflp have been identified, and these are involved in chromatin organisation. Mutations at genes *SPT21* (required for transcription of some histone genes), *SIN3*, *RPD3*, and *CCR4* were found to suppress the Met⁻ phenotype of *cbfl* strains, indicating interactions between Cbflp and the products of these genes. It has been suggested that Cbflp interacts with these proteins to inhibit their repressive effect on the methionine biosynthesis pathway (McKenzie *et al.*, 1993). The Sin3-Rpd3 complex represses transcription using a mechanism which involves histone

deacetylation by Rpd3 (Kadosh and Struhl, 1998). Ccr4 is a component of a multisubunit complex which affects transcription both positively and negatively (Liu *et al.*, 1998).

The function of Cbflp as a chromatin modulator at *MET16*, *MET17*, and some other loci has been clearly established. The earliest observation was made at *TRP1* where two hypersensitive sites are lost in *cbfl* strains, one at the CDEI site and other 190 base pairs downstream of it (Mellor *et al.*, 1990). Two CDEI sites are present at the promoter region of *MET17* and one at *MET16*; the ability of Cbflp to bind to such CDEI sites was demonstrated using their sequences as oligonucleotides in gel shift experiments which produced protein DNA-complexes (Kent, 1994). The chromatin structure at these locations was analysed, using a novel fast non-disrupting method (Kent *et al.*, 1993), in *CBF1* and *cbfl* cells. Clear differences were found between wild type and *cbfl* cells. At *MET17* two MNase cleavage sites are lost at positions -200 and -250 in *cbfl* cells and two new sites are generated in the same cells at positions -290 and -330, mapping to the region of the two CDEI motifs. These changes in MNase cleavage patterns were shown to represent two different states of nucleosomal organization as it was demonstrated using small probes to detect nucleosome position. In one of those states Cbflp maintains a nucleosome free region; in *cbfl* cells a nucleosome is present at this position in both *TRP1* and *MET17* loci. A change in MNase cleavage pattern was also found in *cbfl* cells at *MET16*, in this case also compatible with a nucleosome free region in Cbflp cells, but presenting two nucleosomes in *cbfl* cells (Kent, 1994; Kent *et al.*, 1994). This form of Cbflp function has been compared with that of the general regulatory factor Grf2, which provides a region of 230 bp nucleosome free flanked by positioned nucleosomes at the intergenic *GAL1-10* region (Fedor *et al.*, 1988). This open structure allows other factors involved in transcription to access the DNA, since a Grf2 binding site is able to enhance activation of the *DED1* gene (Chasman *et al.*, 1990). However the ability of Cbflp to modulate chromatin did not show any correlation with transcriptional activity at these loci, since *cbfl* cells retain the ability to de-repress *MET17* and *MET16*. It has been suggested that the nucleosome free regions created by Cbflp could have a role in meiotic sister chromatid adhesion (Kent *et al.*, 1994). An increased accessibility at the region surrounding the CDEI site at *MET16* has been reported in *CBF1* cells upon derepression. It was considered to be a mere consequence of transcriptional activity, rather than chromatin reorganisation due to Cbflp, since the pattern seemed to be similar to that of naked DNA (O'Connell *et al.*, 1995).

A recent study of the Pho4 transcriptional activator has revealed that the presence of the bHLH/ZIP domain of Cbflp, substituting that from Pho4 in a protein chimera, rendered both the chromatin opening and the activation of transcription of *PHO5* promoter, dependent on a specific region on the acidic domain of Pho4, different from that on the wild type Pho4 (McAndrew *et al.*, 1998). These findings suggest that this domain of Cbflp determines, to some extent, how other regions of the same protein interact with other determinants of chromatin structure.

The mechanism of action of Cbflp also remains intriguing, the extensive mutational analysis has revealed that some mutations affect the centromere function but not methionine phenotype and others show the converse behaviour (see Figure 4). This has lead to the suggestion that Cbflp functions in three different ways. At centromeres a DNA bound form is required for optimal function; a non-DNA bound form relieves a repressive effect on gene expression and a third form binds to CDEI sequences and modulates chromatin (Kent, 1994). It is important therefore to further analyse the function of Cbflp in the regulation of chromatin structure.

1.6 Thesis Aims

In summary Cbflp is a bHLH/ZIP protein with the ability to bind and bend CDEI-DNA, interact with bZIP transcription factors and modulate chromatin at various CDEI containing loci *in vivo*. It also interacts with other chromatin modulators and with factors involved in the regulation of transcription. Since previous studies have failed to assign a clear function to Cbflp and analysis of chromatin structure have been done only during the exponential growth phase, it is important to explore other phases of the yeast life cycle. In particular those which seem to be strongly affected in *cbfl* mutants, as in the case of meiosis. Since potential Cbflp binding sites are widespread through the yeast genome, the study of chromatin at these multiple locations is potentially relevant in the general understanding of chromatin dynamics of whole genomes and in the function of Cbflp.

Therefore the research presented in this thesis was developed with two aims:

- 1) To explore the chromatin structure during meiosis at locations known to be modulated by Cbflp.
- 2) To analyse chromatin structure at various other locations through the yeast genome, where Cbflp binding sites are in different contexts, under similar conditions.

Chapter 2

Materials and Methods

2.1 Materials

Chemicals

Salts, sorbitol, water, sugars and solvents of AnalaR grade were obtained from BDH Laboratory Supplies (Poole, UK). Amino acids, Ampicillin, RNase A, agarose, DAPI (4'6-diamidino-2-phenylindole), propidium iodide and Antifade (p-phenylenediamine) were from Sigma-Aldrich Company Ltd (Poole, UK). Yeast extract was from Beta Lab, Surrey, UK. Agar, peptone, tryptone and yeast nitrogen base (YNB) without amino acids, for media preparation, were from Difco Laboratories (Detroit, Michigan, USA). [α - 32 P]dATP was from Amersham International plc (UK). Yeast lytic enzyme was from ICN Biomedicals Ltd, UK. Protogel (premixed 30%, 29:1, acrylamide:bisacrylamide mixture, made by National Diagnostics Inc., Atlanta, GA) was obtained from Flowgen Ltd, UK. CompleteTM protease inhibitors mixture was from Boehringer Mannheim. Micrococcal nuclease (MNase) was from Pharmacia Biotech. Restriction enzymes were obtained from various companies, including: Gibco BRL, New England Biolabs, Promega and Pharmacia Biotech. Klenow *exo*⁻ fragment was from New England Biolabs UK. Plasmid DNA purification columns and total RNA purification columns were obtained from Qiagen.

PCR probes

Probes for the indirect end labelling analysis of the *DRS2* and *GDH3* loci and of the YAL060W open reading frame were produced by PCR and subsequent digestion with restriction enzymes and were a gift from S. Eibert and N. A. Kent. Location of the probes is indicated in the maps accompanying figures.

Plasmids

pSD4 contains a *cbf1* gene version with a *URA3* gene inserted at *MscI* restriction site (Mellor *et al.*, 1990), was used to disrupt the *CBF1* gene in Y55 haploids. pSP73-22BL contains a region from *RsaI* to *HpaI* of the *CBF1* locus (Mellor *et al.*, 1990) and was used to obtain the *CBF1* 1.7Kb fragment probe *BamHI*-*BamHI* for Southern blot analysis. pUC CEN6:45 was used to obtain the probe *HindIII*-*AflIII* (actually *BamHI*-*AflIII* in the plasmid) for indirect end labelling of the centromere of chromosome VI. To obtain the probe for *MET17(25)* the fragment *XbaI*-*EcoRI* was excised from plasmid pM25-17, kindly donated by Y. Surdin-Kerjan. Finally pSPMET16 (Kent, 1994) was used to obtain the probe *MscI*-

EcoRI for indirect end-label analysis of *MET16* locus. Plasmids were amplified, purified and restriction fragments isolated according to standard methods (Sambrook *et al.*, 1989).

Bacterial Strains

All plasmids used in this work were amplified and purified from *Escherichia coli* K12 MC1061 cultures. MC1061: *araD138(ara, leu)7697, lacx74, galU⁻, galK⁻, hsr, hsm⁺, StaA* (Meissner *et al.*, 1987).

Yeast Strains

2226: (a) HOΔpst, *leu2Δ, ura3-n, his4:R, lys2-c*.

2208: (α) HOΔpst, *leu2Δ, ura3-n, ade8-1, can1^R, his6-1*.

2291: (a) HOΔpst, *his-4r, leu2-r, thr4-a, ade1-1, ura3-n, met13-4, lys2-d, can1^R*.

2186: (α) HOΔpst, *ade1, met13-2, lys2-l, cyh2^R*.

VG100: (a) Isogenic with 2226, but *cbf1::URA3*. Constructed through this work.

VG200: (α) Isogenic with 2208, but *cbf1::URA3*. Constructed through this work.

DY55: 2226/2208.

VGD1: 2226/VG200.

VGD2: VG100/VG200.

DBY745: (α) *leu2-3, leu2-112, ura3-52, ade1-100*. Wild type for *CBF1*.

YAG93: (α) Isogenic with DBY745 but with a deletion encompassing most of *cbf1*, except the initial 10 amino acids (Dowell *et al.*, 1992).

YAG214: (a) This strain expresses a Cbf1p protein with a mutation in the basic region: E231A (Mellor *et al.*, 1991).

Strains 2226, 2208, 2291 and 2186 were kindly provided by Dr. Rhona Borts.

Culture Media.

Bacterial cultures were grown in Luria-Bertani broth (LB): 5g/l yeast extract, 10g/l Tryptone and 5g/l NaCl and 2ml/l 1M NaOH made in deionised water. 10g/l of agar were added for plates and 100μg/ml of filter sterilised ampicillin for selective media. Yeast cultures were grown in Yeast Extract, Peptone and Dextrose medium: 20g/l Bactopeptone, 10g/l yeast extract and 20g/l glucose (20g/l agar for plates). Presporulation medium (SPS): 5g/l yeast extract, 10g/l Bactopeptone, 1.7g/l Yeast Nitrogen Base (without amino acids and ammonium sulphate), 10g/l potassium acetate, 5g/l ammonium sulphate and 0.05M potassium biphthalate (to adjust the pH to 5.5). Sporulation medium: 10g/l Potassium acetate supplemented with 0.5 times the normal concentration of amino acids. YPG plates: same content as YPD plates but with 3% v/v glycerol and lacking glucose (Padmore *et al.*, 1991). Phosphate depletion medium: a 10X YNB stock solution was prepared by dissolving

8.5g YNB in 400ml water and precipitating phosphate with 50ml 1M MgSO_4 and 50ml concentrated NH_4OH ; after stirring 30 minutes at room temperature the precipitate was removed by filtration using Whatman No. 1 filter paper and the pH adjusted to 4.7 with HCl (O'Connell and Baker, 1992).

2.2 Microbiological Methods

Storage of strains

Bacterial strains were grown overnight in LB medium at 37°C. Yeast cultures were grown overnight at 30°C in YPD. Aliquots were then added to cryotubes (Nunc) containing sterile glycerol (to attain 15% glycerol for bacteria and 25% for yeast), thoroughly mixed and fast frozen in a dry ice/ethanol mixture. Frozen glycerol stocks were stored at -70°C.

Transformation of bacterial cells

To prepare competent *E. coli* MC1061 cells, 100ml LB cultures were inoculated and grown at 37°C to an OD_{595} of 0.2. Cells were harvested by centrifugation at 3Krpm for 5 minutes at 4°C in a bench top centrifuge. Supernatant was discarded and cells resuspended in 40ml of ice cold 0.1M CaCl_2 and incubated on ice for 10 minutes. Cells were then harvested again and supernatant discarded as above. Cells were then resuspended in 2ml of ice cold 0.1M CaCl_2 /40% glycerol and incubated on ice for 2 to 24 hours prior to transformation. Plasmids were added in 5µl volumes to 100µl aliquots of competent cells, thoroughly mixed and incubated on ice for 10 minutes. Cells were then heat shocked at 42°C for 1 minute. After heat shock cells were resuspended in 1ml of LB and incubated at 37°C for 1 hour. Cells were plated on solid LB medium containing 100µg/ml Ampicillin.

Lithium acetate transformation of *S. cerevisiae*

To disrupt the *cbf1* gene a technique for the replacement of DNA sequences was used (Rothstein, 1983). Single colonies from plates were inoculated in YPD and grown to $2 - 6 \times 10^6$ cells/ml. Cells were harvested, washed with 1X TE (10mM Tris-HCl pH 8.0, 1mM EDTA), resuspended in 0.1 M LiAc/TE to 5×10^8 cells/ml and incubated with agitation at 30°C for 1 hour. 40µg of carrier DNA in 8µl and 20µg of transforming DNA were added to 100µl of cells and incubated 30 minutes at 30°C. Then 0.7ml of 40% PEG (in 0.1 M LiAc/TE) were added and incubated again 30 minutes at 30°C. Finally, cells were heat shocked for 15 minutes at 42° C, washed twice in TE and spread on selective plates.

Sporulation of yeast cells

Cells were streaked from frozen stocks on YPG plates and incubated 3 days at 30°C. Single colonies were inoculated in 5ml of YPD and incubated overnight at 30°C. These cultures were used to inoculate 200ml of SPS to a cell density of 1×10^6 cells/ml. SPS

cultures were grown up to 4×10^7 cells/ml, washed in pre-warmed sporulation media and resuspended in pre-warmed supplemented sporulation media at 2×10^7 cells/ml. Cultures in sporulation medium were incubated at 30°C with vigorous agitation. Aliquots containing 1.5×10^8 cells were then collected at intervals indicated in figures and chromatin digested as indicated below. 0.5ml aliquots were collected and fixed at the same time intervals to monitor meiosis progress by fluorescence microscopy and flow cytometry.

Fluorescence microscopy

Cells were harvested and resuspended in 0.5ml fixation solution (1:3 glacial acetic acid:methanol by volume) for 1 to 24 hours. Cells were then pelleted, supernatant discarded and cells resuspended in sterile water and stored at 4°C . 10 μl samples of cells were stained with 1 μl of DAPI/antifade solution (1 μl of DAPI 1mg/ml plus 99 μl of 10mg/ml antifade solution in water) for 2 minutes. Stained samples were immediately examined using a Zeiss Askioskop and photographed (Film Fuji 400).

Fluorescence Activated Cell Sorter (FACS) analysis

Culture samples containing 2×10^7 cells were concentrated to small volumes by centrifugation and transferred to 1.5ml centrifuge tubes. Cells were spun down again and the supernatant discarded. Cells were washed twice in 1M Sorbitol and supernatant discarded. Cells were then resuspended in 1ml cold 70% ethanol, vortexed for 5 minutes and stored at 4°C . Samples containing $2\text{--}3 \times 10^6$ cells ($\sim 0.3\text{ml}$) were added to 5ml falcon tubes containing 3ml of 3M sodium citrate, mixed and spin at 2,000rpm for 5 minutes at room temperature in a benchtop centrifuge. Supernatant was discarded and cells resuspended in 0.5ml of 50mM sodium citrate containing 0.1mg/ml RNase A and incubated at 37°C for 2 hours. 0.5ml of 50mM sodium citrate containing 4 $\mu\text{g/ml}$ propidium iodide (to achieve a final concentration of 2 $\mu\text{g/ml}$) were then added. Samples were then stored up to 7 days in the dark. Samples were sonicated in the falcon tubes immediately before analysis to prevent clusters of cells which give spurious results.

Mating of Yeast Cells

Cells from overnight cultures were inoculated in fresh YPD at 5×10^6 cell/ml, allowed to grow at 30°C for two hours and then mixed with a similar culture of the opposite mating type. Mixed cultures were incubated at 30°C for four hours without shaking. Samples were then taken to analyse under the microscope for the presence of mating cells and zygotes, once found the cultures were spread on selective plates to isolate diploid colonies.

Dissection of Yeast Asci

Sporulated cultures containing asci were washed with water, resuspended in 1M Sorbitol containing 5µg/ml Yeast Lytic Enzyme (ICN) and incubated at room temperature for 15 minutes. Asci were harvested, washed and resuspended in PBS (pH 7.3). Aliquots of asci were spread in a small line at one side of YPD plates using the wire loop. Asci were then picked up, broken and spores deposited in an ordered manner on the surface of the YPD plate using a Microdisecting Singer MSM System (Series 200). After 2 days incubation of plates at 30°C the resulting colonies were replica-plated on selective plates.

2.3 Nucleic Acid Handling

Plasmid purification from *E. coli* cells

Plasmids were purified from 1ml overnight cultures in media containing the appropriate antibiotic. Bacteria were harvested by centrifugation in Eppendorf tubes and the supernatant discarded. Bacterial pellets were resuspended in 350µl of STET buffer (8% sucrose, 5% Triton-X100, 50mM EDTA, 50mM Tris-HCl pH 8.0), 20µl of 12.5mg/ml lysozyme added and incubated on a boiling bath for 40 seconds. Samples were then centrifuged at 13,000 rpm in a microcentrifuge at room temperature for 10 minutes. The pellet formed was then extracted with a toothpick and discarded. To precipitate the DNA one equal volume of iso-propanol was added to the supernatant, mixed, incubated at -20°C for 10 minutes and centrifuged for 10 minutes. Supernatant was discarded, the inside of the tube wiped out with a cotton bud and DNA resuspended in 20µl of water.

Digestion of DNA with Restriction Enzymes

DNA was digested using Restriction Enzymes supplied by New England Biolabs, Gibco BRL and Pharmacia following the manufacturers instructions. Plasmid digestions were carried out in 10µl volume reaction mixtures containing 200ng/µl DNA, 1X enzyme buffer, 1mg/ml RNase A and 1u/µl enzyme. Reaction mixtures were incubated at 37°C for two hours. Total yeast DNA samples were similarly digested in 25µl reaction mixtures containing 2-2.5u/µl restriction enzyme.

Purification of DNA fragments from agarose gels

After separation of fragments by agarose gel electrophoresis, gels were stained with ethidium bromide and slices of agarose containing the fragments of interest were excised with a razor blade. Three volumes of 6M NaI were added to agarose slices and incubated at 55°C for 5 minutes to melt agarose. 10µl of 100mg/ml silica, 3M NaI suspension (Boyle and Lew, 1995) were added, thoroughly mixed, incubated on ice for 5 minutes and

centrifuged for 10 seconds. Supernatant was discarded and the glass pellet washed twice with 500µl wash buffer (50mM NaCl, 10mM Tris-HCl pH 7.5, 2.5mM EDTA, 50% v/v ethanol). DNA was eluted by adding 10µl of water to the glass pellet and 5 minutes incubation at 55°C. Samples were centrifuged and supernatant, containing the DNA fragment, transferred to a clean tube.

Purification of total DNA from yeast cells

Yeast were grown to a density of $1-2 \times 10^7$ cells/ml in 100ml of YPD; cells were harvested and washed twice in 1M Sorbitol. Cells were resuspended in 10ml 1M Sorbitol/2% Glusulase (Du Pont) and incubated at 30°C with gentle shaking. Once sphaeroplasting was complete, sphaeroplasts were harvested by centrifugation at 2,500rpm for 5 minutes and washed twice in 10ml 1M Sorbitol. To lyse the sphaeroplasts the pellet was gently resuspended in 1ml STES buffer (50mM NaCl, 10mM Tris-HCl pH 8.0, 25mM EDTA, 0.5% SDS). To digest proteins 20µl of 20mg/ml Proteinase K was added and the mixture incubated at 37°C for 30 minutes. This solution was then extracted with one volume of TE saturated phenol/chloroform 1:1. The aqueous phase was transferred to a clean tube and 5µl of 10mg/ml RNaseA was added and the mixture incubated for 10 minutes at 37°C. The DNA was extracted twice more with phenol/chloroform. The DNA was precipitated with 0.2 volumes of 5M ammonium acetate and 1 volume of propan-2-ol, removed and transferred to a clean tube using a pipette tip. The pellet was washed with 70% ethanol, dried and resuspended in TE buffer.

Radiolabelling of probes by Random Priming

The random priming method was used to radiolabel DNA probes for Southern blot, Northern blot and indirect end labelling analysis. 100ng of probe fragments were labelled with 10 µCi of α -[32 P]dATP (3000 mCi/mMol) using the buffer and primers provided by Stratagene and 1 unit of Klenow DNA polymerase lacking exonuclease activity (New England Biolabs, UK) in a 10µl total volume reaction mixture incubated at 37°C for 10 minutes.

Radiolabelling of DNA probes by End-filling reaction

The DNA probes for gel-retardation analysis were labelled by the end-filling reaction using the Klenow fragment of DNA polymerase. Reactions contained 100ng of DNA fragments, 50mM Tris-HCl (pH 8.0), 10mM MgCl₂, 1mM DTT, 1mM each dGTP, dTTP and dCTP, 10µCi [α - 32 P]dATP (3,000Ci/mMol, Amersham) and 2 units of Klenow DNA

polymerase (BRL) in a total volume of 20 μ l; the reaction mixture was incubated at room temperature for 30 minutes.

Purification of radiolabelled probes

Labelling reactions were stopped by adding TE buffer to 100 μ l. Unincorporated radionucleotides were removed by passing the mixture through 1ml Sephadex G-50-medium spin column (Pharmacia).

Gel Electrophoresis of DNA

Electrophoresis of DNA samples was carried out in agarose gels of variable concentration in 1X TBE buffer (0.9 M Tris-borate. 1mM EDTA) at 100 mA.

2.4 Analysis by Southern Blot

Blotting of DNA onto Nylon membranes

DNA fragments were separated by agarose gel electrophoresis as described above. DNA was transferred to nylon membranes by capillary transfer after soaking the gel 15 minutes in denaturation solution (1.5M NaCl, 0.5M NaOH) and 15 minutes in neutralisation solution (1.5M NaCl, 0.5M Tris-HCl pH 7.2). The transference was done over 16 hours using 20X SSC (3M NaCl, 0.3M tri-Sodium citrate, pH 7.0) according to standard methods (Sambrook *et al.*, 1989). DNA was then fixed to nylon membranes by incubating for 2 hours at 80°C

Hybridisation

Denaturation of radiolabelled probe and salmon sperm DNA was carried out by mixing them and boiling for 5 minutes. Membranes were briefly soaked in 2X SSC, rolled up along with a nylon mesh and introduced to an Hybaid Mini-Oven bottle. Hybridisation was carried out by adding 10ml of pre-warmed hybridisation buffer (1.5X SSC, 5X Denhardt's reagent, 0.1% SDS), 0.5ml of 2.5mg/ml of denatured salmon sperm DNA (prepared according to Sambrook *et al.*, 1989) and the purified radiolabelled probe in 100 μ l TE buffer to give 6x10⁸ cpm/ml. Hybridisation reactions were incubated in the rotatory oven at 64° C for 16 to 20 hours.

Washing of hybridised membranes

After hybridisation all filters were washed inside the bottles 3 times for 15 minutes at 64°C in the Hybaid Mini-Oven: twice in 50ml 2X SSC/1%SDS and once in 50ml 0.1X SSC/0.1% SDS. While still wet, blots were heat sealed in plastic bags (BDH). Autoradiography was carried out by exposing the blot to X-OMAT S film in an exposure cassette containing a single Cronex (Dupont) intensifying screen at -70°C for 24-72 hours. To re-probe blots the old probe was stripped out by boiling filters for 15 minutes in

0.1XSSC/0.1%SDS and immediately reprobing them as above or stored at -20°C in sealed plastic bags prior to hybridisation.

2.5 Analysis of Transcription Activity by Northern blot

Purification of total RNA from yeast cells

Total RNA for Northern blots was extracted from samples containing 5×10^7 cells using Qiagen chromatographic columns and following the instructions from the manufacturers.

Denaturing gel electrophoresis of RNA

Electrophoresis of RNA samples was carried out in 1.2% agarose denaturing gels containing 6% formaldehyde, 5mM sodium phosphate (pH 6.5) and 40mM EDTA. Samples were prepared by mixing 50 μg RNA (in 7.5 μl volume) with 30 μl of deionised formamide, 6 μl 10X gel buffer and 10.5 μl formaldehyde. Samples were denatured by heating for 15 minutes at 55°C and then cooled on ice. Prior to loading into the gel wells 6 μl loading buffer (50%glycerol, 1mM EDTA, 4mg/ml bromophenol blue, 4mg/ml xylene-cyanol-FF) was added to each sample and mixed. The running buffer was the same as gel buffer, containing 6% formaldehyde, electrophoresis was carried out at 100mA.

Blotting of RNA onto Nylon membranes

RNA was transferred from gels to membranes by capillary transfer after washing the gels with water. Membranes were boiled for 5 minutes in water prior to transfer. The transference was done over 16 hours using 20X SSC (3M NaCl, 0.3M tri-Sodium citrate, pH 7.0) according to standard methods (Sambrook *et al.*, 1989). RNA was then fixed to membranes by incubating them 2 hours in an oven at 80°C

Hybridisation

Denaturation of radiolabelled probe and salmon sperm DNA was carried out by mixing them and boiling for 5 minutes. Membranes were briefly soaked in 2X SSC, rolled up along with a nylon mesh and introduced to an Hybaid Mini-Oven bottle. Hybridisation was carried out by adding 10ml of pre-warmed hybridisation buffer (1.5X SSC, 5X Denhardt's reagent, 0.1% SDS) and the denatured mixture of 0.5ml of 2.5mg/ml of salmon sperm DNA (prepared according to Sambrook *et al.*, 1990) and the purified radiolabelled probe (labelled by the random priming method, see above) in 100 μl TE buffer to give 6×10^8 cpm/ml. Hybridisation reactions were incubated in a rotatory oven (HybAid) at 42°C for 16 to 20 hours.

Washing of hybridised membranes

Membranes were washed twice with 50ml of a solution containing 1X SSC and 0.1% SDS and once in 0.1X SSC, 0.1% SDS, for 5 minutes each, at 64°C to remove unhybridised

probe. While still wet, blots were heat-sealed in plastic bags (BDH). Autoradiography was carried out by exposing the blot to X-OMAT S film in an exposure cassette containing a single Cronex (Dupont) intensifying screen at -70°C for 72 hours.

2.6 Protein Handling

Extraction of total protein from yeast

After giving yeast cultures the specific treatments indicated in the figures, yeast cells were harvested by centrifugation at 5000g for 5 minutes and washed three times in PBS and once in extraction buffer I (20mM HEPES pH 7.9, 420mM NaCl, 1.5mM MgCl_2 , 0.2mM EDTA, 0.5mM DTT, 25% v/v glycerol, 1x CompleteTM protease inhibitor) or buffer II (20mM Hepes-NaOH, pH 7.5, 0.5M NaCl, 15% glycerol, 10mM β -mercaptoethanol, 1x CompleteTM protease inhibitor, 1mM EDTA); cells were pelleted and supernatant discarded. One pellet volume of glass beads (Sigma, $0.45\mu\text{M}$ diameter) and 100 μl of protein extraction buffer I or II were added. The viscous slurry was vortexed for 30 seconds followed by 1 minute incubation on ice. The vortexing-cooling cycle was repeated three times. The mixture was then pelleted by centrifugation at 5000g at for 5 minutes at 4°C . Supernatant was recovered by careful transfer, without transferring beads, to 1.5ml centrifuge tubes. This crude extract was then centrifuged at 15,000g for 15 minutes at 4°C to discard cell debris. The supernatant was carefully recovered again by transfer to fresh 1.5 ml tubes, snap-frozen in a ethanol/dry ice bath and stored at -70°C .

Gel shift assay

Specific CDEI binding activity was analysed by gel retardation (Baker *et al.*, 1989). The CDEI probe was constructed by annealing 26bp long complementary oligonucleotides containing CDEI sequences. Oligonucleotide sequences are:

5' - GATCCAAATAAGT**CACGT**GATGATAG - 3'

3' - GTTTATTTCAGTGCCTACTATCCTAG - 5'

The annealed probe was labelled with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ by filling in reaction using the Klenow fragment of DNA PolI (Boeringer Mannheim). The binding reactions contained 0.2 μg of labelled probe in a reaction mixture of 20 μl total volume containing 6 μl of yeast protein crude extract with salt free buffer GS (20mM Hepes-NaOH, pH 7.5, 5mM MgCl_2 , 10% glycerol, 0.25mg/ml BSA, 0.05% NP40). Reactions contained a 25 fold excess of non-specific competitor (5 μg of poly[dI-dC]) and controls contained unlabelled probe as specific competitor. The reaction mixture was incubated at room temperature for 30 minutes.

Polyacrylamide gel electrophoresis

For gel shift assays the reaction mixtures were analysed by running them on a 6% acrylamide native 1xTGE (0.025M Tris, 0.19M glycine, 0.1mM EDTA) gel at 110V at room temperature in the same buffer system. Gels were dried down over a Whatman 3MM filter paper under vacuum on a BioRad gel dryer at 80°C for 30 minutes. The protein-DNA complexes were visualized by autoradiography exposing Kodak X-OMAT S film to the dried gels.

2.7 Methods for the Analysis of yeast Chromatin

***In vivo* digestion of chromatin with micrococcal nuclease**

The fast sphaeroplast method for “chromatin snap shots” was used to analyse chromatin *in vivo* (Kent and Mellor, 1995). 1.5×10^8 cells were harvested, resuspended in 1ml of Z buffer (20mg/ml Yeast Lytic Enzyme, 1M sorbitol and 10mM β -mercaptoethanol) and incubated 2 minutes at 25°C. Cells were harvested and washed twice in 1ml of 1M Sorbitol. After the second wash sphaeroplasts were gently and carefully resuspended in 200 μ l of Sphaeroplast Digestion Buffer (1M Sorbitol, 50mM NaCl, 10mM Tris-HCl pH 7.4, 5mM $MgCl_2$, 1mM $CaCl_2$, 1mM β -mercaptoethanol, 0.5mM Spermidine, 0.075% NP-40) and 150u/ml of micrococcal nuclease (MNase). Nuclease digestions were incubated for 4 minutes at 37°C. Reactions were stopped by adding 25 ml of stop buffer (250 mM EDTA, 5% SDS) and mixing immediately. 100 μ l of 1xTE buffered phenol, pH 8.0, and 100 μ l of chloroform were added to the nuclease digested samples (contained in 1.5 ml centrifuge tubes) and mixed by vortexing 10 seconds. The mix was centrifuged at maximum speed during 30 seconds. Top aqueous phase was recovered to a fresh tube, 3 μ l of 10mg/ml RNaseA was added and samples incubated 30 minutes at 37°C. Phenol/chloroform extraction was repeated until no whitish interphase was seen. To precipitate the DNA, 40 μ l of 7.5 M ammonium acetate and 260 μ l of isopropanol were added; samples were mixed, incubated 5 minutes at room temperature and centrifuged 10 minutes at maximum velocity in a microfuge. Pellets were washed with 80% ethanol, dried and resuspended in 20 μ l TE. 2 μ l samples were checked on 1% agarose 1xTBE gels. The remaining 18 μ l of each sample were digested to completion with 10 units of the appropriate restriction enzyme and incubated over night at 37°C. Control samples (to digest naked DNA) were given the same treatment, but without the addition of MNase to sphaeroplasts; after complete DNA purification the nuclease digestion was carried out using MNase at a concentration of 1u/ml and samples incubated at room temperature for

90 seconds, the reaction was stopped by adding phenol-chloroform and DNA re-purified as above. After checking the DNA in agarose gels the remaining of the samples was digested to completion with an appropriate restriction enzyme in a mixture volume of 25 μ l using 40 to 50 units of enzyme per sample. Digested DNA's were run on a 1.5% agarose gel and blotted for indirect end-label analysis.

Indirect end-labelling

This method was used to map the positions of accessible and protected sites in chromatin (Nedospasov and Georgiev, 1980; Wu, 1980). After *in vivo* chromatin digestion, fragments of DNA with a defined end were generated by digesting the purified samples to completion with a restriction enzyme (40u of enzyme per sample). The fragments were size fractionated by agarose gel electrophoresis and then analysed by Southern blot using a small probe which hybridises to fragments stretching from the restriction site to a site cleaved by MNase. The size of the hybridised fragments indicates the position of MNase cleavage sites relative to the restriction site as indicated in the diagram of Figure 9.

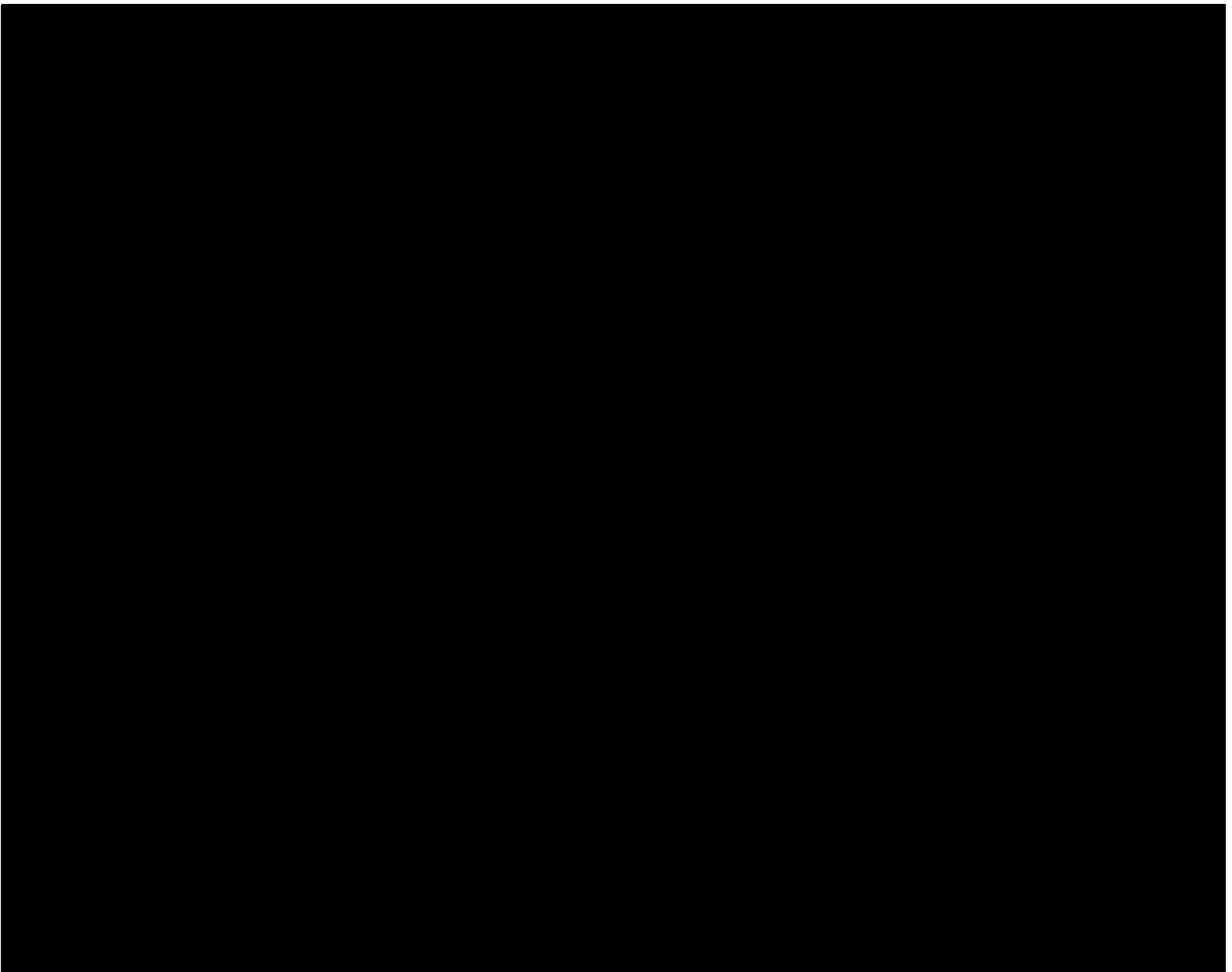


FIGURE 9. Indirect end-labelling (Wu 1980). When naked DNA is partially digested with nucleases they will cut at a number of sensitive sites. However, when chromatin is digested, the presence of protein complexes will cause some sites to be protected and some others to show increased sensitivity. A particular region can be analysed for the presence and relative position of protein complexes. DNA molecules containing the same ends are obtained by cutting the fragments generated by nuclease digestion with restriction enzymes (RE) flanking the region of interest. The fragments are then separated on agarose gels and blotted onto nylon membranes (according to standard methods, Sambrook *et al.*, 1989). A precise map of the presence of protein complexes can then be obtained using a small labelled probe homologous to one of the ends of the region defined by the restriction enzyme sites. Only the fragments between the restriction enzyme sites and containing only one of the ends will be detected (indicated as Fragments in the diagram). The arrows indicate the sites sensitive to the nuclease. The fragments detectable by the probe are represented as vertical lines. The dots below the fragments indicate that these are not generated when digesting chromatin since the corresponding sensitive sites are protected by protein complexes. Therefore these fragments are not found in the chromatin lane of the Southern blot cartoon.

Chapter 3

Chromatin Remodelling at Cbflp binding sites in Cells Undergoing Meiosis

3.1 Introduction

In budding yeast nutrient starvation of diploid cells or $\mathbf{a}/\alpha$ cells by limitation of nitrogen, carbon, phosphate, sulphate, methionine and other compounds leads to meiosis and formation of asci containing four haploid spores as shown in Figure 10. Based on a number of landmark events and the successive expression of various sets of genes, meiosis can be divided into three phases. During the early phase cells enter meiotic prophase in which DNA replication, recombination and formation of the synaptonemal complex occur. Then during the middle phase the two nuclear divisions take place. In the first nuclear division (reductional) sister chromatids must remain together and they are then separated in the second or equational nuclear division. Finally during the late phase the four haploid nuclei are packaged into spores (reviewed by Kupiec *et al.*, 1997; Mitchell, 1994). Centromeres play a key role in this process and the function of its known components has been studied extensively. The integrity of CDEI site and Cbflp binding are absolutely required for proper meiotic function, although there is some disagreement as to which point the process is affected. Cumberledge *et al* were able to show that most mutants lacking this region at only one chromosome (III) are unable to sporulate; only one out of three crosses containing the same mutation was able to sporulate. It did so at a very low efficiency, only 11% of cells sporulated and they showed a low viability (only 48%) and an altered segregation pattern in Meiosis I (Cumberledge and Carbon, 1987). The deletion of CDEI from *CEN6* in Yeast Artificial Chromosomes (YACs) leads to a 10-fold increase in the non-disjunction of sister chromatid and missegregation (Hegemann *et al.*, 1988). When plasmids are used for the same kind of analysis, higher levels of precocious sister chromatid segregation at Meiosis I are found in *cbfl* cells (Cumberledge and Carbon, 1987; Masison and Baker, 1992).

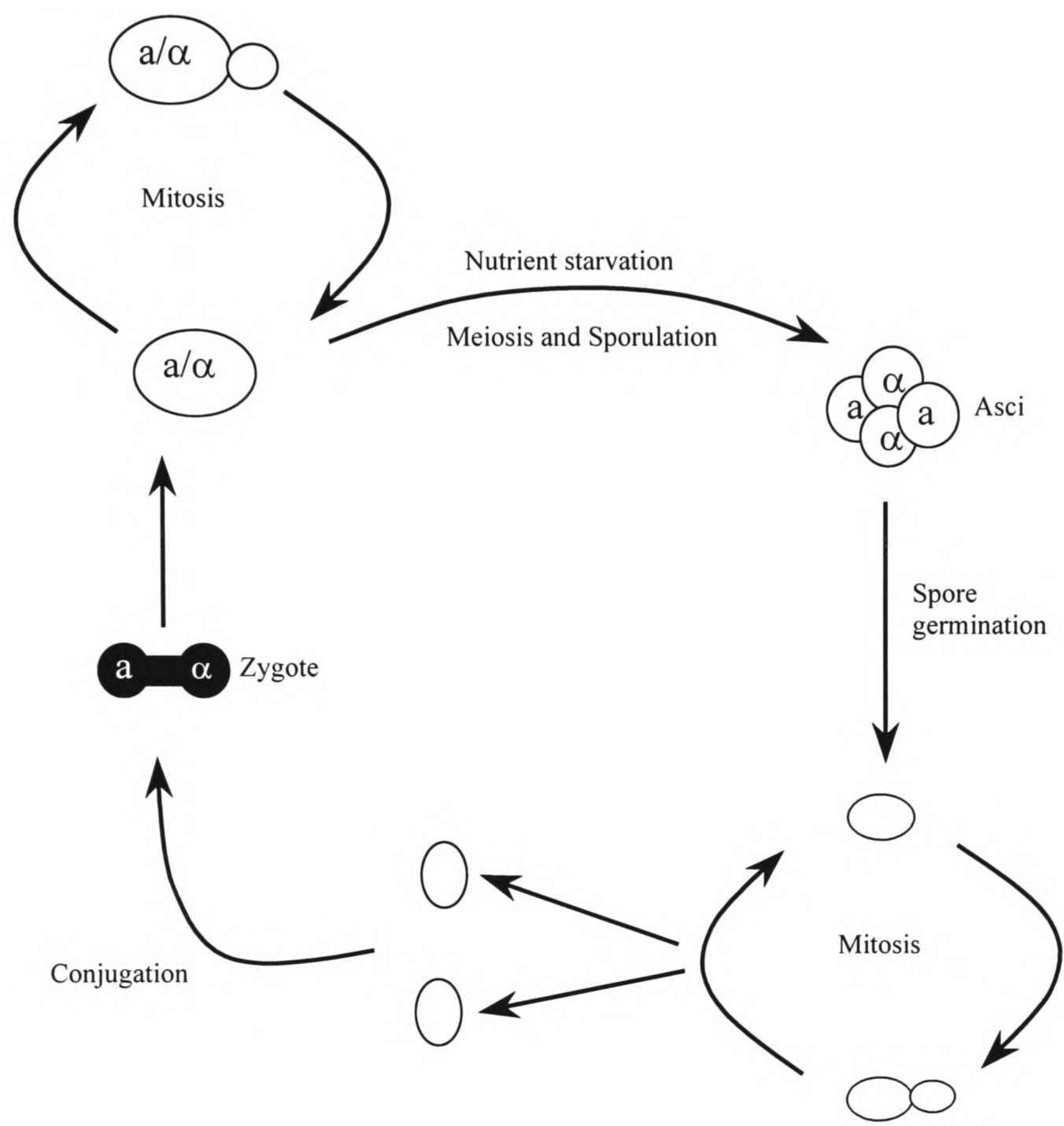


FIGURE 10. Meiosis in the life cycle of *Saccharomyces cerevisiae*. Only one type of cell, the a/α cell can enter meiosis when nutrients are depleted and the only carbon source is a non-fermentable one. Sporulation of diploid a/α cells produces asci containing four spores, in rich medium these spores germinate and the haploid cells enter the mitotic cell cycle. Haploid cells of opposite mating type can conjugate to form zygotes which also enter the mitotic cell cycle in rich medium.

It has been previously demonstrated that *Cbflp* dependent chromatin structures are associated with CDEI sites. The involvement of *Cbflp* in the determination of chromatin structure has only been studied under two conditions: in the presence of rich medium and under de-repressing conditions for the *MET* genes (see Chapter 1). Despite the fact that the role of *Cbflp* and its cognate sequence CDEI at the centromere during meiosis has been extensively studied, no work has been dedicated to study their role during meiosis, at the numerous other locations at which they can be found throughout the yeast genome. In particular, no analysis has been made during other stages of the yeast life cycle at the *MET* loci at which *Cbflp* is known to modulate chromatin (see Chapter 1). This is of prime importance in the understanding of the function of this factor/element set, since the effect of *cis* or *trans* mutations of the pair on meiosis can not be explained from the effect they have during mitosis. The aim of this chapter is to investigate whether the chromatin structure maintained by *Cbflp* at some CDEI sites, whose chromatin structure is already known for *cbfl* and *CBF1* cells under exponential growth, remains stable or becomes modified during meiosis.

3.2 Chromatin structure at CDEI sites in cells undergoing meiosis

To perform the analysis of chromatin structure during meiosis a wild type strain Y55 (McCusker and Haber, 1988), which can undergo rapid and synchronous meiosis was used. The diploid Y55 cells can be synchronized at G1 by exposing them for a number of generations to the pre-sporulation medium described in Chapter 2. After transferring the cells to sporulation medium, most of the cells complete the meiotic process within 12 hours. Samples were taken every 90 minutes after transfer to sporulation medium and treated according to the rapid, non-lytic method described by Kent and Mellor (1995). Aliquots were taken simultaneously and fixed (as described in Chapter 2) to monitor the progression of cells into meiosis using fluorescence activated cell sorter (FACS) analysis and fluorescence microscopy (after DAPI staining).

The chromatin structure at *MET16* locus is transiently remodelled during meiosis

It has been previously shown that the CDEI site located at position -175 of the *MET16* UAS can be bound by *Cbflp*, since a probe containing this region produces a protein/DNA complex in a gel retardation assay. It has been also shown that a specific chromatin structure is maintained *in vivo* by *Cbflp* at this region. Figure 11 shows the analysis of chromatin structure at *MET16* locus in *CBF1/CBF1* cells undergoing meiosis.

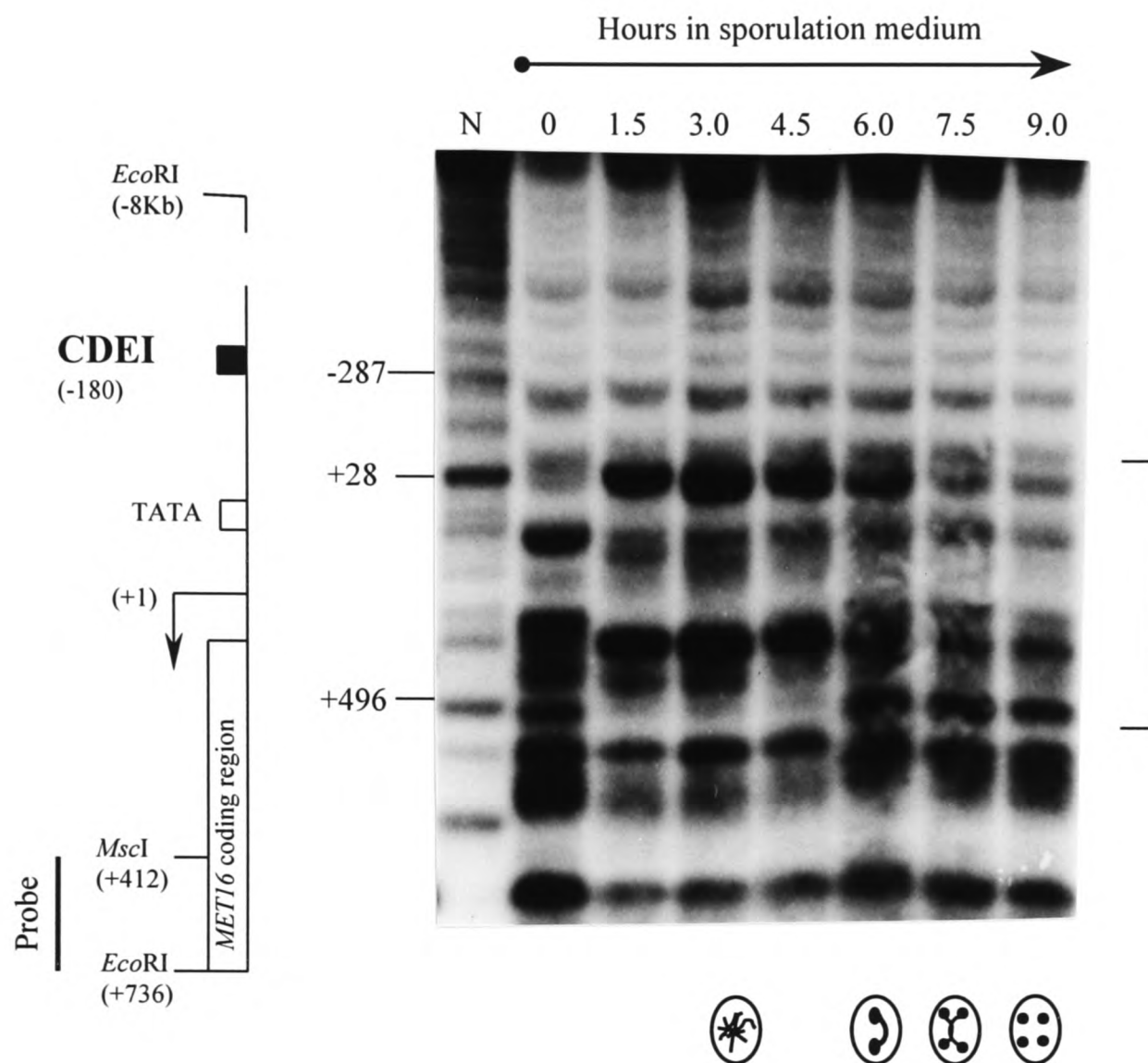


FIGURE 11. Chromatin structure at *MET16* during meiosis in *CBF1/CBF1* cells. Time course analysis by indirect end labelling. Top : N, naked DNA; numbers indicate the time points, in hours, at which samples were collected after transfer to sporulation medium. Samples were sphaeroplasted and chromatin digested *in vivo* with 150u/ml MNase (as described in Chapter 2). After phenol extraction each DNA sample was digested to completion with 10 units of *EcoRI* and restriction fragments separated on a 1.5% agarose gel. DNA was then blotted and hybridised to the restriction fragment probe indicated in the left diagram. Relative position of some sites cut by MNase in naked DNA is indicated to the left of N lane. The bracket indicates a region at which a transient chromatin structure is observed. Cartoons at the bottom represent the state of nuclei at various significant stages, as seen by fluorescence microscopy.

The indirect end label analysis reveals a clear change in MNase cleavage pattern, different from that found in *CBF1CBF1* cells growing in rich medium: the chromatin structure at time point zero is the same as found in exponentially growing *CBF1* cells. However, a reorganisation is seen at 90 minutes after transfer into sporulation medium. It is stable up to 4.5 hours but returns to a similar pattern as the initial state after 6 hours; by 7.5 hours the chromatin structure is similar as that observed when cells are grown in rich medium, despite the fact that they are still in sporulation medium. However, the initial state is not fully regained even after 9 hours. The 5' end of this chromatin reorganisation maps at the CDEI site and extends into the coding region of this locus. This remodelling is seen as a number of MNase sensitive sites becoming protected and some others becoming accessible compared with time zero chromatin structure and with naked DNA. This pattern and the timing of remodelling is highly reproducible (n=3). To determine whether the MNase accessibility patterns obtained could be affected by MNase concentration (although standard concentrations were used), an experiment was performed where three aliquots of cells were taken after growing in pre-sporulation medium and after 1.5 hours in sporulation medium. Three different concentrations of MNase were used to digest chromatin. As can be clearly seen in Figure 12 the pattern is reproducible and independent of MNase concentration. Therefore, the new pattern found is related to the metabolic conditions in which the cells are growing and reflects some aspect of the chromatin organisation at this locus.

The chromatin structure at *MET17* is transiently remodelled during meiosis

It has also been shown that Cbflp dependent chromatin structures are found at the *MET17* locus, which presents two CDEI sites at its promoter but whose transcriptional regulation is not dependent on Cbflp (Kent *et al.*, 1994). As shown in Figure 13 a new MNase cleavage pattern was also found at this locus during meiosis. Again, the chromatin structure found at time point zero is the same found for this locus in exponentially growing *CBF1* cells. The accessibility pattern of lanes 1.5 and 3.0 hours is clearly different from the initial pattern. A third pattern is also evident in the lanes from 6 to 9 hours, with an intermediate pattern at 4.5 hours. These patterns differing from the initial one are also different from the pattern obtained with naked DNA, compare for instance lanes 1.5 and 6 with N lane. These differences suggest a reorganisation of the components of chromatin. The transient nature of the reorganisation at *MET17* is coincident with that found at *MET16*, following a similar dynamics in time.

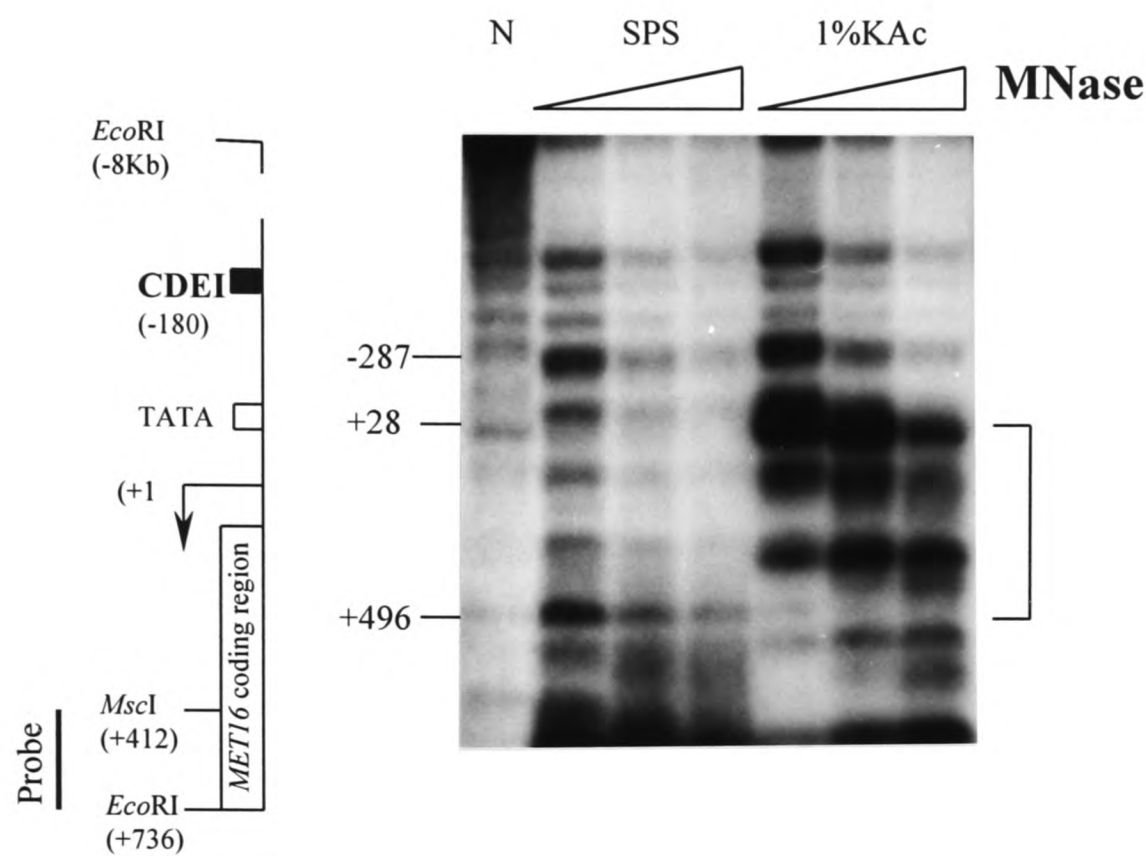


FIGURE 12. Effect of MNase concentration on accessibility patterns at *MET16*. Chromatin was prepared using three concentrations of MNase for each set of samples: 75, 150 and 300 units/ml respectively. SPS lanes are from *CBF1/CBF1* cells incubated in preporulation medium, equivalent to time point zero of meiosis experiments. 1%KAc lanes are from cells harvested after 90 minutes incubation in sporulation medium.

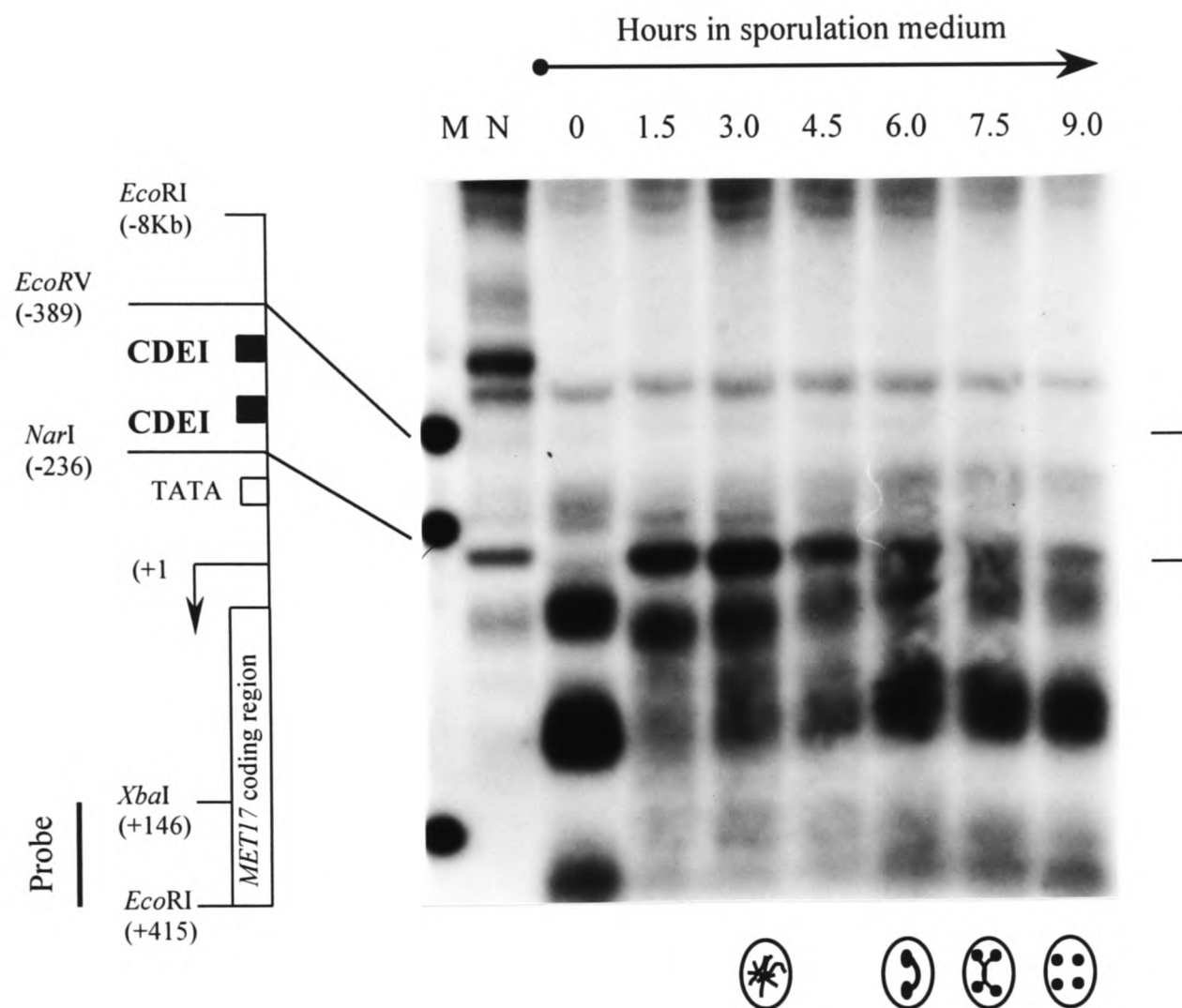


FIGURE 13. Chromatin structure at *MET17* during meiosis in *CBF1/CBF1* cells. Time course analysis by indirect end labelling. Top: M, markers; N, naked DNA; numbers indicate the time points at which samples were collected after transfer to sporulation medium. Samples taken at the time points indicated were sphaeroplasted and chromatin digested *in vivo* with 150u/ml MNase (as described in Chapter 2). After phenol extraction each DNA sample was digested to completion with 10 units of *EcoRI* and restriction fragments separated on a 1.5% agarose gel. DNA was then blotted and hybridised to the restriction fragment probe indicated in the left diagram. The bracket indicates a region at which a transient chromatin structure is observed. Cartoons at the bottom represent the state of nuclei at various significant stages, as seen by fluorescence microscopy.

In this case the remodelling involves most of the promoter region but also extends into the coding region of the gene. It is important to note that the pattern in lanes from 6 to 9 hours in sporulation medium is not similar to the initial state, it is however, similar to the pattern found in log phase *cbfl* cells (see below).

Chromatin structure at *TRP1* locus

It has been previously shown that a specific Cbflp dependent chromatin structure exist at the *TRP1* locus; two hypersensitive sites are lost in *cbfl* strains from this pattern, one at the CDEI site and one 190 base pairs downstream of it (Kent *et al.*, 1994; Mellor *et al.*, 1990). This locus has been analysed during the mitotic cell cycle, it was found that the region at which Cbflp seems to be determinant for chromatin structure remains stable during this process in wild type cells (Kent and Mellor, 1995). Figure 14 shows the chromatin structure at this locus during meiosis. It does not show any modification of the MNase cleavage pattern at the region previously shown to be modified in the absence of Cbflp or downstream to the CDEI site. It does, however, show a change in the region upstream to the CDEI site, asterisks indicate the bands involved in this modification. Both, the structure at time zero and that at 1.5 hours in sporulation medium are different from the pattern in naked DNA. The chromatin structure of this upstream region is not altered in *cbfl* cells, it has the same structure in these cells as in *CBF1* cells (Kent and Mellor, 1995). Therefore, although a modification of the accessibility pattern occurs at *TRP1* locus, this is located more than 300bp upstream of the CDEI site.

Chromatin structure at *CEN6* during meiosis

The chromatin structure of yeast centromeres has been previously analysed during meiosis. A method was used in which nuclei are purified from cells and the chromatin samples are prepared by a method more than one hour long (Yeh, 1985; cited in Bloom *et al.*, 1986) and therefore unable to detect minor changes in chromatin structure existing for short periods of time. The analysis of the chromatin structure of the *CEN6* locus was performed here using the rapid method designed by Kent and Mellor (1995), which takes only 8 minutes to complete. In Figure 15 a time course of meiotic induced cells shows perhaps only a slight change at the centromere, a small reduction in the size of the nuclease resistant core (lane 1.5 hours). The change occurs at the side of the nuclease resistant core at which Cbflp binds to the CDEI site. The resistant core recovers its initial size and remains stable through the rest of the process.

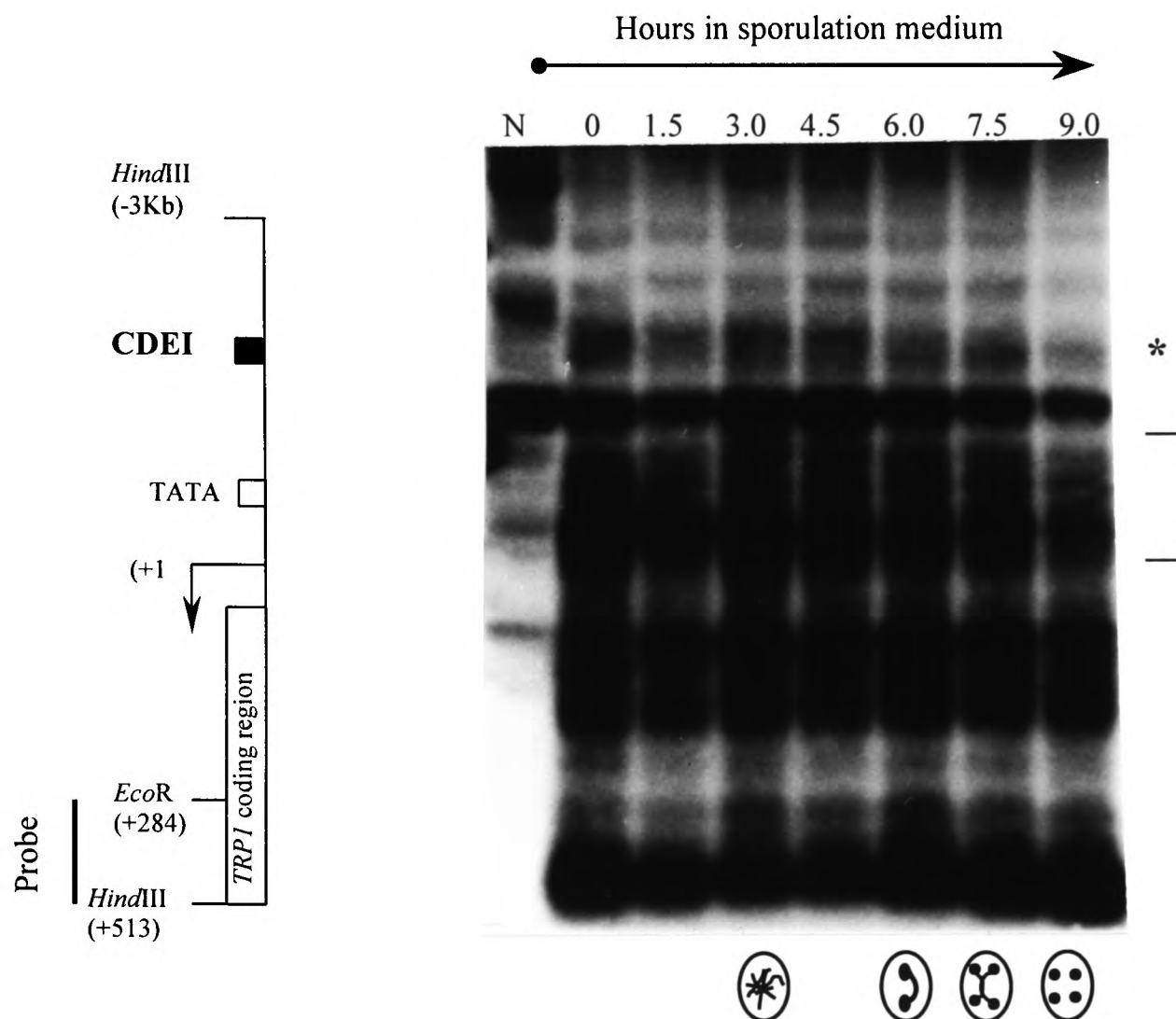


FIGURE 14. Chromatin structure at the *TRP1* locus during meiosis in *CBF1/CBF1* cells. Time course analysis by indirect end labelling. Top: N, naked DNA; numbers indicate the time points at which samples were collected after transfer to sporulation medium. Samples were sphaeroplasted and chromatin digested in vivo with 150u/ml MNase (as described in Chapter 2). After phenol extraction each DNA sample was digested to completion with 10 units of *Hind*III and restriction fragments separated on a 1.5% agarose gel. DNA was then blotted and hybridised to the restriction fragment probe indicated in the left diagram. A bracket indicates the region at which specific chromatin structure is associated in *CBF1* cells (Mellor *et al.*, 1990; Kent, 1994). The asterisk indicates a region, upstream the CDEI site, which appears to be modified. Cartoons at the bottom represent the state of nuclei at various significant stages, as seen by fluorescence microscopy.

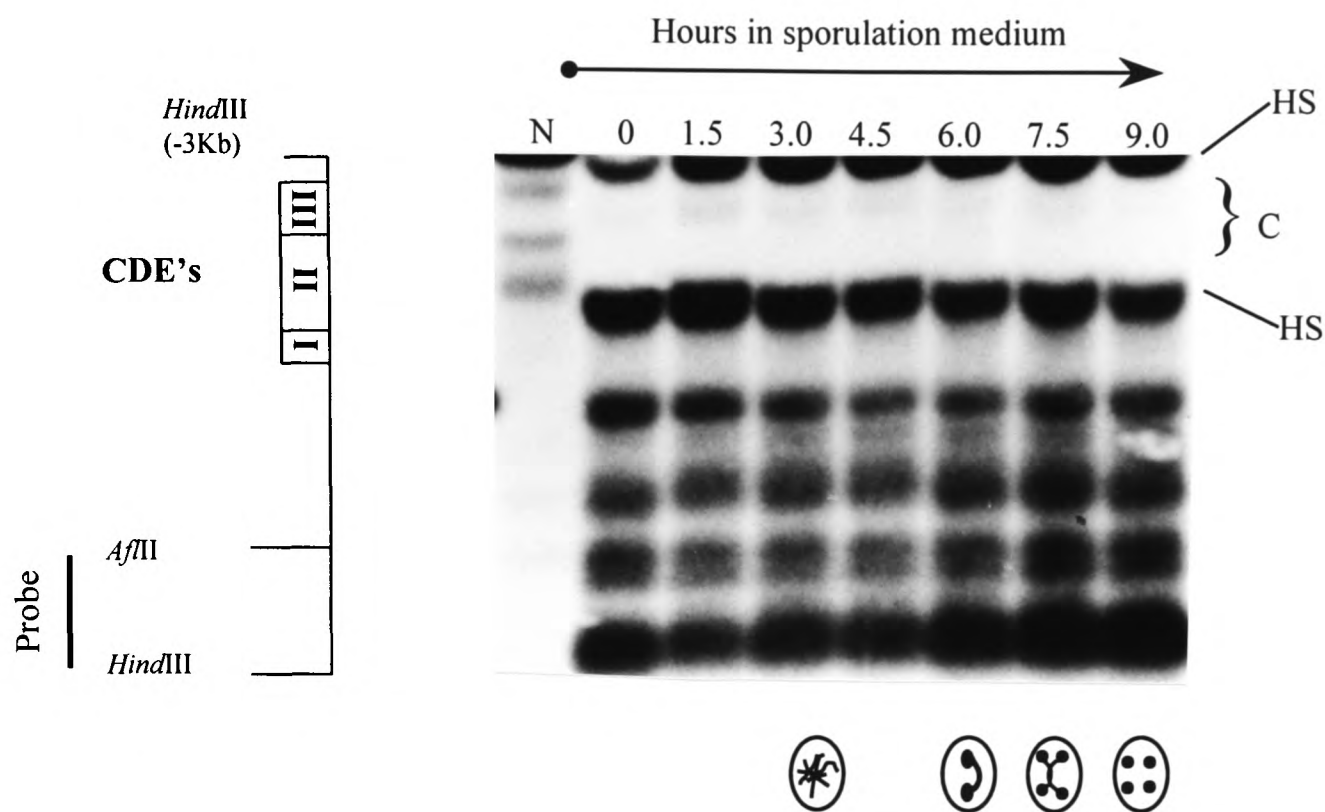


FIGURE 15. The centromere chromatin structure during meiosis in *CBF1/CBF1* cells. Time course analysis of *CEN6* region during meiosis by indirect end labelling. Top: N, naked DNA; numbers indicate the time points, in hours, at which samples were collected after transfer to sporulation medium. C, nuclease resistant core; HS, hypersensitive site. Samples were sphaeroplasted and chromatin digested in vivo with 150u/ml MNase (as described in Chapter 2). After phenol extraction each DNA sample was digested to completion with 10 units of *HindIII* and restriction fragments separated on a 1.5% agarose gel. DNA was then blotted and hybridised to the restriction fragment probe indicated in the left diagram. The bracket indicates the nuclease resistant core. Cartoons at the bottom represent the state of nuclei at various significant stages, as seen by fluorescence microscopy.

It has been suggested that during the replication of the centromere DNA its chromatin structure could be different (Hegemann and Fleig, 1993). The small and short-lived change found in this work may therefore be related with the fact that centromeres are replicated early during the synthesis of DNA (McCarroll and Fangman, 1988).

3.3 Discussion

The results presented in this chapter show a clear chromatin remodelling at and around CDEI sites at two *MET* genes, detected as transient changes in the pattern of MNase cleavage sites; these changes are very distinctive (for instance compare lanes for 0 and 1.5 hours in figures 11 and 13). At the *TRP1* locus a change also occurs but is relatively far from the CDEI site. An even more subtle modification may occur at the CDEI site of *CEN6*, but is short lived and much less extensive than those found at *MET* genes.

Some of the bands found in the new pattern at *MET16* are present in the naked DNA, but their intensities are very different compared to that found in naked DNA or at time zero. Some of the sites present in naked DNA and time zero become protected in the new pattern. This suggests that this region does not simply become deproteinised, but rather reflects a reorganisation of the protein-DNA complex, which makes some sites more prone to the activity of the MNase than when they are in naked DNA. This probably reflects a different conformation of the DNA molecule in the reorganised chromatin. Although a slight change in nuclease accessibility has been previously reported, it was considered to be equivalent to naked DNA and due to transcriptional activity upon derepression (O'Connell *et al.*, 1995). The novel chromatin structure found at *MET16* during meiosis is not equivalent to naked DNA, and therefore represents an overall reorganisation of protein components of chromatin over the DNA molecule. There is the possibility that during meiosis *Cbflp* could detach from its site at this locus and the structure could therefore be equivalent to that found in *cbfl* cells. Figure 16 shows that this is not the case; the patterns found during meiosis are aligned with those of *cbfl* and *CBF1* cells in log phase, these reproduced here with kind permission (Kent, 1994). First, the pattern in lane 1 or time zero point is similar to that in lane 4 of DBY745 *CBF1* strain; second and more important, the pattern in lane 2 or 1.5 hours in sporulation medium is clearly different from the pattern of exponentially growing *cbfl* cells, lane 3. Therefore, this locus can exist in at least three different chromatin conformations: as it is found in exponentially growing

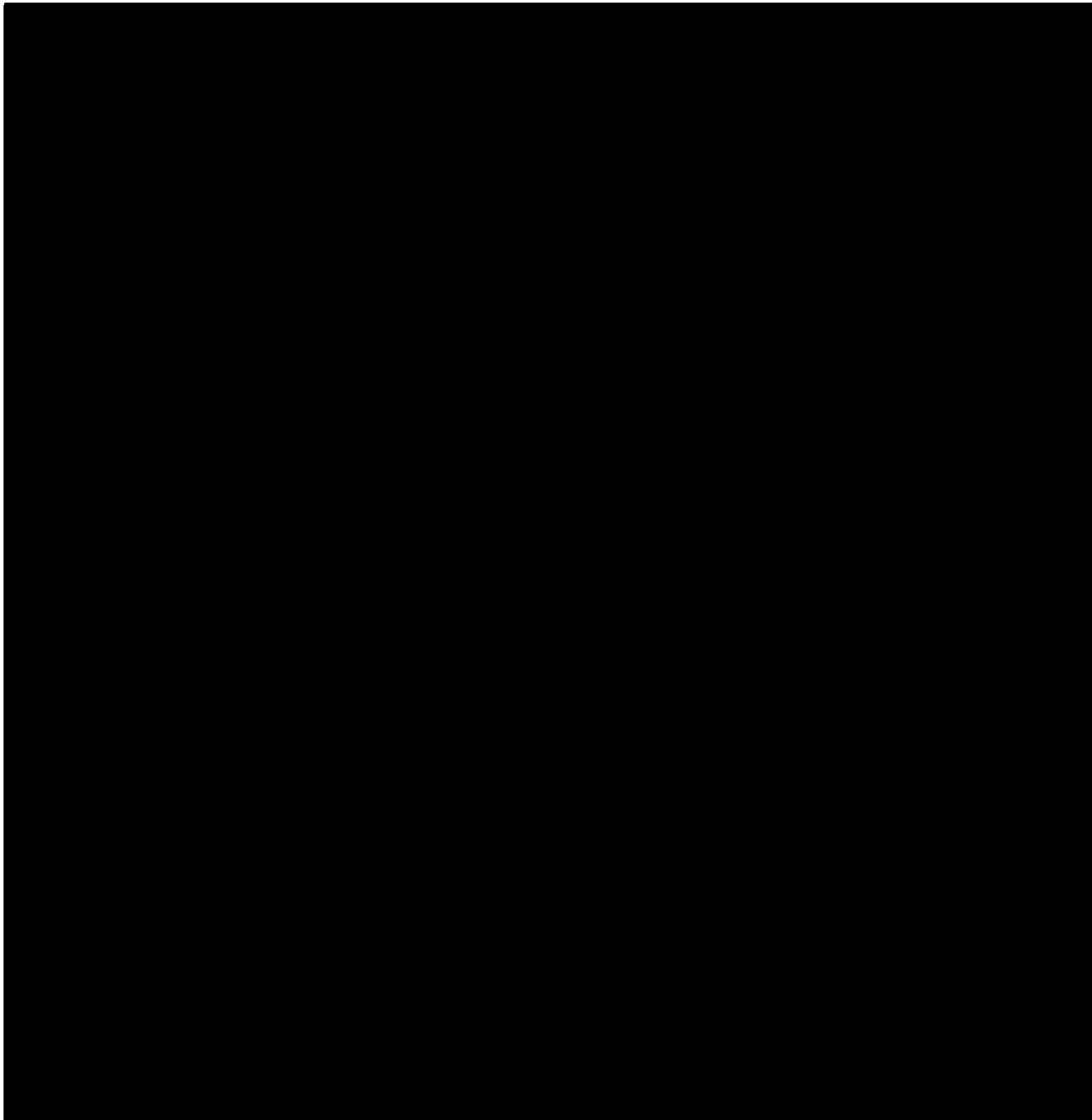


FIGURE 16. Alignment of MNase accessibility patterns at *MET16* locus. The chromatin structures found in cells undergoing meiosis are compared with those previously described for exponentially growing *CBF1* and *cbfl* cells. N, naked DNA; 1, *CBF1/CBF1* cells incubated in pre-sporulation medium; 2, *CBF1/CBF1* cells incubated 1.5 hours in sporulation medium; 3, exponentially growing *cbfl* cells (YAG93); 4, exponentially growing *CBF1* cells (DBY745). Lanes N, 1 and 2 reproduced from Figure 11; lanes 3 and 4 reproduced with kind permission (Kent, 1994).

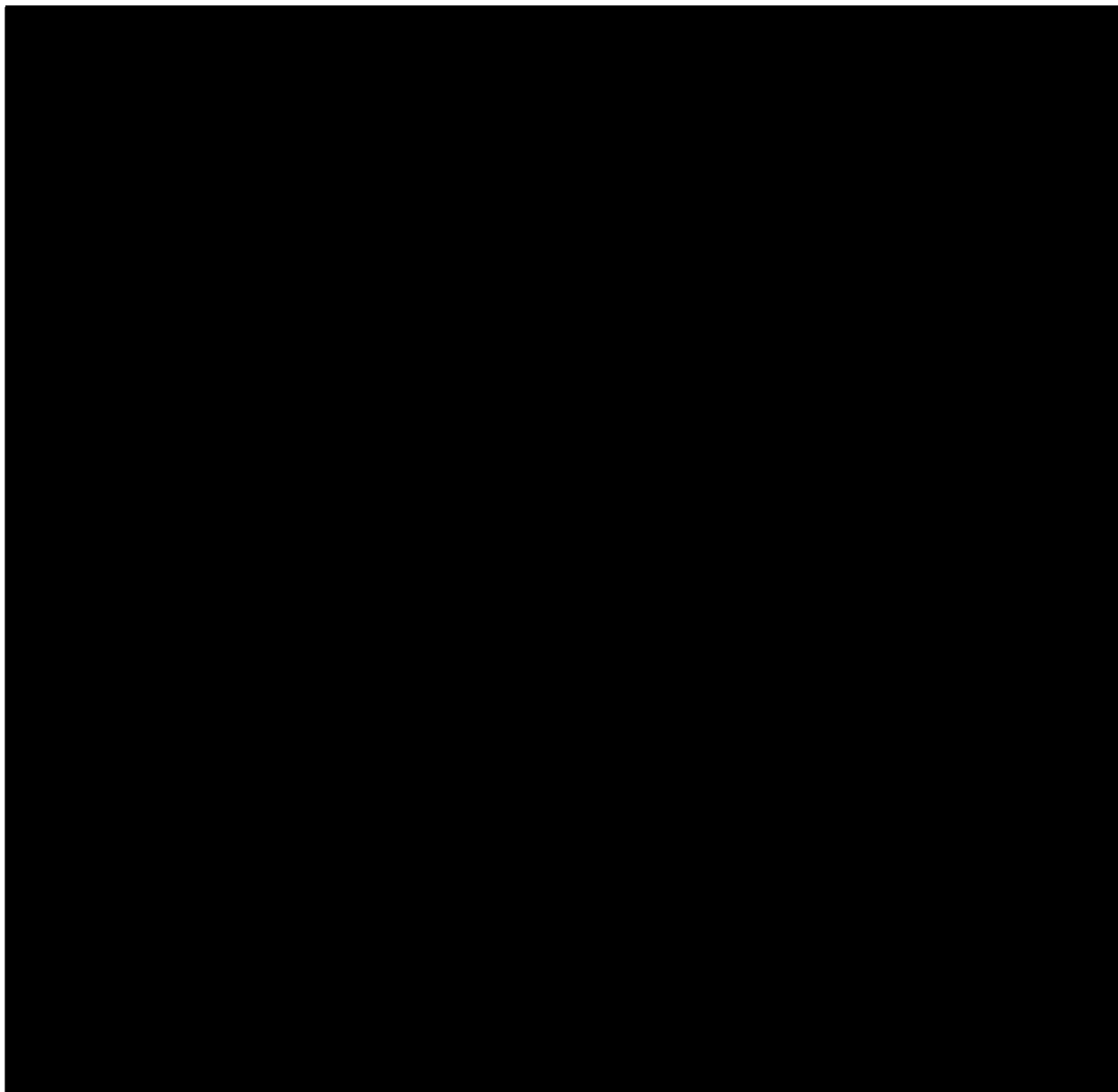


FIGURE 17. Alignment of MNase accessibility patterns at *MET17* locus. The chromatin structures found in cells undergoing meiosis are compared with those previously described for exponentially growing *CBF1* and *cbfl* cells. M, markers; N, naked DNA; 1, *CBF1/CBF1* cells incubated in pre-sporulation medium; 2, *CBF1/CBF1* cells incubated 1.5 hours in sporulation medium; 3, exponentially growing *cbflΔ* cells (YAG93); 4, exponentially growing *CBF1* cells (DBY745); 5, cells incubated 9 hours in sporulation medium. Lanes M, N, 1, 2 and 5 reproduced from Figure 13; lanes 3 and 4 reproduced with kind permission (Kent, 1994).

CBF1 cells; as it is found in exponentially growing *cbfl* cells and in the newly found conformation, described here, during meiosis in *CBF1/CBF1* cells.

At *MET17* there is also a reorganisation at and downstream to the region containing the two CDEI sites. This new organisation also becomes apparent from 1.5 hours in sporulation medium and begins to display a pattern similar to the initial one at 7.5 hours, following the same dynamics in time as the reorganisation at *MET16*. At *MET17* all the patterns found through meiosis are different from that of naked DNA, indicating that they correspond to differences in the organisation of the chromatin components. These patterns are also compared with those of *cbfl* cells in Figure 17. The patterns in lanes 1, time zero point in meiotic analysis, and 4, log phase *CBF1* cells, are similar. The pattern in lane 2, 1.5 hours in sporulation medium, is different from those and from lane 3, which is the pattern displayed by exponentially growing *cbfl* cells. Again, this indicates that the elements of chromatin have been organised in a new way, generating a different pattern of MNase accessibility. Also compare lane 3, *cbfl* cells, with lane 5 *CBF1/CBF1* cells, 9 hours in sporulation medium; these two patterns are similar, suggesting that at least Cbflp may be absent from chromatin at this point during the process. It is possible then, that a mechanism to reset the initial chromatin structure, which operates later in the process, may be involved at this locus.

In summary the results presented in this Chapter show that during meiosis chromatin structure is modified in the vicinity of CDEI sites. A clear and extensive chromatin remodelling occurs at *MET16* and *MET17*, which maps at and downstream their CDEI sites. A number of questions arise from the findings presented in this chapter, but three are immediately obvious. Is the chromatin reorganisation found specific for the meiotic process? Since Cbflp binds to these sites, is it relevant for this chromatin reorganisation? Does remodelling of chromatin occur at the other multiple CDEI sites at which Cbflp can potentially bind? These questions will be addressed in the next chapter.

Chapter 4

Cbflp-dependent Chromatin Remodelling at Multiple Loci

4.1 Introduction

In Chapter 3 it was shown that reorganisation of chromatin occurs at and around CDEI sites, this reorganisation is particularly extensive at two *MET* genes. At other locations specific factors are required for chromatin reorganisation, for instance the bHLH transcription factor Pho4 is known to be required for remodelling of the *PHO5* promoter which contains two binding sites for this factor (see Section 1.5). Cbflp has been shown to bind to the CDEI sequences of *MET16* and *MET17* *in vitro* and Cbflp dependent chromatin structures associated at these sites *in vivo* (Kent *et al.*, 1994). An important question following the finding that chromatin is reorganised at those CDEI sites when cells are starved is whether Cbflp is involved in that chromatin remodelling. In order to answer this question a first step is to analyse the same loci under similar conditions in cells lacking a functional Cbflp protein. In this chapter the possible involvement of Cbflp in the remodelling of chromatin observed during meiosis is investigated. First a *cbfl/cbfl* strain isogenic with the highly efficient meiotic strain is constructed; then these cells are subjected to the same treatment for induction of meiosis and chromatin is analysed at the two *MET* loci.

An early study calculated that there are at least 500-600 Cbflp molecules per cell and considered this as an underestimation (Baker *et al.*, 1989). If Cbflp binds as a homodimer at all yeast centromeres and to the sites at the CDEI containing loci *MET16*, *MET17*, *TRP1*, *CYT1* and *QCR8*, there should be another at least 200-250 homodimers or 400-500 Cbflp containing heterodimers or a combination of both doing something elsewhere in the genome of the yeast cell. The yeast genome is known to contain numerous potential Cbflp binding sites. A recent analysis of the complete yeast genome sequence has been performed by N. A. Kent ; it revealed the presence of 4,821 CACRTG motifs, of which 953 are high affinity CACGTG sites. These may be found not only at promoters but also at coding and intergenic regions; in some cases these sites are present as small clusters of two or three motifs (Kent N. A., personal communication). A number of loci with different classes of potential Cbflp binding sites located at different positions with respect

to initiation of transcription site, but within 1Kb from the ATG, have been analysed. The ability of Cbflp to bind to these sites *in vitro* has been demonstrated and it has also been shown that at these locations specific chromatin structures are maintained *in vivo* in the presence of Cbflp (Kent *et al.*, 1999). In view of the findings that chromatin is remodelled at CDEI sites in starved cells, one attractive possibility is that reorganisation of chromatin structure during nutrient starvation may be occurring at multiple Cbflp binding sites, possibly affecting the chromatin structure of a significant portion of the yeast genome. To investigate this possibility, a number of loci containing various numbers and classes of potential Cbflp binding sites at diverse locations, among those analysed by Kent *et al.* (1999), were selected to analyse their chromatin structure under the starvation conditions leading to meiosis. The chromatin structure is analysed in wild type and *cbfl* cells in exponential growth phase and starved cultures.

4.2 Chromatin structure in *cbfl/cbfl* cells induced to enter meiosis

According to the results presented in the previous chapter there is a clear chromatin remodelling at *MET16* and *MET17* loci during meiosis. The 5' end of these remodelling events maps to the CDEI sites in both cases. In order to determine if Cbflp is involved in this reorganisation of chromatin, an isogenic *cbfl/cbfl* diploid strain was constructed to analyse the chromatin structure under the same conditions.

Construction of cells with a gene disruption at *CBF1* locus

To construct an isogenic diploid strain with a disrupted *cbfl* gene the two haploid parental strains of DY55 were used. Y55 strains 2226 (**a**, *ura3:n*) and 2208(**α**, *ura3:n*) were transformed by the lithium acetate method (Gietz *et al.*, 1992) with a *Bam*HI-*Bam*HI fragment bearing a *cbfl* allele with a *URA3* gene inserted at the *Msc*I site, in the middle of the coding region (see Chapter 2). After transformation, cells were grown on synthetic complete selective plates lacking uracil. Because *cbfl*Δ leads to a Met⁻ phenotype, colonies were restreaked to test for the methionine auxotrophy and uracil prototrophy. DNA of methionine auxotrophs was extracted, digested with BamHI and analysed by Southern blot to confirm the *URA3* insertion at the *cbfl* locus. To obtain the homozygotic diploids, haploid cells were mated, grown on selective plates and then DNA from diploids was also extracted and analysed by southern blot. The blots (Figure 18) confirm that the diploid cells contain both *cbfl* alleles disrupted. These cells were subsequently used to perform experiments in order to investigate the role of Cbflp in the chromatin reorganisation described in the previous sections.

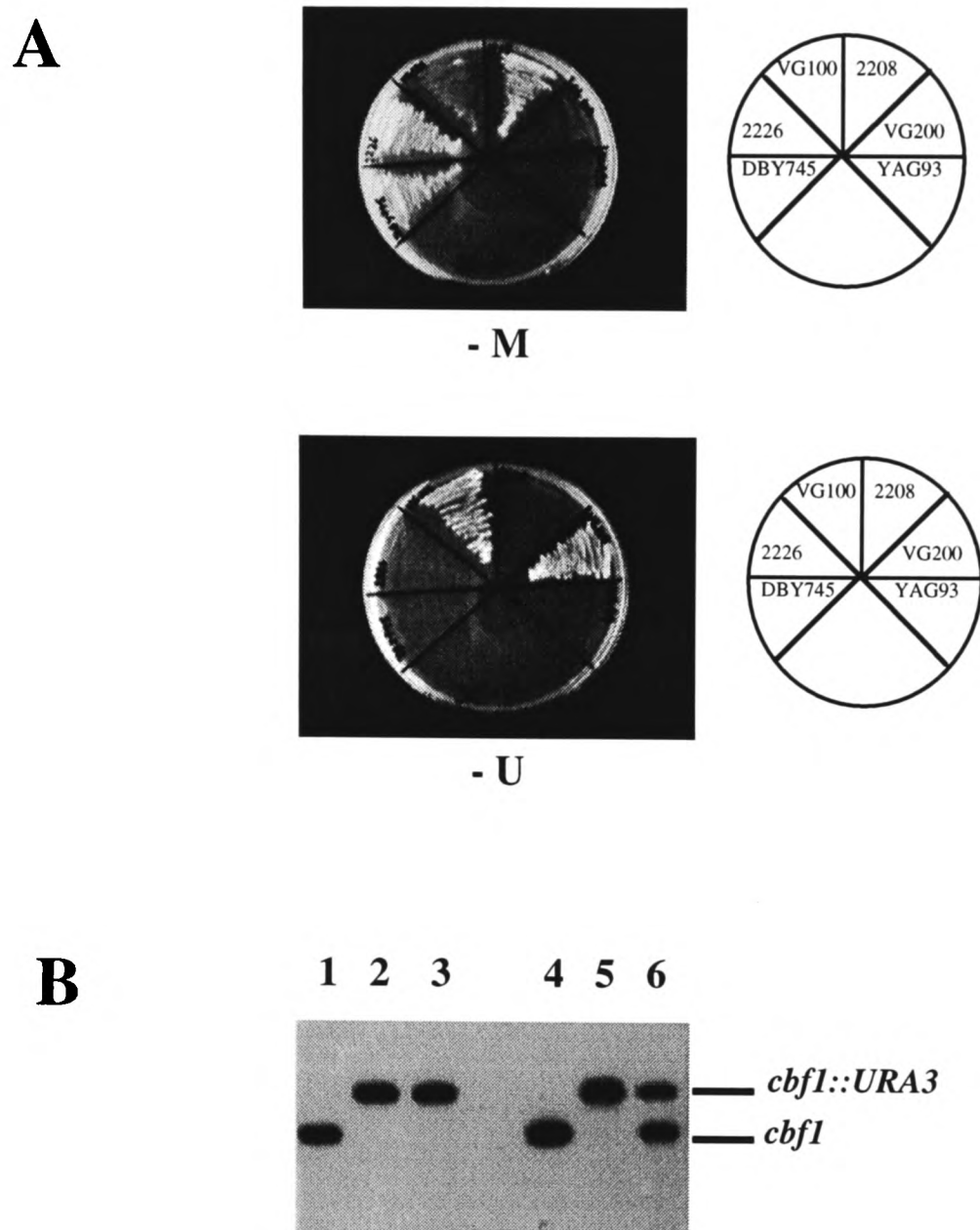


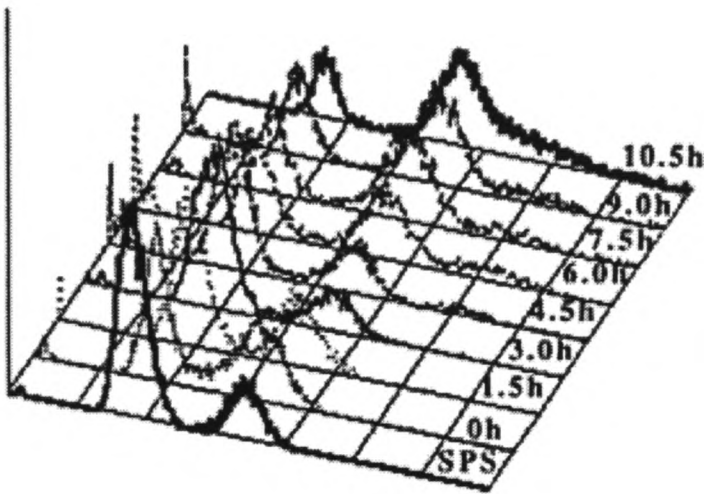
FIGURE 18. Disruption of the *CBF1* gene in Y55 strains. **A.** Transformed and non transformed haploid cells grown on selective plates to test for methionine and uracil auxotrophy or prototrophy. Cells were grown on SC plates lacking methionine (-M) or lacking uracil (-U). In both plates, from bottom left and clockwise the strains are: DBY745, 2226, VG100, 2208, VG200 and YAG93, as indicated in the diagrams at the right. **B.** Southern blot analysis of haploid and diploid Y55 derivative strains. Total DNA was extracted and digested to completion with BamHI, separated on a 1% agarose gel, transferred to a membrane and probed with a BamHI-BamHI fragment of the *CBF1* gene. Lanes: 1, 2226; 2, VG100; 3, VG200; 4, DY55 (*a/α*, 2226/2208, *CBF1/CBF1*); 5, VGD2 (*a/α*, VG100/VG200, *cbf1::URA3/cbf1::URA3*); 6 VGD1 (*a/α*, 2226/VG200, *CBF1/cbf1::URA3*).

Chromatin structure at *MET16* in *cbfl* cells induced to enter meiosis

Previous studies have shown that *cbfl/cbfl* yeast cells sporulate poorly (about 10% sporulated cells). However, it is not known which point of the meiotic cell cycle is impaired. Flow cytometry profiles show that most *CBF1/CBF1* cells complete pre-meiotic DNA replication by 7.5 hours after transfer to sporulation medium, since the 2n peak has reached its minimal height and the 4n peak reaches its maximum (Figure 19). Fluorescence microscopy shows 60% of the *CBF1/CBF1* cells have completed the second nuclear division by 9 hours. In contrast, after 120 hours in sporulation medium the profiles of 2n and 4n peaks of *cbfl/cbfl* cells remain unchanged, longer incubation leads to degradation of DNA (Figure 19). No evidence of meiotic nuclear division is seen in *cbfl/cbfl* cells by fluorescence microscopy and many cells with small buds appear during the whole time of incubation in sporulation medium, indicating that cells do not synchronise at G1 as wild type cells do after 18 hours in pre-sporulation medium. These data suggest that *cbfl/cbfl* cells become arrested prior to the initiation of pre-meiotic DNA replication.

Figure 20 shows that when the chromatin structure at this locus was analysed in *cbfl/cbfl* cells subject to the same treatment described in Chapter 3, no change in MNase accessibility pattern is observed, it remain similar to that previously found for *cbfl* log phase cells (Kent *et al.*, 1994). The inability of *cbfl/cbfl* cells to remodel *MET16* gene chromatin in sporulation medium in this experiment can be explained in two ways. Firstly, the process may require Cbflp. However, because, as demonstrated above, *cbfl/cbfl* cells arrest before entering meiosis it is thus possible that meiosis *per se* is required. To discern between these two possibilities *CBF1* and *cbfl* haploid cells were subject to the same treatment and chromatin structure analysed.

CBF1



cbf1

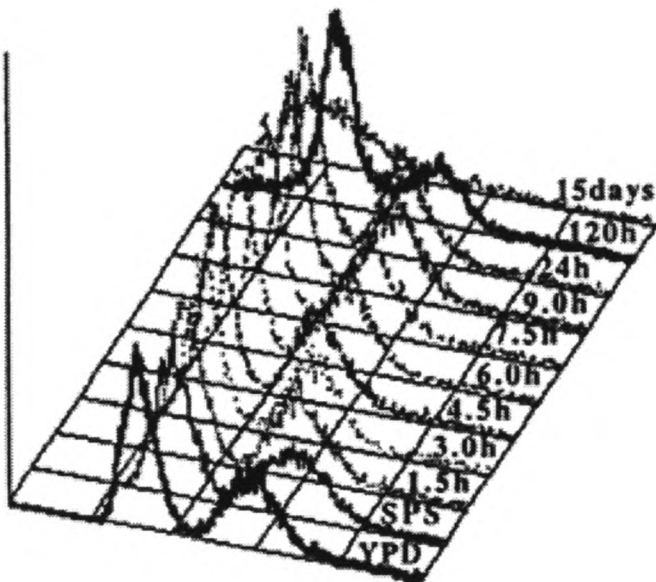


FIGURE 19. Flow cytometry analysis of *CBF1/CBF1* and *cbf1/cbf1* cells in sporulation medium. DY55 (*CBF1/CBF1*) and VGD2 (*cbf1/cbf1*) cultures were induced to enter meiosis in sporulation medium after 18 hours incubation in pre-sporulation medium. Samples were taken at the time intervals indicated (in hours). Cells were fixed and stored at 4° C prior to analysis. YPD, rich medium; SPS, pre-sporulation medium; h, hours.

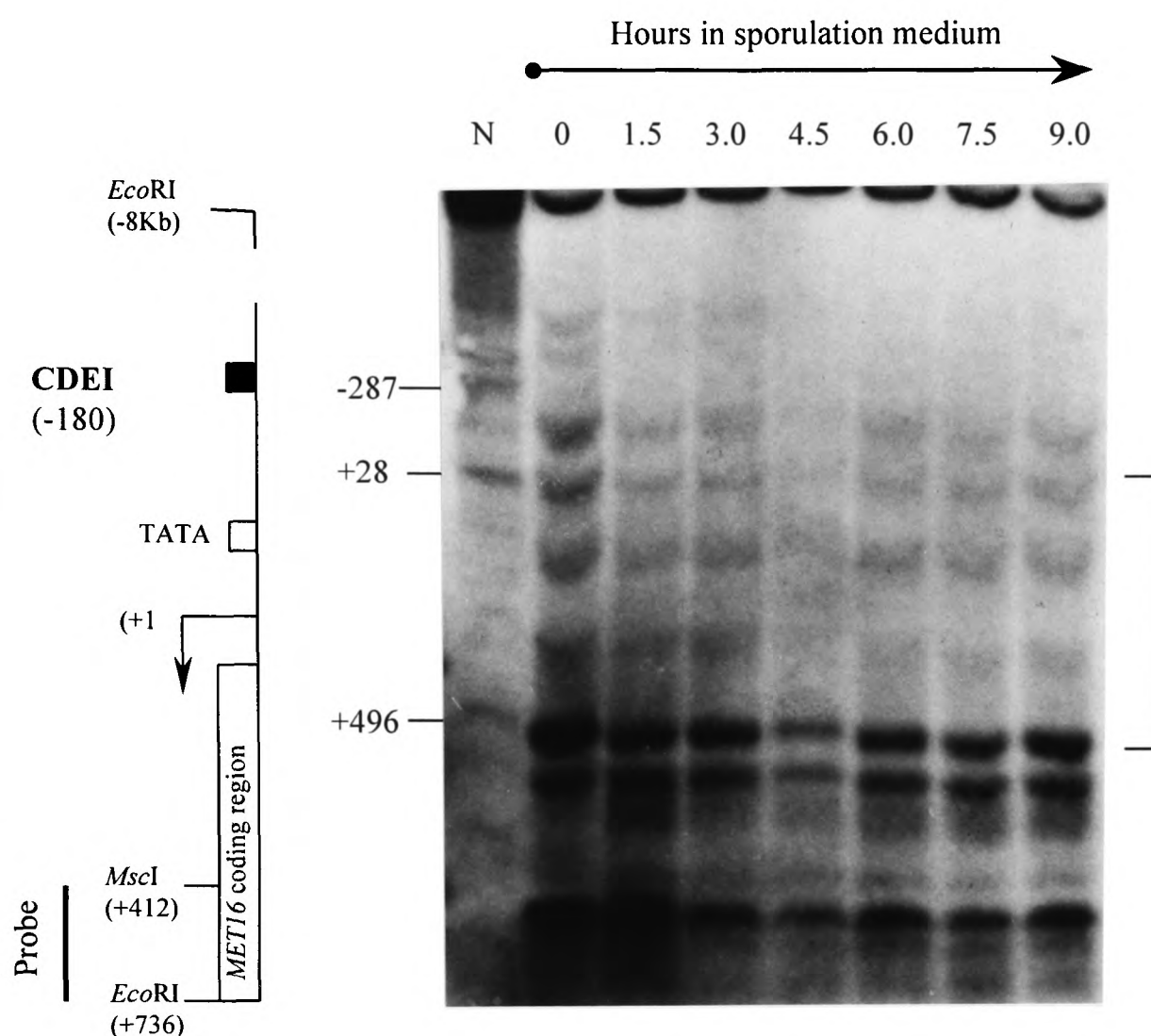


FIGURE 20. Chromatin structure at *MET16* locus in *cbfl/cbfl* cells induced to enter meiosis. Time course analysis by indirect end labelling. N, naked DNA; numbers indicate the time points at which samples were collected after transfer to sporulation medium. Samples were sphaeroplasted and chromatin digested *in vivo* with 150u/ml MNase (as described in Chapter 2). After phenol extraction each DNA sample was digested to completion with 10 units of *EcoRI* and restriction fragments separated on a 1.5% agarose gel. DNA was then blotted and hybridised to the restriction fragment probe indicated in the left diagram. Relative position of some sites cut by MNase in naked DNA is indicated to the left of N lane.

4.3 Chromatin structure at *MET16* and *MET17* in 1N starved cells

The chromatin structure at *MET16* was analysed in haploid cells under the same conditions which lead to sporulation in diploid cells. As shown in Figure 21 *CBF1* cells also display a change in the MNase cleavage pattern when they are starved. This change occurs from 45 minutes in starvation conditions. The new MNase accessibility pattern found in haploid cells is the same one produced by starvation of diploid cells. The chromatin remodelling is therefore not specific for meiosis. Rather it depends on the nutrients condition of the media. The analysis of haploid *cbf1* cells under starvation shown in Figure 22 confirms that the chromatin remodelling is dependent on Cbflp, since no change is seen upon transfer to sporulation medium in these cells. In this figure the three possible chromatin conformations at this locus can be clearly appreciated. One is that of the *CBF1* cells in the log phase, the second is the new pattern found when *CBF1* cells are starved and the third one is the already known structure found in *cbf1* cells which does not become modified upon starvation.

The *MET17* loci was analysed in haploid cells under similar conditions as above, in this case the chromatin structure is also reorganised and dependent on Cbflp as shown in Figure 23. Again, it is clear from these experiments that the reorganisation of chromatin is dependent on the presence of a functional Cbflp being expressed in the cells. The three possible chromatin conformations at this locus are clearly discernible. These results indicate that the chromatin reorganisation is triggered by starvation and is not unique to the meiotic process. More importantly, it is clearly dependent on Cbflp. Therefore, Cbflp is involved in the reorganisation of chromatin structure at these two loci through an unknown mechanism.

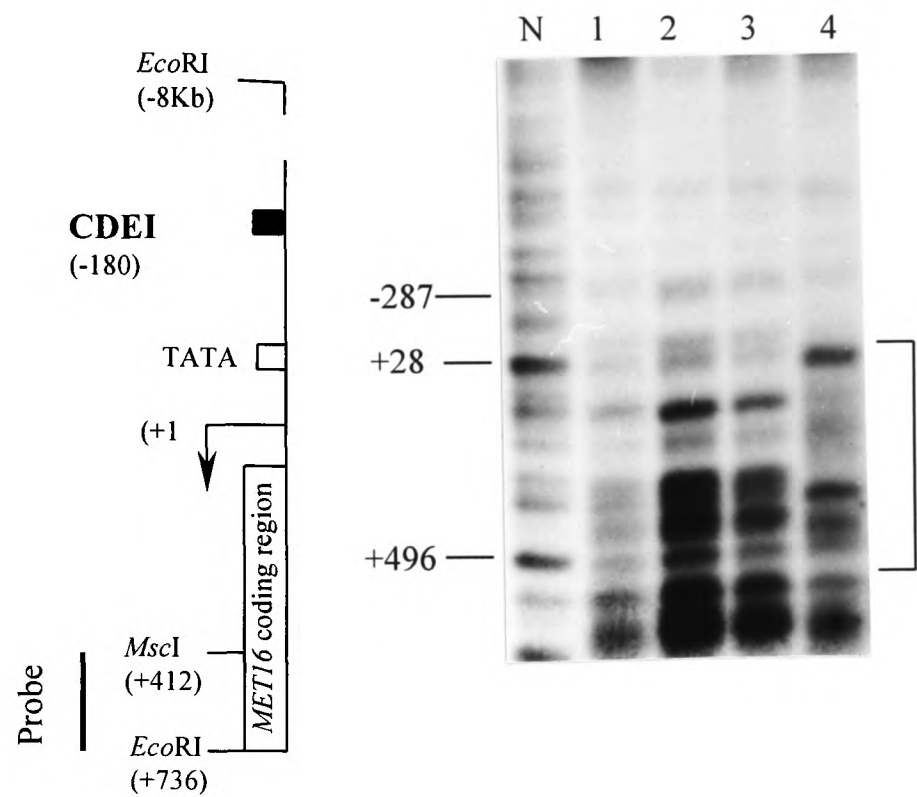


FIGURE 21. Chromatin structure at *MET16* locus in haploid *CBF1* starved cells. 2208 Y55 cells were subjected to the same treatment as diploid cells, including incubation in pre-sporulation medium. Cells were harvested and chromatin digested with MNase as described in Chapter 2. N, naked DNA; lane 1, exponentially growing cells; lane 2, cells harvested after 18 hours incubation in pre-sporulation medium; lane 3, time point zero in sporulation medium (resuspended in sporulation medium and immediately harvested and sphaeroplasted); lane 4, 45 minutes incubation in sporulation medium.

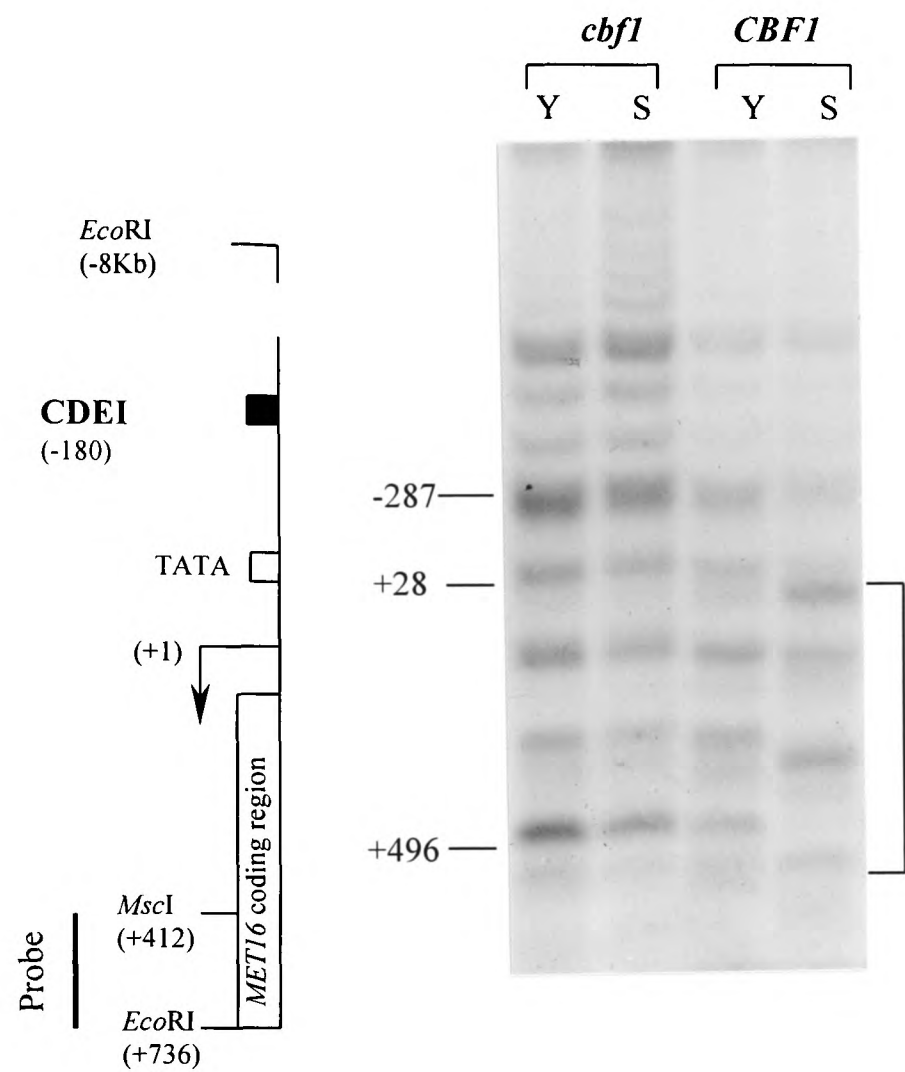


FIGURE 22. Chromatin structure at *MET16* locus in haploid *cbfl* starved cells. VG100 Y55 cells were subjected to the same treatment as haploid *CBF1* cells. Cells were harvested and chromatin digested with MNase as described in Chapter 2; samples from *CBF1* cells are included for comparison. Y lanes, exponentially growing cells; S lanes, cells harvested after 45 minutes in sporulation medium. The bracket indicates the region at which reorganisation of chromatin is seen upon starvation in *CBF1* cells.

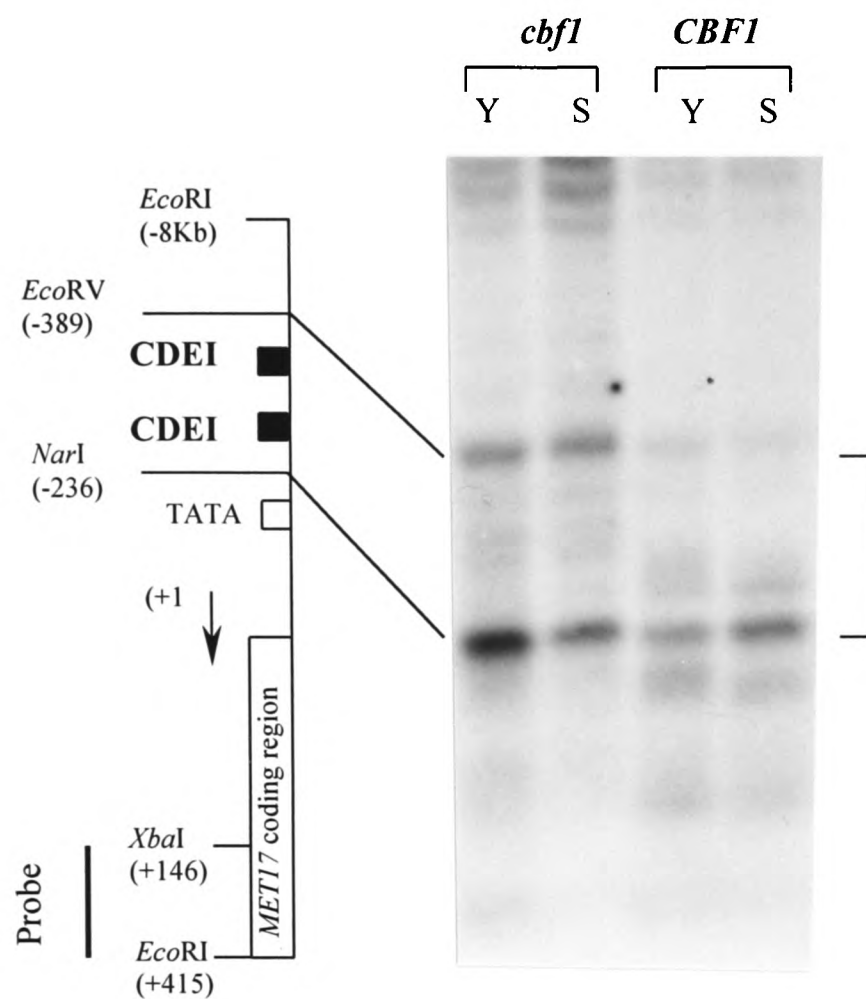


FIGURE 23. Chromatin structure at *MET17* locus in haploid *cbf1* cells. VG100 (*cbf1::URA3*) cells were subjected to the same treatment as haploid *CBF1* cells. Cells were harvested and chromatin digested with MNase as described in Chapter 2; samples from *CBF1* cells are included for comparison. Y lanes, exponentially growing cells; S lanes, cells harvested after 45 minutes in sporulation medium. The bracket indicates the region at which a reorganisation of chromatin is seen upon starvation only in *CBF1* cells.

4.4 Chromatin structure at loci containing CDEI-like sites in starved cells

The chromatin structure of various other non-*MET* loci was analysed in starved cells. A number of genes containing potential Cbflp binding sites of various categories and located on Chromosome I were selected for this analysis. Three of them (*DRS2*, *GDH3* and the YAL060W open reading frame) have been shown to present Cbflp dependent chromatin structures associated with their potential Cbflp binding sites (Kent *et al.*, 1999). Since it was found that chromatin remodelling in diploid cells is detectable from 90 minutes, cells were incubated in sporulation medium for 90 minutes after being harvested from log phase cultures and washed twice in water. In order to be able to establish the Cbflp dependence of possible chromatin modifications at these loci, both *CBF1* and *cbfl* cells were analysed in each case. The differences between *CBF1* and *cbfl* cells (Kent *et al.*, 1999) can be observed here, but only the differences between exponentially growing and starved *CBF1* cells are highlighted by dots in the figures presented in this work.

Chromatin structure at *DRS2* locus

This gene codes for a membrane spanning Ca(2+) transporting ATPase implicated in ribosome assembly (Ripmaster *et al.*, 1993) and is located on Chromosome I. This locus contains three potential Cbflp binding sites, one in the promoter region, one in the coding region and one in what could be still the promoter but also could form part of the intergenic region. Two of these sites, at positions -480 and +120, conform to the canonical CACGTG CDEI sequence; the third site, at position -185, has the CACATG sequence. Chromatin was analysed in diploid DY55 cells after 90 minutes exposure to nutrient starvation. A Cbflp dependent chromatin structure has been observed at this locus by comparing exponentially growing *CBF1* and *cbfl* cells (Kent *et al.*, 1999), these two different patterns can be clearly appreciated also here in Figure 24. This figure also shows that there is a Cbflp dependent chromatin reorganisation under nutrient starvation. Compare lanes *CBF1Y* and *CBF1K*; the new pattern in the second one is also different from the pattern in *cbfl* cells, which remains unchanged upon starvation. The reorganisation of chromatin in *CBF1* starved cells extends into the coding region, apparently beyond the CDEI site located at position +120. As for *MET16* and *MET17*, three different chromatin conformations can be found at this locus. These conformations are also different from the pattern of digested naked DNA, indicating again, that these patterns reflect some aspect of the organisation of the protein and DNA components of chromatin. Therefore, remodelling of chromatin also occurs at this locus in a Cbflp dependent manner.

Chromatin structure at the YAL060W locus

The YAL060 ORF is also located on Chromosome I (Bussey *et al.*, 1995), its deduced amino acid sequence has high similarity to the alcohol/sorbitol dehydrogenase but it is not known in which metabolic process it actually participates. This locus contains two potential Cbf1p binding sites at the 5' end of its putative coding region; one of these, at position +274 has the CACATG sequence and the other, at position +356, is a canonical CDEI site with the CACGTG sequence. Kent *et al* (1999) have shown that a Cbf1p dependent chromatin structure is associated at this locus, mapping at the region where the two potential binding sites are found. As shown in Figure 25, a Cbf1p dependent chromatin reorganisation occurs also at this locus. Once more, in addition to the patterns displayed by *CBF1* and *cbf1* cells, respectively, a third MNase accessibility pattern is generated by starving *CBF1* cells. In the new pattern two sites become less accessible and one site becomes more accessible. No alteration of the cleavage pattern is observed in starved *cbf1* cells, indicating that the reorganisation observed in *CBF1* cells is dependent on Cbf1p.

Chromatin structure at *GDH3* locus

The *GDH3* gene codes for an NADP-glutamate dehydrogenase which catalyses the reaction between ammonium ions and α -ketoglutarate to form L-glutamate in the glutamate biosynthesis pathway (Wilkinson *et al.*, 1996). Mutations on this gene cause a wrinkled colony phenotype; it is located on Chromosome I and contains a single potential DNA binding site of the type CACGTG at position +143, at the 5' end within the coding region. Due to polymorphism the *Sall* site used to generate the probe is not present in the DY55 genome. Therefore the analysis was performed in this case using the DBY745 (*CBF1*) strain and its isogenic *cbf1* counterpart, YAG93. This gene also presents Cbf1p dependent chromatin reorganisation, when cells are starved, as clearly observed in Figure 26; compare lanes *CBF1Y* with *CBF1K*, some sites are lost and some become less sensitive in the second one. The previously described differences between the patterns of exponentially growing *CBF1* and *cbf1* cells (Kent *et al.*, 1999) are also reproduced by this experiment, compare the two Y lanes. No changes are observed after starving *cbf1* cells, compare lanes Y and K for *cbf1* cells. This result indicates that the modifications in MNase accessibility patterns found in starved wild type cells are Cbf1p dependent.

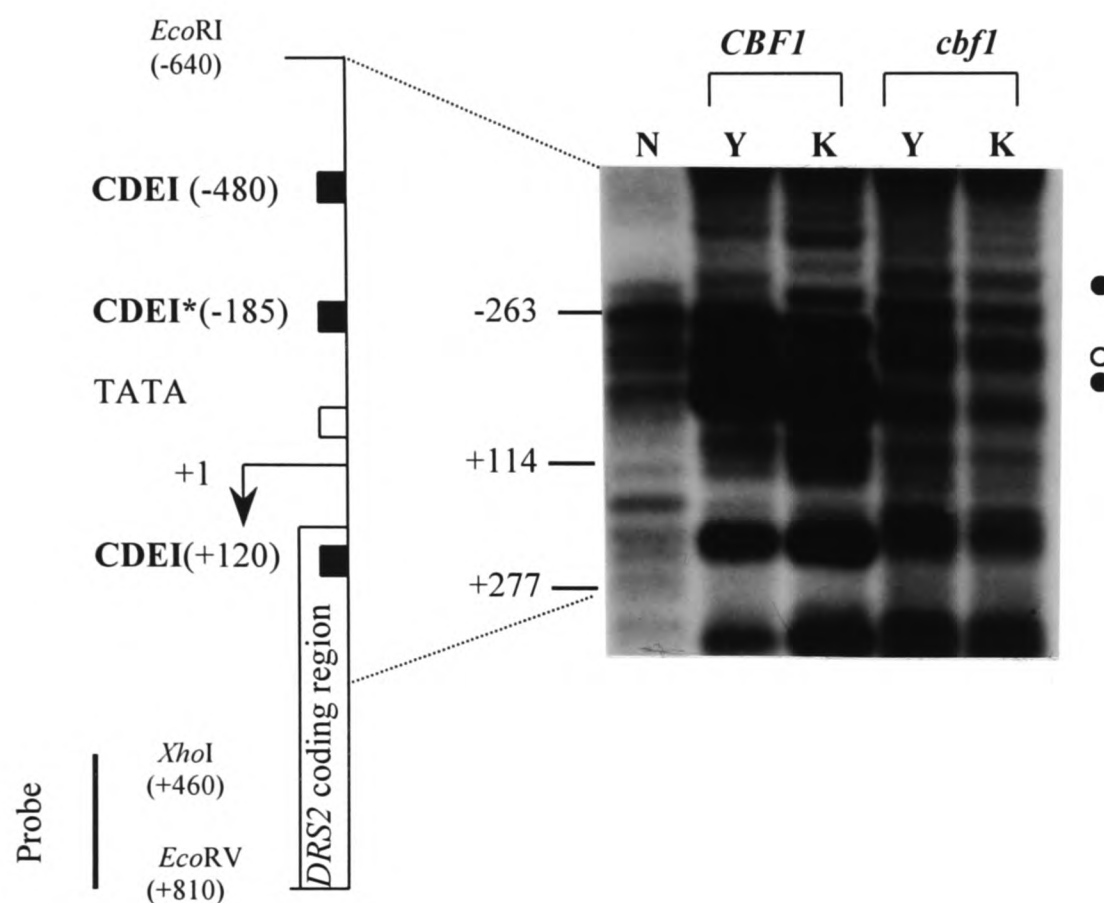


FIGURE 24. Chromatin structure at the *DRS2* locus in response to starvation in *CBF1* and *cbf1* cells. *CBF1*, wild type diploid cells; *cbf1*, *cbf1::URA3* diploid cells; N naked DNA; Y, rich YPD medium; K, sporulation medium, 1% potassium acetate. Exponentially growing cells were used for Y lanes; cells were starved for 90 minutes to prepare chromatin in K lanes. Chromatin was digested with MNase as described in Chapter 2. After phenol extraction each DNA sample was digested to completion with 10 units of *EcoRV* and restriction fragments separated on a 1.5% agarose gel. DNA was then transferred to a membrane and hybridised to the restriction fragment probe indicated in the left diagram. White dot indicates a site with decreased or lost accessibility and black dot indicates a new sites or a site with increased accessibility when *CBF1/CBF1* cells are transferred to nutrient starvation conditions. Asterisk denotes a CACATG site.

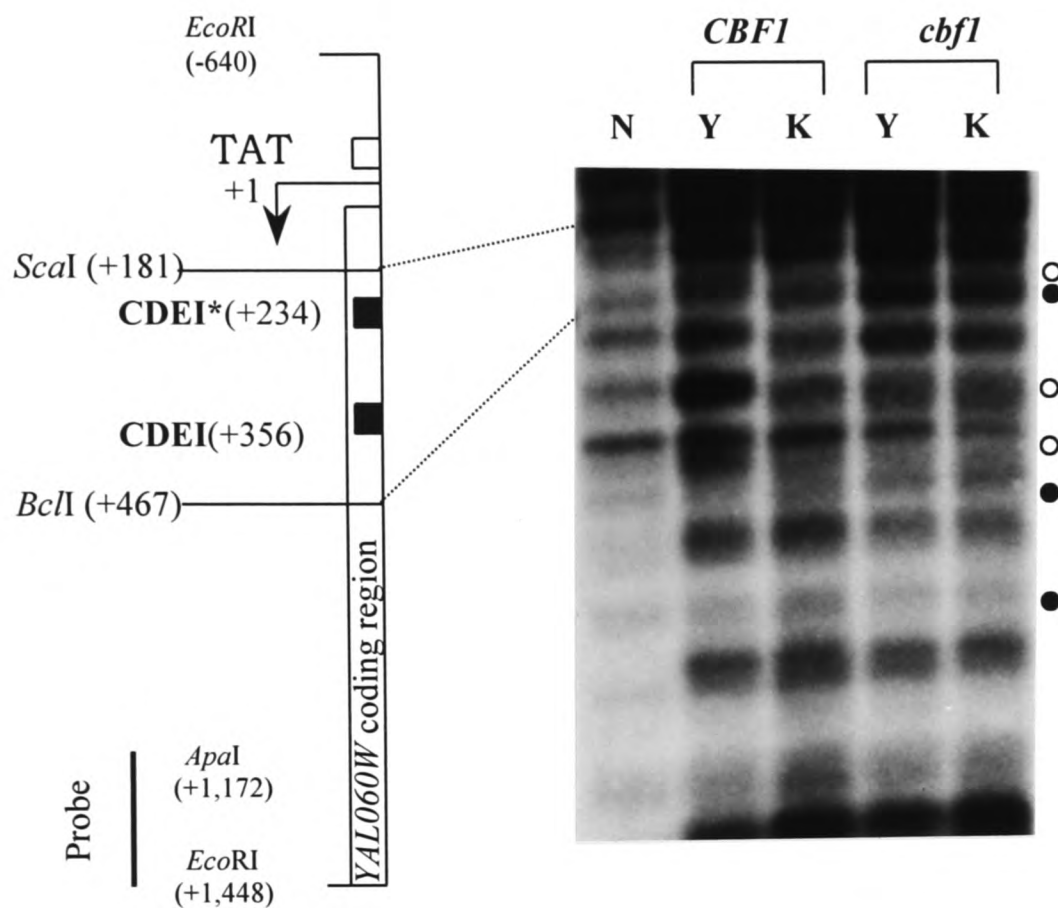


FIGURE 25. Chromatin structure at the YAL060W open reading frame in response to starvation in *CBF1/CBF1* and *cbf1/cbf1* cells. *CBF1*, wild type cells; *cbf1*, *cbf1::URA3* cells; M, markers; N naked DNA; Y, rich YPD medium; K, sporulation medium, 1%KAc. Exponentially growing cells were used for Y lanes; cells were starved for 90 minutes to prepare chromatin in K lanes. Chromatin was digested with MNase as described in Chapter 2. After phenol extraction each DNA sample was digested to completion with 10 units of *EcoRI* and restriction fragments separated on a 1.5% agarose gel. DNA was then transferred to a membrane and hybridised to the restriction fragment probe indicated in the left diagram. White dots indicate sites accessible to MNase which are lost in *CBF1/CBF1* cells when transferred to nutrient starvation conditions, black dots indicate sites with increased sensitivity. The asterisk indicates that the Cbf1p binding site contains the CACATG sequence.

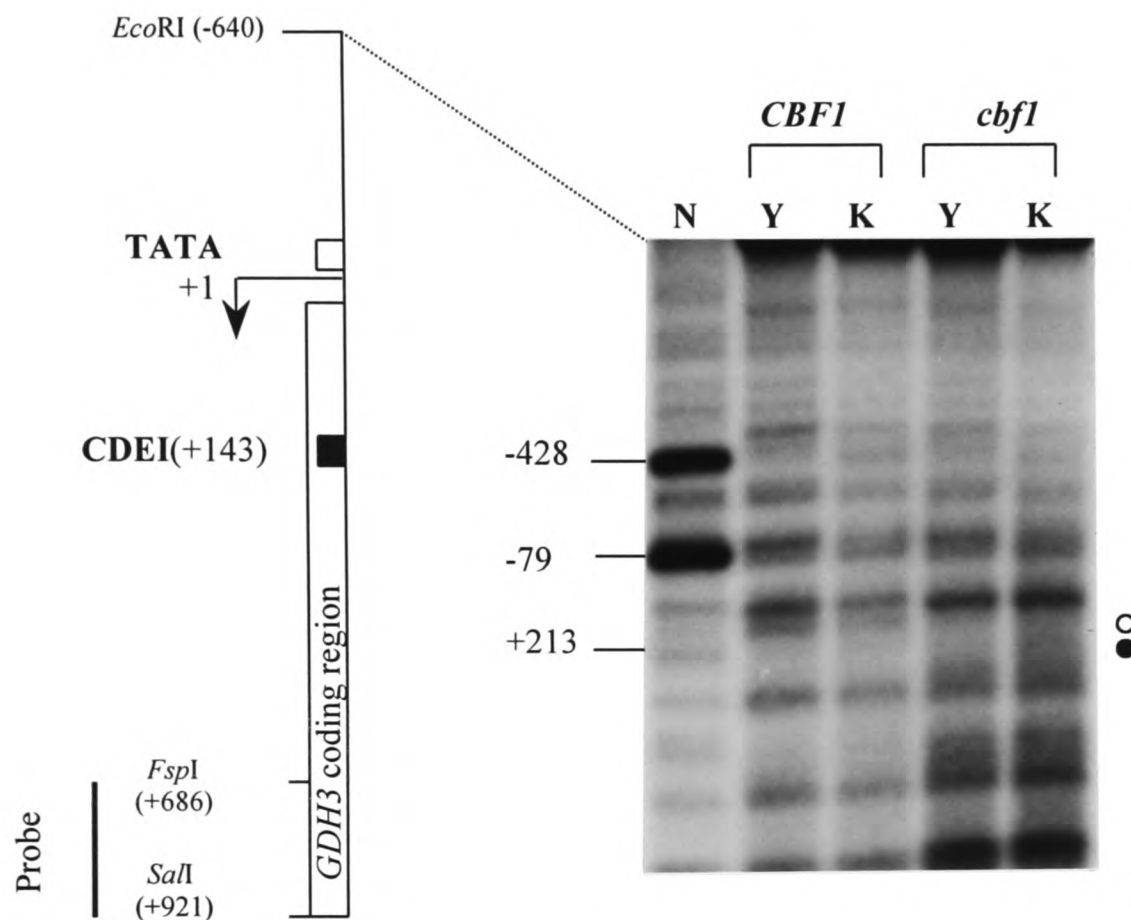


FIGURE 26. Chromatin structure at the *GDH3* locus in response to starvation in *CBF1* and *cbf1* cells. *CBF1*, DBY745 wild type cells; *cbf1*, null strain YAG93; M, markers; N naked DNA; Y, rich YPD medium; K, sporulation medium, 1%KAc. Exponentially growing cells were used for Y lanes; cells were starved for 90 minutes to prepare chromatin in K lanes. Chromatin was digested with MNase as described in Chapter 2. After phenol extraction each DNA sample was digested to completion with 10 units of *SalI* and restriction fragments separated on a 1.5% agarose gel. DNA was then transferred to a membrane and hybridised to the restriction fragment probe indicated in the left diagram. White dots indicate sites with decreased or lost accessibility and black dots indicate new sites or sites with increased accessibility when *CBF1* cells transferred to nutrient starvation conditions.

4.5 Chromatin structure at a locus lacking Cbflp-binding sites: *HIS3*

The analysis of all previous loci shows that a Cbflp dependent chromatin reorganisation occurs at and around various types of potential Cbflp binding sites. However, it is possible that under these conditions the chromatin is generally reorganised in a Cbflp dependent manner regardless whether a specific locus has CDEI sites or not. The promoter region of the *HIS3* locus does not contain any potential Cbflp binding motifs and it has been shown that its chromatin structure is not altered in *cbfl* cells (Kent, 1994); it is located on chromosome XV. Its chromatin structure was analysed under starvation conditions; no difference in the chromatin structure, as reflected by the MNase accessibility patterns, was found between *CBF1* and *cbfl* cells nor between log phase and starved cells at this locus, as can be seen in Figure 27. The MNase accessibility patterns are the same in all four lanes representing four different conditions.

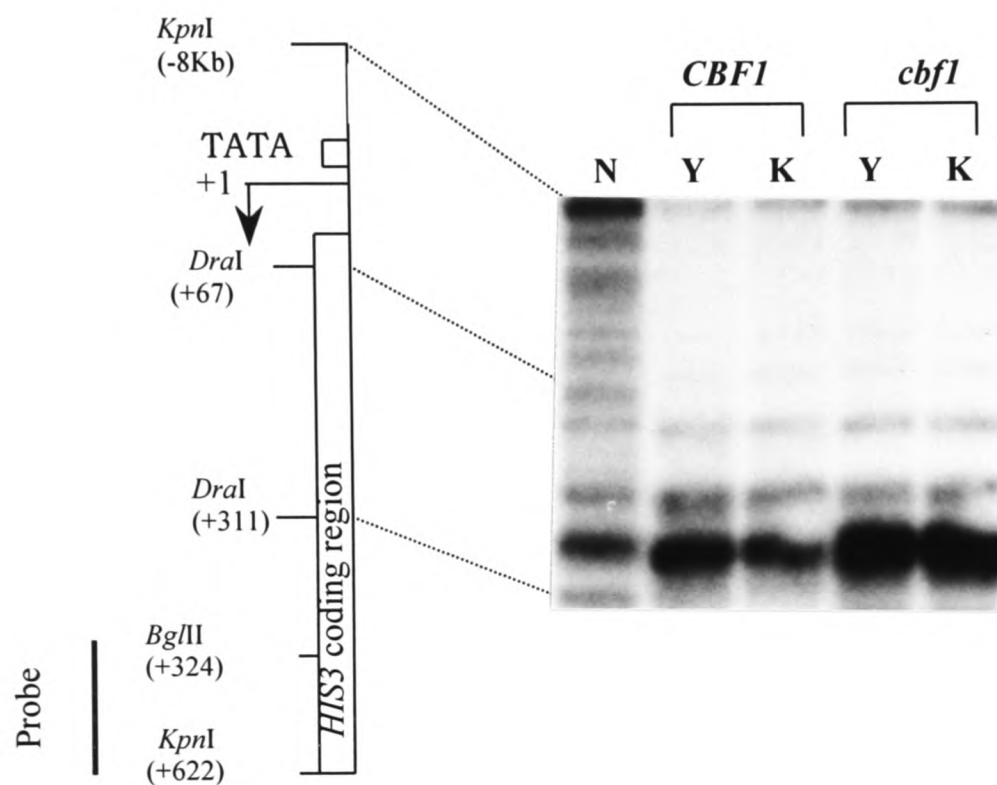


FIGURE 27. Chromatin structure at the *HIS3* locus in response to starvation in *CBF1/CBF1* and *cbf1/cbf1* cells. *CBF1*, wild type cells; *cbf1*, *cbf1::URA3* cells; M, markers; N, naked DNA; Y, rich YPD medium; K, sporulation medium, 1%KAc. Exponentially growing cells were used for Y lanes; cells were starved for 90 minutes to prepare chromatin in K lanes. Chromatin was digested with MNase as described in Chapter 2. After phenol extraction each DNA sample was digested to completion with 10 units of *KpnI* and the restriction fragments separated on a 1.5% agarose gel. DNA was then transferred to a membrane and hybridised to the restriction fragment probe indicated in the left diagram.

4.6 Discussion

Although it is known that the lack of a fully functional Cbf1p strongly affects the meiotic process, no attempts have been made to follow the process and determine at which point cells become impaired. Figure 19 shows that the disruption of the *CBF1* gene renders cells otherwise highly efficient in meiosis unable to enter the pre-meiotic S phase. This is consistent with previous findings that only 10% of colonies, produced by mating identical haploid *cbf1* strains, are able to sporulate (Cumberledge and Carbon, 1987). The VGD2 *cbf1/cbf1* cells used here appear to be blocked at the G1 to S phase transition and the 4N cells appearing in the FACS profile are in fact G2 mitotic cells, since fluorescence microscopy shows many budding cells (data not shown). It has been previously shown that mutations in Cbf1p affect the cell cycle increasing the generation time in null mutants (Mellor *et al.*, 1990) and increasing the growth rate when the putative phosphorylation site S226 is mutated (Nissom, 1998). This may explain the inability of *cbf1/cbf1* cells to synchronise at G1 in pre-sporulation medium, but does not explain their inability to enter meiosis once cells reach G1 at different times when transferred to sporulation medium. It is possible that *cbf1/cbf1* cells fail to sense the nutrient status in their environment or fail to transfer the signal, which in wild type cells leads either, to an eventual G1 arrest when cells are in pre-sporulation medium, or to pre-meiotic DNA replication when cells are in sporulation medium.

Results presented in the previous chapter suggest that chromatin remodelling occurs at CDEI sites in a/α cells undergoing meiosis; here is shown that such reorganisation is in fact dependent on the presence of Cbf1p. Both diploid cells, which can undergo meiosis and haploid cells, which cannot, display a modification of the MNase cleavage patterns at *MET16* and *MET17* CDEI sites upon starvation. In contrast diploid *cbf1/cbf1* cells, isogenic with those used in the previous chapter, do not present any modification of chromatin structure at *MET16* locus over time when they are starved in sporulation medium. The pattern remains unchanged over a similar period as that covered for wild type cells. In haploid wild type cells, subject to nutrient starvation, patterns similar to those found in diploid cells undergoing meiosis are also generated (see Figures 21-23). The fact that similar chromatin rearrangements occur in haploid cells at the two *MET* loci indicates that these changes are not specific for meiotic cells. Haploid *cbf1* cells are also unable to display any modification of the MNase accessibility patterns at *MET16* and *MET17* when cells are starved. Not only an analysis of the *MET* loci was carried out in

haploid cells, the *GDH3* locus was also analysed in this type of cells and in a different genetic background (strain DBY745). Therefore, the reorganisation of chromatin seems to be triggered by nutrient conditions and it occurs regardless of cell type, suggesting that it is part of a more general response. The data presented here for other loci containing potential Cbf1p binding sites extend the results presented in Chapter 3; they also confirm that the generation of new MNase cleavage patterns at these sites is dependent on Cbf1p. Cells bearing a disrupted or deleted *cbf1* gene are unable to display this reorganisation upon starvation at the loci analysed, this suggests that the sensing of the environmental nutrient conditions and/or the response involving the chromatin reorganisation are disrupted or destroyed in *cbf1* cells.

In all cases the new MNase accessibility patterns generated when cells are exposed to starvation medium are clearly different from the patterns of digested naked DNA, suggesting that some aspect of the chromatin organisation becomes modified under these conditions. In all loci containing potential Cbf1p binding sites the new patterns originated by nutrient starvation are dependent on Cbf1p. In contrast, under the same conditions, no modification in the MNase cleavage patterns is observed at the promoter region of the *HIS3* locus, which does not contain binding sites for Cbf1p. This suggests that the reorganisation detected at the other loci is specific for Cbf1p binding sites.

The proteins encoded by 3 of the 5 genes analysed here seem to be unrelated both amongst themselves, and with the *MET* genes, since they participate apparently in quite diverse cellular functions. They also fall into various categories regarding the specific sequence of the potential Cbf1p binding site(s), its neighbouring sequences, the actual number of potential Cbf1p sites and their location respect to the putative initiation of transcription site (including various locations within the coding region). These varied features indicate that the Cbf1p dependent reorganisation of chromatin is independent of many characteristics of the loci, with the only condition being the presence of at least one Cbf1p binding site. This suggests that Cbf1p may have a general function, involving the reorganisation of chromatin at the multiple loci which present single sites and clusters of potential Cbf1p binding sites, that have been found through the yeast genome (Kent *et al.*, 1999). This Cbf1p dependent chromatin reorganisation at and around multiple potential Cbf1p binding sites occurs when yeast cells are starved. This might suggest that Cbf1p participates in the sensing of the nutritional state of cells and that it is required for a response involving the reorganisation of chromatin at many locations. The inability of most *cbf1* cells to enter meiosis may be related to the inability of these cells to remodel

chromatin. This Cbf1p dependent chromatin remodelling may be somehow required to initiate pre-meiotic DNA replication. The main conclusions of this chapter are that the structure of chromatin at and around potential Cbf1p-binding sites found at various loci becomes modified when yeast cells are starved and that this reorganisation of chromatin is dependent on Cbf1p.

The sporulation medium used to conduct the experiments shown in this and the previous chapter starves cells for almost all nutrients; an analysis of the possible involvement of various key nutrients in signalling this rearrangement is presented in the following chapter. Since the remodelling of chromatin is dependent on Cbf1p, it is important to initiate an investigation to determine which of its functional domains are required for this reorganisation to occur, this is also presented in the following chapter.

Chapter 5

Functional Analysis

5.1 Introduction

Cbflp is known to be required to maintain specific chromatin structures at CDEI sites *in vivo* (Kent *et al.*, 1994; Mellor *et al.*, 1990). One of the modes of action of Cbflp proposed by Kent *et al* involves a form of Cbflp bound to DNA required for such chromatin structures to be maintained *in vivo*. In addition to that, rather passive function, Cbflp plays a key role in the *in vivo* dynamic reorganisation of chromatin at multiple loci containing Cbflp binding sites in the yeast genome, as shown by data presented in Chapters 3 and 4. As the accessibility to the DNA is modified at and around Cbflp binding sites when cells are starved, an obvious question is whether Cbflp remains bound to DNA during this process and whether the DNA binding activity is required for the reorganisation of chromatin. Cbflp is a modular protein containing various domains whose function has been studied by sequence and mutational analysis, it contains a basic DNA binding region as part of the bHLH domain (see Section 1.3). Although DNA binding activity of Cbflp is not required to maintain methionine prototrophy some weak DNA binding mutants are unable to maintain a wild type chromatin structure at *MET16* and *MET17* loci (Kent *et al.*, 1994). In this Chapter attempts are made to determine the relevance of the DNA binding domain and activity of Cbflp for the remodelling of chromatin observed at Cbflp binding sites.

The alignment of the basic region of the two known Cbflp proteins (from *S. cerevisiae* and *Kluyveromyces lactis*) and the putative Cbflp from *Schizosaccharomyces pombe* with USF, Max and Pho4 has revealed a serine residue conserved among these proteins (Nissom, 1998). In Pho4, a yeast activator of transcription, this residue is phosphorylated by cAMP dependent kinase (PKA) to regulate its activity in chromatin remodelling and activation of transcription (Fisher and Goding, 1992); mutations at this position also have an effect on its DNA binding activity (see Section 1.5). It has been shown that Cbflp is phosphorylated *in vitro* (Niedenthal *et al.*, 1995); a residue at position 226 in Cbflp has been revealed as a putative phosphorylation site when its primary sequence was analysed with the Scan Prosite program (Nissom, 1998). This serine residue has been mutated either to alanine or glutamic acid in order to mimic a protein unable to be phosphorylated and one permanently phosphorylated, respectively. These mutations do not

alter the ability of Cbflp to bind specifically to CDEI DNA *in vitro*, but show alterations of the cell cycle *in vivo* (Nissom, 1998). Since Cbflp seems to be participating in a pathway which involves sensing the nutrient content in the environment of cells and a response involving chromatin reorganisation, an interesting possibility is that a signal is transmitted to Cbflp by phosphorylation. The two mutants S226A and S226E are used here to explore the relevance of this putative phosphorylation site in the chromatin reorganisation described in the previous two chapters.

When cells are transferred to sporulation medium they are starved for every nutrient, except a carbon source. In order to determine the lack of which nutrient(s) triggers the chromatin remodelling, various media were tested. Since the transcriptional activity of *MET16* is dependent on the content of methionine in the medium, the experiments were carried out using different media with or without methionine, in order to analyse the relationship between the reorganisation of chromatin structure described in previous chapters and the transcriptional activity at this locus.

5.2 CDEI DNA binding activity during chromatin remodelling

The CDEI binding activity of Cbflp was analysed during the meiotic process by taking samples at the same time points as for the chromatin analysis. This activity was tested using an electrophoretic mobility shift assay (EMSA; Dent and Latchman, 1991). A double stranded synthetic oligonucleotide containing both the CACGTG recognition site and the flanking sequences required for specific binding (Fisher and Goding, 1992) was used in these experiments (see Chapter 2). Crude protein extracts were made in high salt buffer to release DNA bound proteins and the reactions were made in salt free binding buffer; non denaturing TGE gels were used to analyse the DNA-protein complexes formed. Figure 28A shows that a fast migrating protein-DNA complex is formed in protein extracts from cells growth in pre-sporulation medium and in cells undergoing meiosis, these two media have in common the presence of potassium acetate as carbon source. The complex is specific, since it is not competed by a 1000 fold excess unlabeled non-specific competitor in all reactions but is competed off by a 500 fold excess of unlabeled specific oligonucleotide (S lanes). This complex is of a lower mobility than those produced by a protein extract from a strain grown in rich medium (lane R).

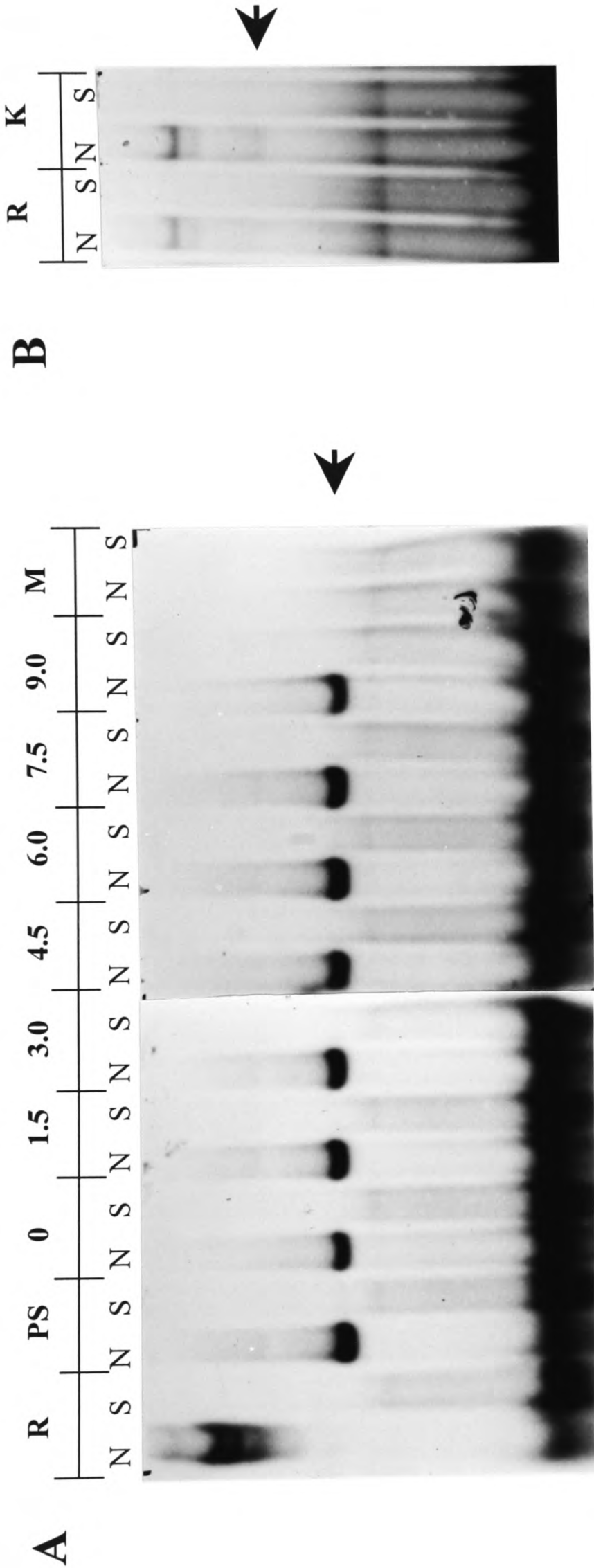


FIGURE 28. Specific CDEI DNA binding activity. (A) CDEI DNA binding activity in DY55 cells undergoing meiosis. Gel shift assays were carried out using protein crude extracts. R, cells growing in rich medium; PS, cells grown in pre-sporulation medium; numbers indicate the time intervals cells were incubated in sporulation medium; M, mock reaction. (B) CDEI DNA binding activity in 2208 Y55 haploid cells. R, protein crude extract from cells incubated in rich medium; K protein crude extract from cells incubated in potassium acetate for 45 minutes. N, non-specific DNA competitor; S, specific DNA competitor (unlabelled CDEI probe). Arrows indicate specific CDEI DNA-protein complexes present only in protein crude extracts from cells incubated in potassium acetate. Protein crude extracts were prepared as described in Chapter 2.

Since the chromatin reorganisation also occurs in haploid cells the DNA binding activity in these cells under starvation was also analysed. A different protein extraction buffer was used in the analysis of the formation of these DNA-protein complexes. Figure 28B shows that the CDEI DNA binding activity is also maintained in haploid cells under the conditions that trigger the remodelling of chromatin. Two DNA-protein complexes are evident in the sample from cells grown in rich medium. A third complex of faster mobility is formed in the sample from starved cells, similar to that of diploid cells grown in pre-sporulation or sporulation medium; this form clearly retains the DNA-binding domain and specific DNA-binding activity of Cbflp. These findings indicate that a particular form of Cbflp, the one with faster mobility, is predominant in cells grown in medium containing potassium acetate as carbon source and in starved cells, suggesting some processing of this factor under these conditions. To test the requirement of CDEI DNA binding activity in chromatin remodelling, the chromatin structure of the non-DNA binding mutant YAG214 (Cbflp_{E231A}; Mellor *et al.*, 1991) was analysed under starvation. The mutant YAG214 does not produce protein-DNA complex in gel shift assays, it is however, a methionine prototroph and it has been therefore suggested that a form of non-bound Cbflp functions to maintain methionine independent growth; *in vivo* the YAG214 mutant is unable to modulate chromatin structure at *MET* genes (Kent *et al.*, 1994). Figure 29 show that there is no chromatin remodelling in starved YAG214 cells, chromatin structure remains unchanged during starvation. This implies that the DNA binding activity of Cbflp is required to remodel chromatin at *MET16*.

To further investigate the correlation between chromatin remodelling and meiosis competence, a diploid α/α 214 strain able to undergo rapid and synchronous meiosis was produced by crossing YAG214 strains with Y55 carrying *cbf1::URA3* insertions. *cbf1::URA3*/214 diploid cells sporulate efficiently. Asci were dissected and haploid cells with the 214 version of Cbflp were selected. These 214 haploid strains were mated and diploid colonies isolated in selective medium. The diploid condition was confirmed by FACS analysis (Figure 30). These diploid 214/214 cells were induced to enter meiosis using the same method as for previous experiments. None of the 214/214 (VGA1 strain) cultures was able to enter meiosis. This suggests that the DNA binding activity required for the Cbflp dependent chromatin remodelling is also required for cells to enter meiosis and that the inability of *cbf1* cells to enter meiosis may not be related to the methionine phenotype, since the YAG214 cells with the E231A mutation are methionine prototrophs.

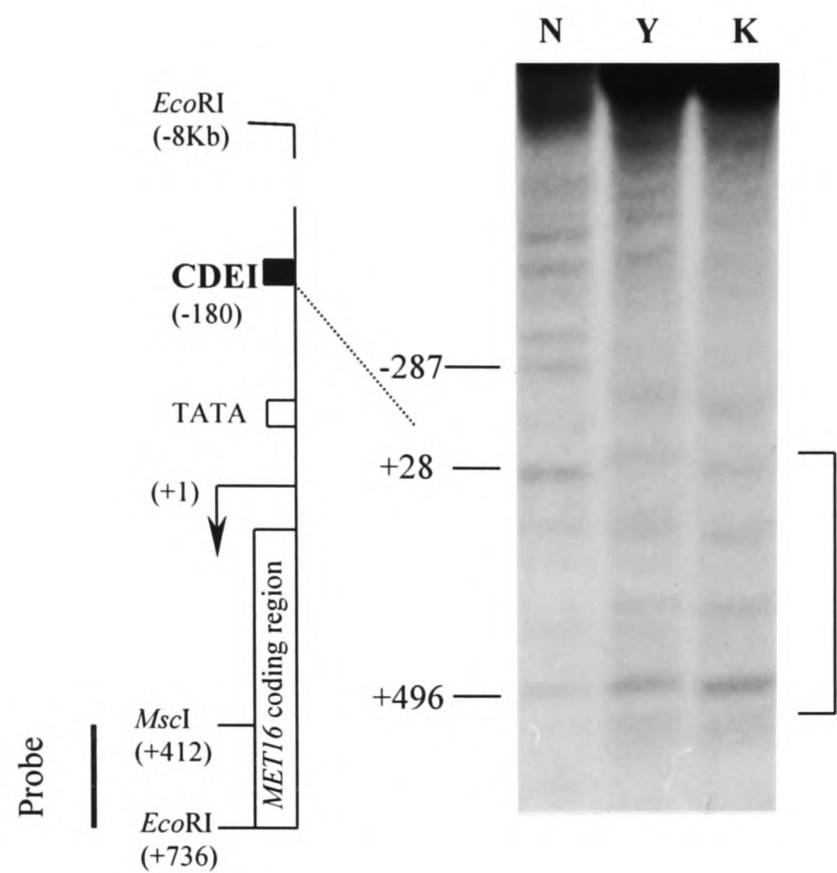


FIGURE 29. Chromatin structure at *MET16* locus in starved cells expressing the 214 mutant Cbflp. N, naked DNA; lane Y, exponentially growing cells; lane K, cells harvested after 45 minutes incubation in sporulation medium. Chromatin was digested with MNase as described in Chapter 2. After phenol extraction DNA samples were digested to completion with *EcoRI* and restriction fragments separated on a 1.5% agarose gel. DNA was then transferred to a membrane and hybridised to the restriction fragment probe indicated in the left side diagram. The bracket indicates the region at which modification of cleavage pattern is seen in starved wild type cells.

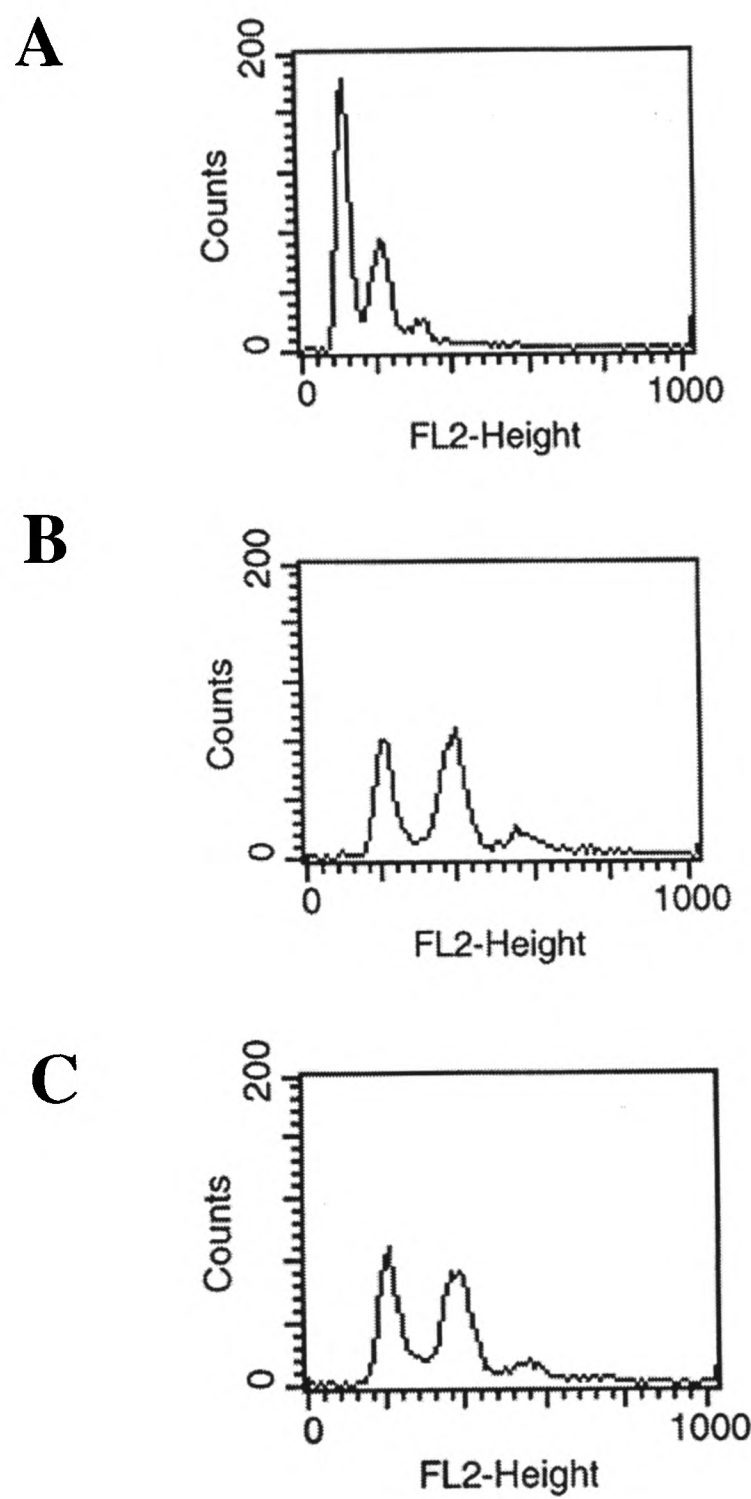


FIGURE 30. Flow cytometry analysis of 214 diploid cells (VGA1). **A.** Starved haploid cells; these cells show a big 1N peak and small 2N peak. **B.** and **C.** Two different 214/214 diploid clones; these cells show 2N and 4N peaks, but are unable to enter meiosis and sporulate.

5.3 A potential phosphorylation switch for the regulation of the Cbf1p dependent chromatin remodelling

Phosphorylation is a signal frequently used to control the activity of proteins. For instance, the activity of the bHLH activator of transcription Pho4 is regulated through phosphorylation of serine residues by the cyclin-cdk complex Pho80-Pho85 (Fisher and Goding, 1992) and by PKA. The alignment of Pho4 and Cpf1 has revealed that Cpf1 has a serine residue at a position (226) homologous to that which is phosphorylated by PKA in Pho4. This residue was mutated to alanine and to glutamic acid in Cbf1p; cells bearing these mutations were a gift from P. Nissom and they show alteration of the cell cycle (Nissom, 1998). Both of these mutants are able to bind DNA in gel shift assays. As Cbf1p seems to be involved in the remodelling of chromatin at multiple sites under starvation, experiments were performed to determine whether this residue is relevant for such activity. The ability of these two mutants to maintain and modify chromatin structure at *MET16* was tested by giving the same treatment to cells expressing each one. Their expression was induced in the YAG93 *cbf1* null mutant from pYG *CEN* based plasmids, with the *CBF1* gene under the control of the *GAL* promoter (Nissom, 1998).

Figure 31 shows that mutant S226A is able to restore the chromatin structure observed in wild type cells at *MET16* when its expression is induced. However, when cells are transferred to starvation medium they are unable to display a remodelled chromatin structure. These data indicate that the S226A mutant is able to function, as the wild type protein, in maintaining the chromatin structure associated at this locus, but cells bearing it are unable to sense starvation conditions and/or to trigger the chromatin remodelling arising in wild type starved cells. In contrast, upon induction of expression of the S226E mutant in rich medium, it presents a chromatin structure, which matches the remodelled pattern found in wild type starved cells (Figure 32, lane +/C). When starved, these cells maintain this same chromatin structure. Therefore, the mutant S226A seems to be always correlating with a non-remodelled state of chromatin structure whereas the mutant S226E correlates with a remodelled chromatin structure. These results suggest that to maintain the basal chromatin structure, found in exponentially growing cells, a non-phosphorylated state of Cbf1p residue S226 is required. They also suggest that this residue has to be modified in order to achieve the chromatin reorganisation triggered by starvation.

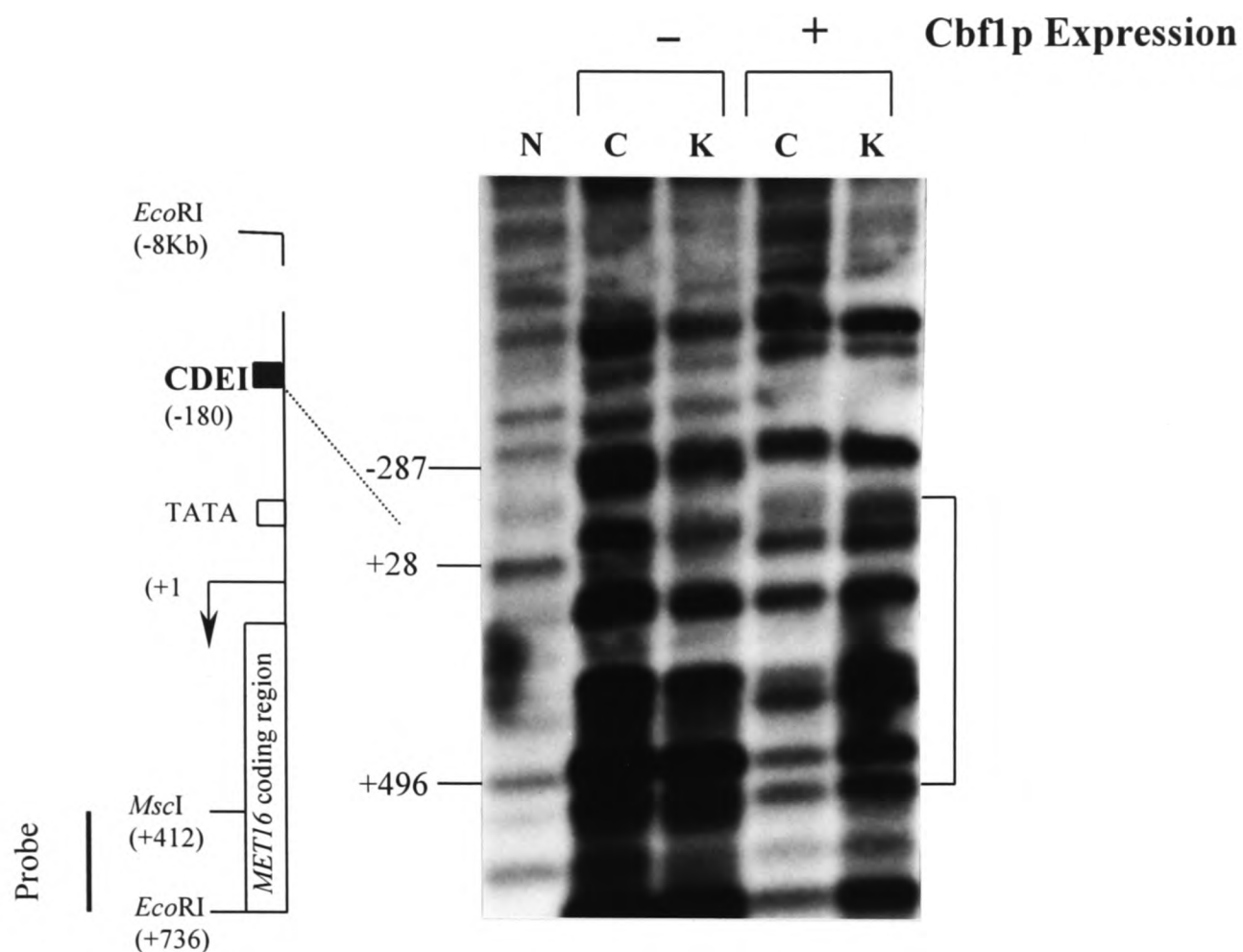


FIGURE 31. Chromatin structure at *MET16* locus in cells bearing a plasmid coding for the S226A mutant version of Cbf1p. Analysis by indirect end labelling. Top: N, naked DNA; -, cells non induced to express Cbf1p protein; +, cells induced to express Cbf1p protein; C, cells grown in synthetic complete medium; K, after incubation in inducing or non inducing synthetic complete medium cells were starved in 1% potassium acetate for 45 minutes. Cells were harvested, sphaeroplasted and chromatin digested with 150u/ml MNase (as described in Chapter 2). After phenol extraction each sample was digested to completion with *EcoRI* and the restriction fragments separated on a 1.5% agarose gel. DNA was then transferred to a membrane and hybridised to the probe indicated in the diagram to the left. The bracket indicates the region at which starved wild type cells display a modification of the MNase accessibility pattern.

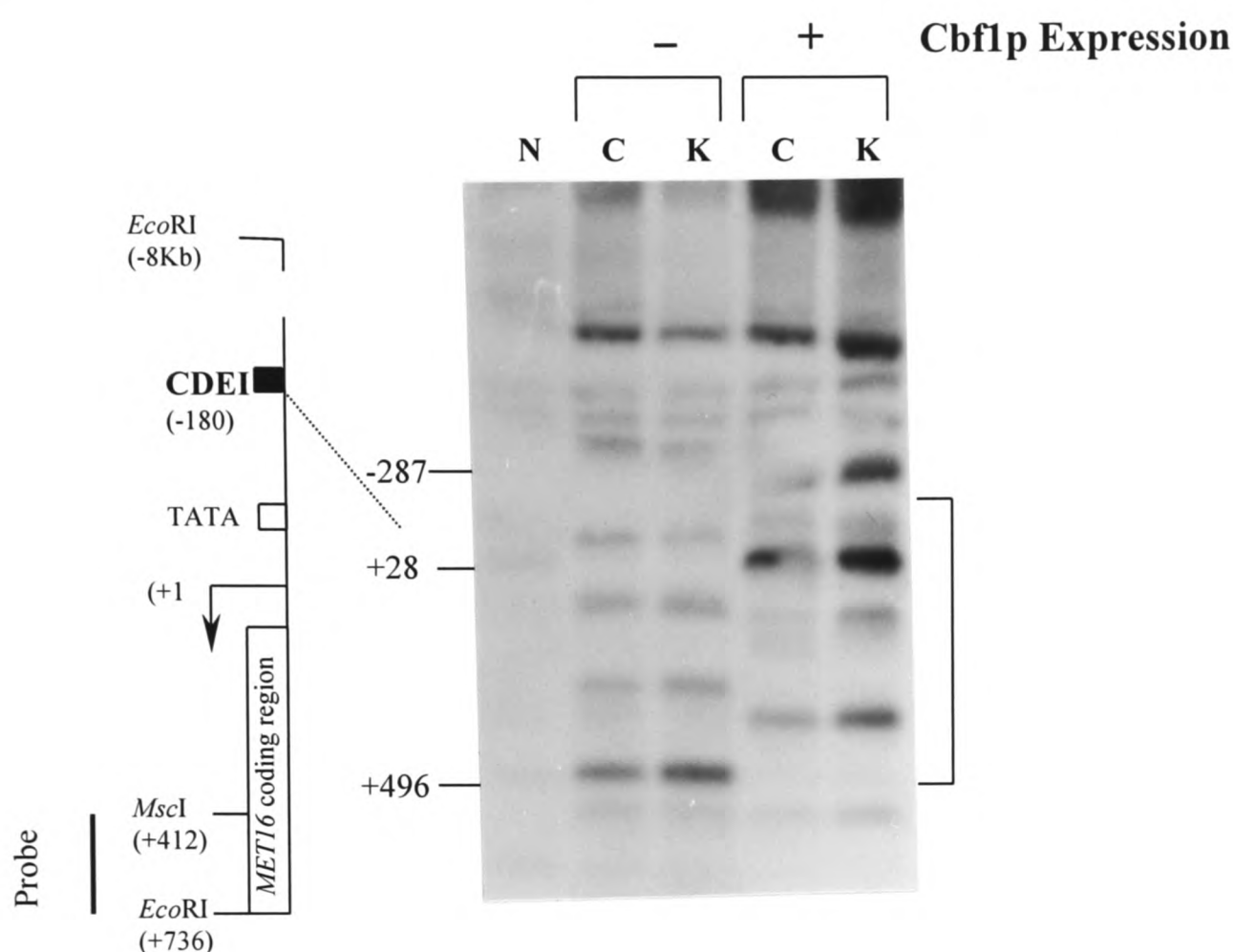


FIGURE 32. Chromatin structure at *MET16* locus in cells bearing centromeric plasmids coding for the S226E mutant version of Cbflp. Analysis by indirect end labelling. Top: N, naked DNA; -, cells non induced to express Cbflp protein; +, cells induced to express Cbflp protein; C, cells grown in synthetic complete medium; K, after incubation in inducing or non inducing synthetic complete medium cells were starved in 1% potassium acetate for 45 minutes. Cells were harvested, sphaeroplasted and chromatin digested with 150u/ml MNase (as described in Chapter 2). After phenol extraction each sample was digested to completion with *EcoRI* and the restriction fragments separated on a 1.5% agarose gel. DNA was then transferred to a membrane and hybridised to the probe indicated in the diagram to the left. The bracket indicates the region at which starved wild type cells display a modification of the MNase accessibility pattern.

5.4 Nutritional analysis

In order to determine lack of which nutrients trigger the remodelling of chromatin, Cbf1p cells were incubated in a variety of media, each lacking a particular key nutrient. As the chromatin was analysed at *MET16* and starvation for methionine also induces sporulation, both repressing and derepressing conditions for the transcriptional activation of this gene were used in combination with the nutrient analysis. For this analysis, cells were grown in rich medium (YPD) to exponential growth phase, harvested, washed twice in sterile water, resuspended in the indicated medium and incubated at 30°C for 90 minutes before *in vivo* digestion of chromatin.

Effect of phosphate content

Following the results in the previous section showing that a the putative phosphorylation site is required for the chromatin remodelling and the suggestion that a cross talk between sulfur and phosphate metabolism may be occurring (O'Connell and Baker, 1992), it seemed relevant to analyse the effect of inorganic phosphate content on the regulation of chromatin structure at this location. Figure 33 shows that phosphate content does not have any effect on chromatin structure at this location, chromatin structure is the same in low phosphate as it is in high phosphate and in rich medium. Surprisingly only the absence of methionine in both conditions, high and low phosphate content, is able to trigger the chromatin reorganisation. Therefore, the signal regulating the chromatin remodelling is not directly dependent on phosphate content in the cells. The high phosphate experiment is equivalent to incubating cells in rich medium since it contains glucose as carbon source, withdrawing methionine under this condition is therefore equivalent to de-repressing the *MET16* gene. It is surprising that the sole absence of methionine triggers a reorganisation of chromatin, particularly considering that previous efforts reported a chromatin structure similar to naked DNA under these conditions (O'Connell *et al.*, 1995).

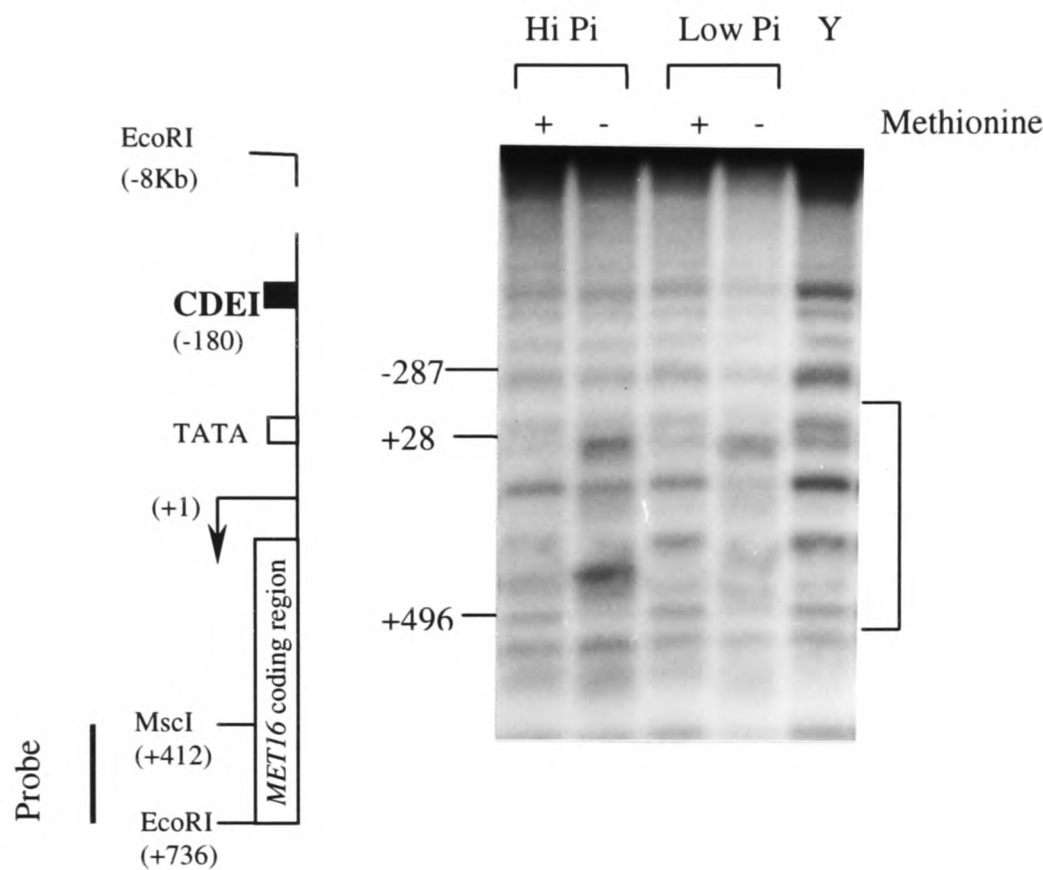


FIGURE 33. Effect of phosphate content on the chromatin structure at *MET16* under repressing and de-repressing conditions. Top: N, naked DNA; Y, YPD. Cells were incubated in synthetic complete medium (see Chapter 2) containing inorganic phosphate either at high (Hi Pi) or low concentration (Low Pi) as indicated. The presence (+) of 1mM methionine or its absence (-) is also indicated. For each condition a culture was incubated for 90 minutes. Chromatin was digested *in vivo* with 150u/ml MNase (as described in Chapter 2). Each purified DNA sample was digested to completion with EcoRI and the restriction fragments separated on a 1.5% agarose gel. DNA was transferred to a membrane and hybridised to the probe indicated in the left diagram. The bracket indicates the region at which chromatin becomes modified.

Effect of carbon source on chromatin structure

Potassium acetate. Synthetic complete media with potassium acetate as carbon source was used to incubate cells for 90 minutes. Figure 34 shows that the sole presence of potassium acetate as part of a complete medium does not trigger the chromatin remodelling. Under these conditions only the absence of methionine causes a remodelling of *MET16* chromatin.

Glucose. A synthetic complete medium was used with glucose as carbon source, either having or lacking methionine. Figure 35 confirms that the sole absence of methionine induces reorganisation of chromatin at *MET16*, as suggested by the high phosphate result (see Figure 34).

Galactose. A synthetic complete medium was used to test the effect of galactose as carbon source in the presence or absence of methionine. Figure 36 shows that when galactose is the carbon source in medium containing methionine, the chromatin structure at *MET16* is similar to that found in exponentially growing cells when glucose is the carbon source. However, in contrast with media containing glucose as carbon source, when cells are incubated in medium with galactose and lacking methionine no modification of the chromatin structure is observed at *MET16*.

5.5 Transcriptional activity and chromatin remodelling at *MET16*

The fact that the sole lack of methionine triggers reorganisation of chromatin in media containing glucose or acetate as carbon source suggest that derepression of the *MET16* promoter may correlate with the remodelling of chromatin. Therefore, the transcriptional activity of this locus was analysed under those conditions. Figure 37 shows that a basal level of *MET16* mRNA is maintained in the presence of glucose as carbon source and that it is indeed increased in these cells in the same medium in the absence of methionine, since the mRNA increases (five fold) as seen by Northern blot analysis. This suggests that chromatin remodelling at *MET16* and an increase in transcription activity are concomitant events under these conditions. When galactose is the carbon source the increase in transcription activity in the absence of methionine is only slightly higher (two fold) than basal level. In synthetic medium containing acetate as carbon source the lack of methionine triggers reorganisation of chromatin, but the increase in transcription activity is only similar to that in galactose (where no chromatin reorganisation is observed). These suggest that under particular conditions these two events can be independent and that the control of transcription at this locus is influenced by carbon source.

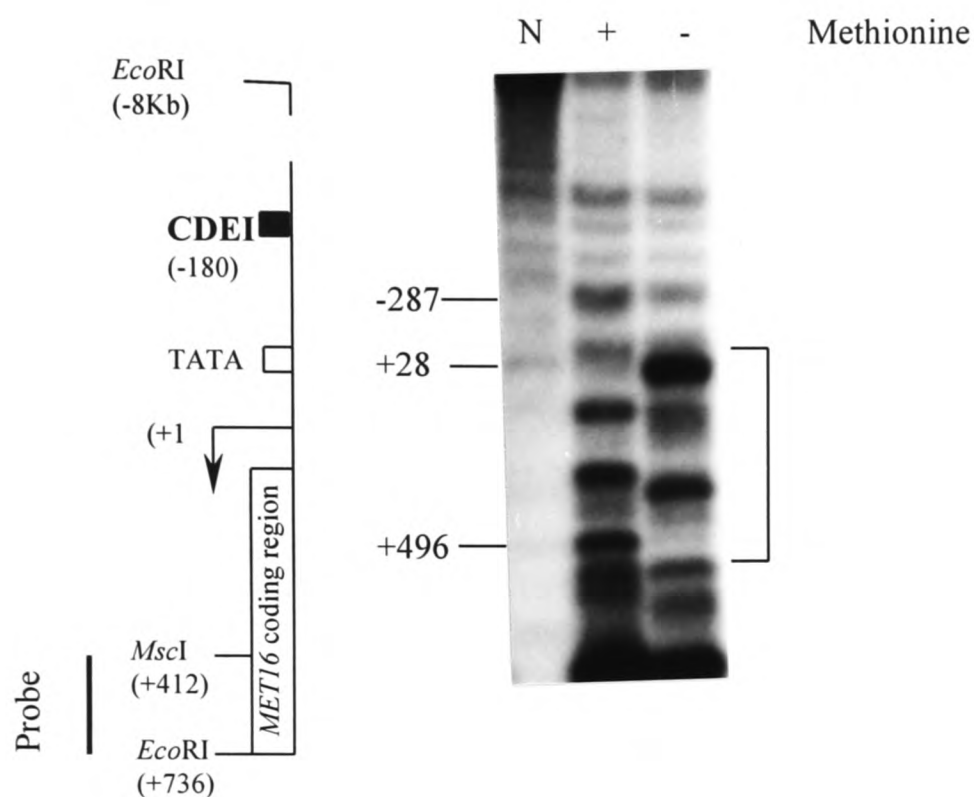


FIGURE 34. Effect of potassium acetate as carbon source on the chromatin structure at *MET16* locus under repressing and derepressing conditions. Cells were incubated in YPD and then washed and transferred to synthetic media containing potassium acetate 1% as carbon source. Top: N, naked DNA. The presence (+) of methionine 1mM or its absence (-) is also indicated. Chromatin was digested *in vivo* with 150u/ml MNase (as described in Chapter 2). Each purified DNA sample was digested to completion with *EcoRI* and the restriction fragments separated on a 1.5% agarose gel. DNA was transferred to a membrane and hybridised to the probe indicated in the left diagram. The bracket indicates the region at which chromatin becomes modified.

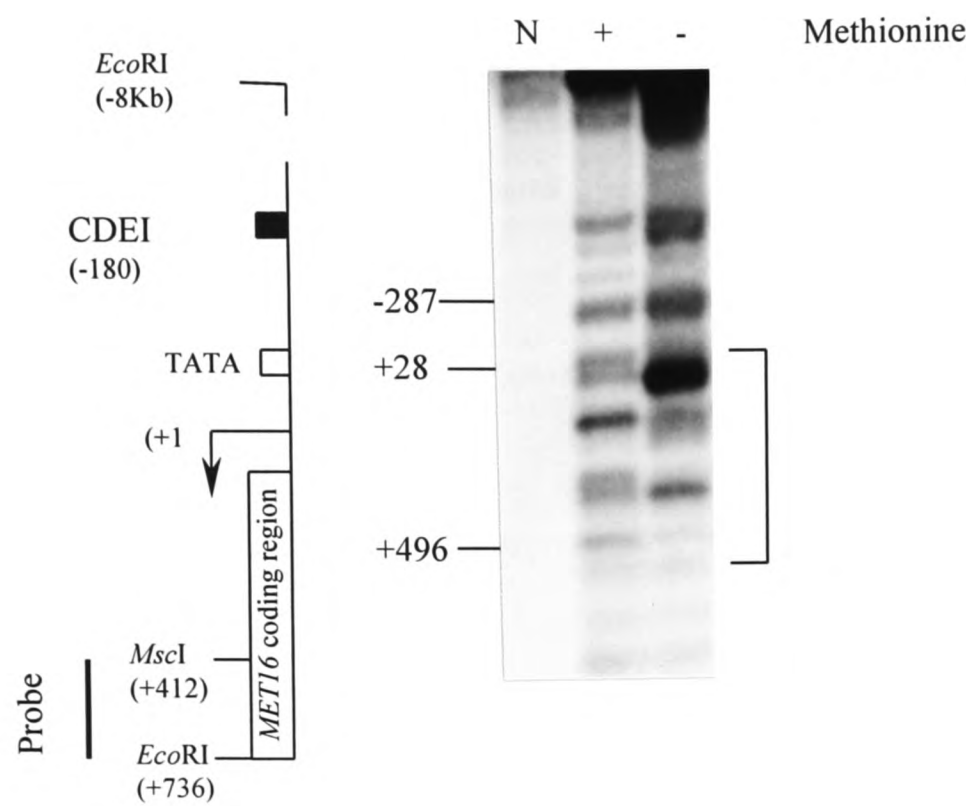


FIGURE 35. Chromatin structure at the *MET16* locus. Chromatin structure at *MET16* analysed in rich medium containing repressing (+) or derepressing (-) concentrations of methionine. Cells were grown in synthetic complete medium containing all nutrients and amino acids at normal concentrations including glucose as carbon source. Cells in lane (-) were incubated for 90 minutes in medium containing a de-repressing concentration of methionine.

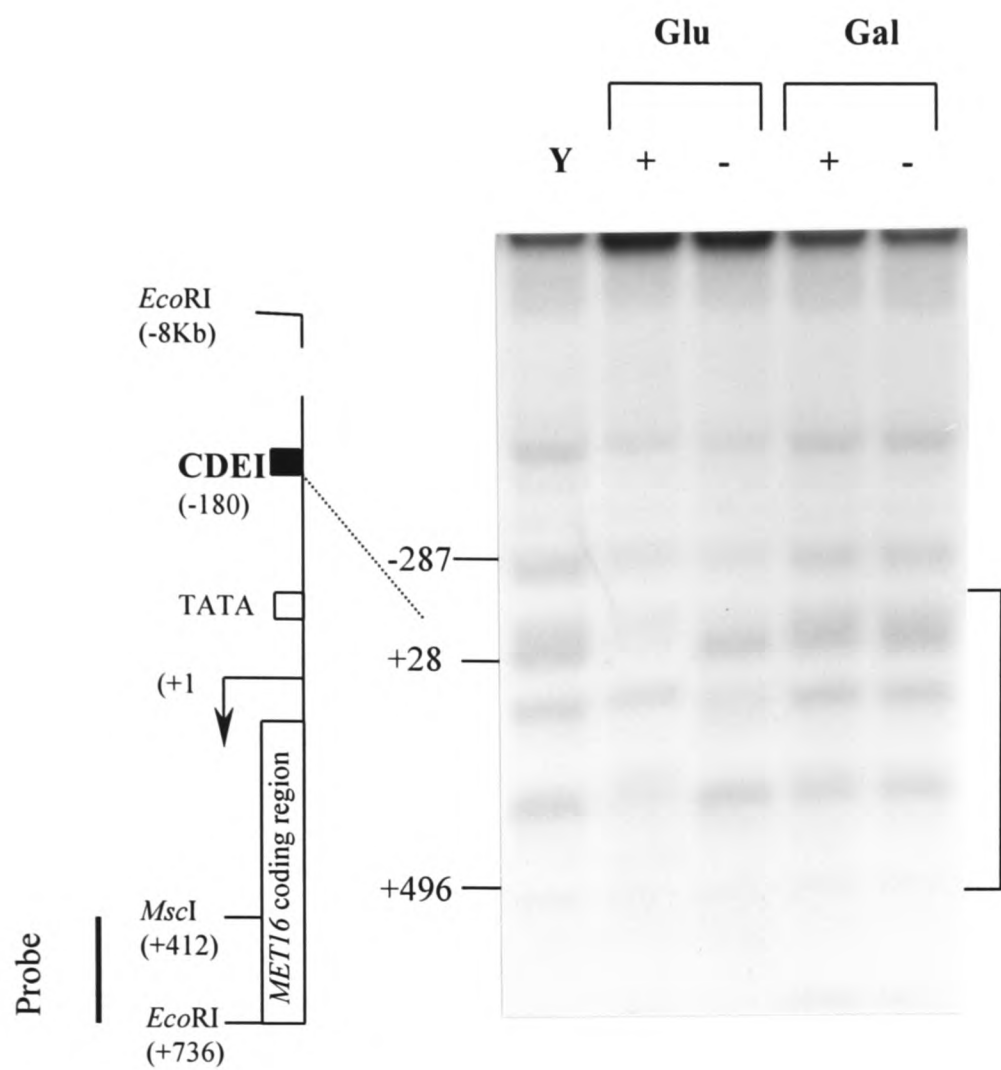


FIGURE 36. Chromatin structure at the *MET16* locus in cells incubated in media containing galactose as carbon source. Chromatin structure at *MET16* was analysed in rich medium containing repressing (+) or derepressing (-) concentrations of methionine. Cells were grown in synthetic complete medium containing all nutrients and amino acids at normal concentrations including galactose as carbon source. Cells in lane (-) were incubated for 90 minutes in medium containing a de-repressing concentration of methionine. Chromatin preparations from cells similarly treated in media containing glucose as carbon source are included for comparison. The brackett indicates a region of chromatin that becomes reorganised in cells undergoing meiosis.

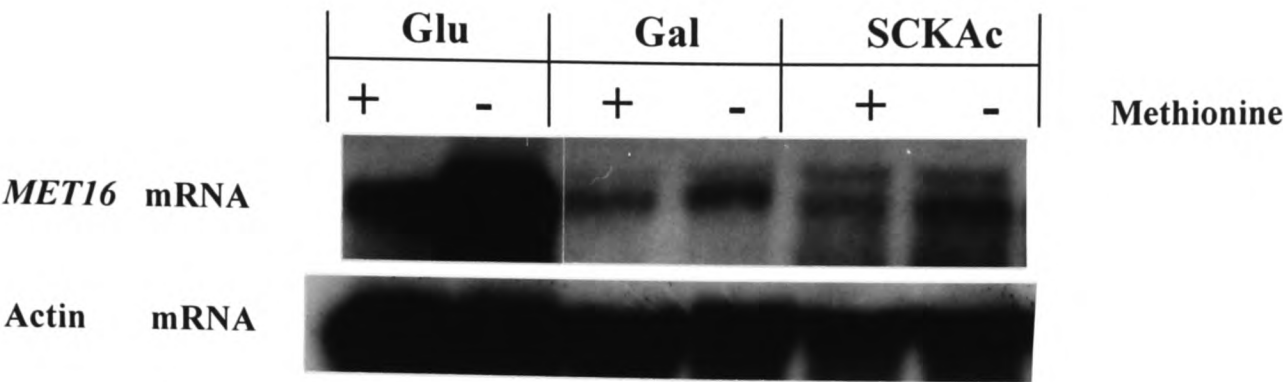


FIGURE 37. Northern blot analysis of *MET16* mRNA. Cells were harvested from cultures containing the same concentrations of methionine as for the chromatin analysis in different carbon sources and total RNA extracted as described in Chapter 2. RNA's were separated on denaturing agarose gels, transferred to membranes and hybridised using the same DNA probe (*MscI-EcoRI* fragment of the *MET16* coding region) as for the indirect end labelling experiments (upper bands in some lanes for *MET16* mRNA are due to a defect of the gel).

5.6 Discussion

The purification of Cbflp by various groups has revealed the presence of at least three different versions of the protein with apparent molecular masses 16, 37 and 65KDa in yeast extracts (Baker and Masison, 1990; Cai and Davis, 1989; Mellor *et al.*, 1990). The shorter ones are N-terminal truncated forms retaining the DNA-binding and dimerization domains that produce DNA-protein binary complexes in DNA-binding assays (Dowell *et al.*, 1992). The transcription activity of the gene coding for the cytochrome *c1* is regulated in response to oxygen and carbon source and the UAS_{CYT1} contains a Cbflp-binding site. Using a DNA fragment containing the complete UAS_{CYT1} in EMSA experiments with nuclear extracts from cells grown under various conditions Oechsner and Bandlow (1998) have shown that extracts from cultures grown under purified N₂ form an additional fast migrating DNA-protein complex (C_{IV}) not present under standard growing conditions or under a CO₂ atmosphere. This has led to the conclusion that a growth regulated function of Cbflp is the basis for the occurrence of the variety of Cbflp forms present in yeast protein extracts (Oechsner and Bandlow, 1998). When using a DNA fragment containing the Cbflp-binding site and flanking sequences required for Cbflp binding, the protein crude extracts from cells grown in media containing potassium acetate as carbon source produce a fast migrating DNA-protein binary complex (Figure 28), this suggest that a modification and processing of Cbflp may be occurring under these conditions. It is possible that a consequence of a metabolic change involved in the growth of cells in a non-fermentable carbon source as potassium acetate may modify the processing of Cbflp leading to the formation of the fast migrating form. This form clearly retains the DNA-binding domain and specific DNA-binding activity of Cbflp.

The fact that protein extracts from cells undergoing meiosis and from haploid cells under starvation both produce specific protein-DNA complexes indicates that the DNA binding activity of Cbflp remains intact during the remodelling of chromatin and may be required for it. Further support for this requirement comes from the analysis of chromatin structure in the YAG214 cells (Cbflp_{E231A}, weak DNA binding mutant). Although this mutant is able to maintain methionine independent growth, the chromatin structure at *MET16* in these cells during exponential growth phase is the same as that found in null mutant *cbfl* cells (Kent *et al.*, 1994). Figure 29 shows that YAG214 cells also fail to reorganise chromatin when starved, this indicates that the Cbflp dependent chromatin remodelling may require a DNA bound form of Cbflp. When a/α 214 cells are transferred

to sporulation medium, they fail to enter meiosis; since this mutant can sustain methionine independent growth, such failure suggest a correlation between Cbflp-dependent chromatin remodelling at Cbflp-binding sites and meiosis competence.

A second important requirement for the Cbflp dependent chromatin remodelling at its binding sites is the preservation of a putative phosphorylation site: the serine residue at position 226. The results presented in this chapter show that when this residue is mutated to a non phosphorylable one, S226A, no change in the MNase cleavage pattern is observed when cells expressing it are starved. When mutated to a residue that mimics permanent phosphorylation, S226E, the strain displays a chromatin structure matching the reorganisation found in wild type starved cells, upon induction of expression in synthetic complete medium. This suggests that the starvation signal that triggers the chromatin reorganisation at Cbflp binding sites may be transmitted to Cbflp through the phosphorylation of this serine residue by an, as yet, unidentified protein kinase. It is possible that in S226E mutant cells chromatin structure at *MET16* is never in the conformation found in wild type log phase cells; instead, as soon as Cbflp_{S226E} binds to DNA, it brings about the elements required for the chromatin conformation corresponding to starved cells.

None of the conditions used to test the relationship between deprivation for a key compound, media composition and the chromatin structure was able to trigger the chromatin reorganisation. However, the lack of methionine seems to play a central role in triggering such remodelling and this role is parallel to the effect of methionine on meiosis. Freese *et al* (1984) investigated a variety of nutrient deprivation conditions leading to sporulation in yeast; under all those conditions the addition of methionine or S-adenosylmethionine (SAM, AdoMet) suppressed the process. Deprivation for methionine, on the contrary, caused the entry into meiosis and sporulation; since in the absence of this amino acid the concentration of SAM decreases it has been suggested that this decrease is responsible for the initiation of meiosis (Freese *et al.*, 1984). No chromatin reorganisation was observed when cells were incubated in low phosphate medium, unless methionine was withdrawn. Previous efforts have been made to find evidence of a cross talk between phosphate and sulfur metabolism (O'Connell and Baker, 1992), from the results presented in this chapter it does not seem to be a direct link through Cbflp at the chromatin level. Although a negative charge at the putative phosphorylation site S226 seems to be required for chromatin reorganisation, it does not seem to be directly activated by a low concentration of inorganic phosphate in the culture medium. Phosphate deprivation was

carried out in media containing glucose as carbon source; it is known that phosphate deprivation does not lead to meiosis whenever glucose is present (Freese *et al.*, 1982). The medium used to assay deprivation for phosphate also contained methionine, which also inhibits meiosis (Freese *et al.*, 1984).

When cells are incubated in medium containing potassium acetate as carbon source no modification of chromatin organisation is observed unless methionine is withdrawn, this medium composition is similar to that of pre-sporulation medium in which no reorganisation of chromatin occurs. It has been reported that when cells are transferred to a synthetic medium, with similar composition to the one used here, and containing different concentrations of acetate, no spore formation was observed (Freese *et al.*, 1982). Thus, again, the absence of chromatin modification parallels the absence of sporulation in cells incubated in synthetic medium containing potassium acetate as carbon source.

In media containing glucose as carbon source the sole absence of methionine triggers reorganisation of chromatin at *MET16*. It is not known how the starvation signals are integrated to regulate the two main regulators of meiosis-specific transcription Ime1 and Ime2. At least three different points in the meiotic process are inhibited by glucose: the transcription of *IME1*, the transcription of *IME2* and the entry into late meiotic phase (Honigberg and Lee, 1998). Although glucose inhibits meiosis, cells pre-grown in glucose are able to enter meiosis once they have consumed the ethanol produced from glucose (Freese *et al.*, 1982). It is possible that, in the absence of methionine, the chromatin modification occurs and cells are prepared to enter meiosis once the carbon source has been consumed and the inhibitory effect of glucose relieved.

No reorganisation of chromatin was observed in medium containing 100mM galactose as carbon source and derepressing concentrations of methionine (Figure 36). This carbon source is known to inhibit meiosis at concentrations above 2mM. Therefore, when transferred to a medium containing galactose and lacking methionine cells may be confronted with two conflicting signals. After the growth of cells in media containing methionine, SAM is accumulated in the vacuole; a slow release of SAM into the cytoplasm from the vacuole has been observed in a SAM auxotroph (Nakamura and Schlenk, 1974). At high concentrations of galactose, above 2mM, including the concentration used here, the doubling time increases to 2.5 hours (Freese *et al.*, 1982). The slow growth in galactose may be responsible for a slow consumption of SAM, maintaining an intracellular concentration such that the entry into meiosis is inhibited and the chromatin is not reorganised despite the lack of methionine in the growth medium. This does not rule out

the possibility of a more direct effect of this carbon source on the mechanism directly responsible for the reorganisation of chromatin and on the initiation of meiosis. In any case, the fact that, under these conditions inhibitory for meiosis, no chromatin remodelling is observed, suggests again a correlation between chromatin structure reorganisation at Cbflp-binding sites and entry into meiosis.

Neither of the conditions analysed above leads to meiosis on its own (Freese *et al.*, 1982) and none of them was able to trigger chromatin reorganisation. The role that the lack of methionine plays in triggering meiosis and the presence of it inhibits the process is paralleled by the role of this amino acid in triggering or inhibiting reorganisation of chromatin. These findings reinforce the view that a correlation exists between the modification of chromatin structure and the entrance into meiotic process. In this set of experiments, however, there was found to be an association between the derepression of the *MET16* gene and a modified MNase cleavage pattern. This is interesting, since it has been reported that no modification of chromatin structure occurs at *MET16* locus under similar conditions (nor at *MET17*) upon derepression in *CBF1* cells (Kent *et al.*, 1994) and a pattern similar to naked DNA has been found by others (O'Connell *et al.*, 1995). In medium containing galactose, the lack of methionine does not modify chromatin and the transcription activity from *MET16* is not significantly increased under the same conditions (Figure 37). The reason for this lack of a significant increase in transcription activity may be the same as for the inhibition of meiosis: the potential slow release of SAM maintains repressive intracellular concentrations. Although these findings may suggest a correlation between reorganisation of chromatin and transcription activity at this locus, when cells are incubated in synthetic complete medium with potassium acetate as carbon source and lacking methionine the chromatin structure is modified, but only a slight increase in *MET16* mRNA, similar to that in galactose, is registered. The fact that YAG214 does not reorganise chromatin but maintains methionine independent growth, also cast some doubts over this apparent correlation.

Kent *et al* (1994) had shown that Cbflp is involved in maintaining specific chromatin structures at CDEI sites. Cbflp has also been shown to act as a tethering factor interacting with additional bZIP proteins; for instance, at *MET16* it interacts with the Met4 transcription factor and with Met28, to form a multiprotein complex able to promote transcription activity (Kuras *et al.*, 1997). A third role for Cbflp seems to be as an active player in the dynamic reorganisation of chromatin structure; the modification of its S226 residue may enable it to modify and/or sustain additional interactions, possibly with factors

directly responsible for the remodelling of chromatin structure. In summary, the data presented in this chapter show that the remodelling of chromatin at Cbf1p binding sites requires the DNA binding activity of Cbf1p and a negative charge at S226 residue, which is perhaps the target of a protein kinase. Cbf1p may also be the subject of some processing resulting from the growth of cells in a non-fermentable carbon source, such as potassium acetate. A correlation between the Cbf1p-dependent chromatin remodelling and the conditions which trigger meiosis was also found. These findings suggest that Cbf1p is the target of a starvation signal and that it may interact with proteins and protein complexes involved in the remodelling of chromatin.

Chapter 6

General Discussion

6.1 Cbflp-dependent Chromatin Remodelling

Specific chromatin structures have been found to be associated at multiple sites to which Cbflp has potential to bind to *in vivo* (Kent *et al.*, 1999; O'Connell *et al.*, 1995; Kent *et al.*, 1994; Mellor *et al.*, 1990). The results presented in this thesis show that chromatin structure at and around those sites becomes further reorganised when yeast cells are starved. This is shown by the changing MNase accessibility patterns that clearly differ from those of exponentially growing *cbfl* and *CBF1* cells and from the patterns generated by digestion of naked DNA. This indicates a modification of interactions between protein and nucleic acid components of chromatin and not simply de-proteinisation.

From the patterns obtained in this work for the *MET16* locus in *CBF1* starved cells it seems that protein complexes, most probably nucleosomes, are being moved from their initial position when cells are starved. The new patterns obtained could be compatible with these complexes not being completely removed from DNA, since this should result in a pattern similar to that of naked DNA, but this is not the case. Instead, it seems as if protein complexes were changing position; a movement in a downstream direction will account for some of the downstream sites becoming protected. Although the MNase cleavage pattern at *MET16* in *CBF1* log phase cells has been described as similar to that of naked DNA (Kent *et al.*, 1994), Figure 11 shows that these two patterns are different. The overall new pattern is also different from naked DNA. For instance, a site immediately downstream to the one marked as +28 is accessible in log phase cells and it becomes significantly less sensitive in the new pattern, as do others further 3' to this region, into the ORF. Nevertheless, when contrasted with the structure in exponentially growing cells, it certainly seems that there are more protected sites in *cbfl* cells than in *CBF1* cells. As for the chromatin structure upstream of the CDEI site at *MET16*, this work shows that a sensitive site in naked DNA is protected in all three patterns found in *CBF1* and *cbfl* exponentially growing cells and in *CBF1* starved cells (Figure 21, Chapter 4). This indicates that this region does not become modified either in the absence of Cbflp or when cells are starved. Therefore, the Cbflp-dependent reorganisation of chromatin at *MET16*,

in starved and meiotic cells, seems to occur at and downstream to the CDEI site, to which Cbflp is able to bind.

Along with the *MET* genes, other loci are found to display chromatin reorganisation at their Cbflp binding sites upon starvation. These are unrelated *DRS2* and *GDH3* loci and the YAL060W ORF (see Chapter 4). These three loci have been previously analysed in log phase *cbfl* and *CBF1* cells and Cbflp dependent specific chromatin structures associated with their potential Cbflp binding sites have been described (Kent *et al.*, 1999). These differences between *cbfl* and *CBF1* cells are also reproduced in the experiments shown in Chapter 4. Although in all five cases, including the two *MET* genes, there are bands which become protected and sites which become accessible upon starvation, it is difficult to classify them into particular categories since the patterns differ from one gene to the next. This does not rule out the possibility that Cbflp is playing the same role in the chromatin transitions at all these sites; a similar process may be occurring, the differences reflecting a combination of the number and class of Cbflp-binding sites, the sequence specificity of the MNase and/or of the assembly of different protein complexes specific for each particular gene. Other factors with the ability to modify chromatin structure have been also shown to produce different structures at different locations. For instance, at the *PHO5* promoter Pho4 displaces 4 nucleosomes, but at *PHO8* it seems to only partially destabilise nucleosomes, with the modification detected up to 800bp downstream to the Pho4 binding site (Barbaric *et al.*, 1992).

Evidence for the involvement of Cbflp in the remodelling at its binding sites is also presented here; at all loci, where chromatin is modified when cells are starved, no change was observed in *cbfl* cells under the same conditions (Chapter 4), indicating that the reorganisation is dependent on Cbflp. This is further supported by the fact that the mutant YAG214, expressing a weak binding version of Cbflp which sustains methionine independent growth, is unable to display the reorganisation of chromatin at *MET16* upon starvation (Chapter 5). Furthermore, the S226A point mutant that restores the wild type chromatin structure associated to the Cbflp binding site at *MET16* is unable to remodel chromatin upon starvation. The absolute dependence on Cbflp for the chromatin transitions observed at these loci suggests that Cbflp is involved in an, as yet, unknown chromatin remodelling mechanism. However, it is unlikely that Cbflp carries out the chromatin remodelling on its own, since it does not contain domains related to known proteins with the ability to remodel chromatin and this function is frequently carried out by multiprotein complexes (see Section 1.5). Nevertheless, through genetic analysis it has

been shown that Cbf1p may interact with some components of chromatin and with factors involved in chromatin dynamic reorganisation. For instance, Sin3 and Rpd3, components of a complex with the ability to de-acetylate histones, have been suggested to interact with Cbf1p since mutations in any of them relieve the methionine auxotrophy caused by deleting the *cbf1* gene (McKenzie *et al.*, 1993). Furthermore, using GST tagged versions of Cbf1p, high molecular weight multiprotein complexes have been recently isolated whose composition is still under analysis but are known to contain histone acetyl transferase activity (Nissom, 1998).

Three types of protein complexes containing Cbf1p are formed *in vitro* over the *CYT1* locus at the CDEI-like site after incubation of cells under anaerobic conditions; Cbf1p is subject of phosphorylation/dephosphorylation events according to growth conditions especially on non-fermentable carbon sources (Oechsner and Bandlow, 1998). The inability of the non-phosphorylatable mutant S226A (Chapter 5) to reorganise chromatin upon starvation suggests that covalent modification of the serine residue at position 226 may be required for the protein to be processed to produce the fast mobility form detected in cells incubated in sporulation medium (Figure 28). The fact that Cbf1p retains its DNA binding activity during the chromatin transition (Chapter 5) suggest that it remains bound to its target sites. Taken together, these may suggest that Cbf1p is a component of a multiprotein complex whose function is to reorganise chromatin or, alternatively, that it may contribute to target such complexes to its multiple binding sites in response to starvation. Recent experiments, done in this lab by N. A. Kent, show that a *gcn5Δ* strain is unable to reorganise chromatin structure under conditions which trigger such Cbf1p-dependent remodelling at *MET16* in a wild type strain (personal communication).

A few examples of loci containing Cbf1p binding sites through the yeast genome have been analysed in the present work and found that, after shifting to starvation conditions, there are clear changes of chromatin structure, dependent upon the presence of Cbf1p in the cells. It seems reasonable to expect that reorganisation of chromatin may also happen at the other numerous potential Cbf1p binding sites through the yeast genome, regardless of the function of proteins encoded by particular genes containing these sites. It is therefore tempting to suggest that this may have consequences involving the reorganisation of chromatin on a genomic scale.

6.2 Cbf1p-dependent Chromatin Remodelling and Transcription

As chromatin reorganisation is frequently related to DNA transactions and particularly to transcription, the question arises as to whether the Cbf1p dependent chromatin reorganisation found at multiple sites could be related to transcription. While doing this work the transcriptional program of yeast sporulation was published (Chu *et al.*, 1998); although the strain used by these authors completes meiosis faster than the strain used in this work, the data are useful since the chromatin changes observed here occur from 90 minutes in sporulation medium, which is comparable with the early metabolic phase indicated by those authors. If the primary function of the chromatin reorganisation at Cbf1p binding sites were the activation of transcription, then some uniformity in the transcription activity should be observed among these loci, but this is not the case. Transcriptional activity of *MET16*, *MET17* and *GDH3*, increases during the metabolic transition preceding the pre-meiotic S phase. However, the transcriptional activity of *DRS2* does not increase during the early phase, it increases only during the late phase of meiosis, but chromatin reorganisation is detected during the early phase (from 90 minutes in sporulation medium). On the other extreme is the ORF YAL060W, transcription activity for this locus actually decreases and this only during the middle phase of meiosis, much later than when the chromatin modification is detected. As shown in Chapter 4 a Cbf1p dependent chromatin reorganisation occurs at this locus after 90 minutes in sporulation medium. This suggests that the Cbf1p chromatin remodelling is not necessarily correlated with an increase in transcriptional activity. In fact, a repressive effect of Cbf1p has also been reported at some other loci as in the case of *QCR8*, where it was also found that a specific chromatin structure is dependent on intact Cbf1p and Cbf1p binding site (de Winde and Grivell, 1992), and *CYT1* (Oechsner and Bandlow, 1996); in the last case the chromatin structure is unknown. Furthermore, both *MET16* and *MET17* are expressed during meiosis and they seem to have very different expression kinetics (Chu *et al.*, 1998; <http://cmgm.stanford.edu/pbrown/sporulation/>), the dynamics of their chromatin transitions, however, is very similar.

Following the finding that Cbf1p maintains specific chromatin structures at its binding sites, chromatin structure at *MET16* and *MET17* loci upon derepression was studied (Kent *et al.*, 1994; O'Connell *et al.*, 1995). The *MET17* locus was reported as not presenting any modification of its chromatin structure under de-repressing conditions (Kent *et al.*, 1994); however here it is shown to be reorganised in *CBF1* haploid and

diploid starved cells, in the absence of methionine. Two groups have demonstrated that *MET17* is transcribed in *cbf1* cells in the absence of methionine (Kent *et al.*, 1994; Kuras and Thomas, 1995). If the new MNase cleavage patterns found in *CBF1* cells under nutrient starvation were a mere consequence of transcriptional activity and a de-proteinised region, the same patterns should also be displayed by *cbf1* cells. This however, is not the case, indicating that although chromatin remodelling and transcription occur concurrently during meiosis the former does not seem to be either required for the latter or a consequence of it. This implies that chromatin remodelling and transcriptional activity are separable events occurring at this locus and that Cbf1p is essential only for the chromatin remodelling.

In Chapter 5 it was also shown that the chromatin structure at *MET16* becomes reorganised upon derepression, under various different nutrient conditions, in particular in rich medium just lacking methionine, and that a concomitant increase in *MET16* mRNA occurs. The new MNase cleavage pattern found here upon derepression is similar to one of the new patterns found during meiosis. The chromatin structure at the *MET16* promoter presented by O'Connell *et al* (1995) under de-repressing conditions for this gene was found to be similar to that of naked DNA (O'Connell *et al.*, 1995); it is possible that the method used to analyse chromatin in that case was disruptive enough to devoid this region from some of its chromatin components, displaying therefore the structure of naked DNA. Although Kent *et al* (1994), used a fast, newly designed and far less disruptive method (subsequently used for this work), they reported no modification of chromatin structure under similar conditions (Kent *et al.*, 1994). However, a careful analysis of the patterns presented by these authors reveals that, in fact, some of the changes described in this work are also present in their figures (Kent, 1994; Kent *et al.*, 1994). It has recently been found that the strain used in those studies is actually temperature sensitive, growing much better at 25°C (as judged by morphology and growth curves, N. A. Kent, personal communication; Nissom, 1998); the experiments referred to were carried out at the standard temperature of 30°C, which may have affected the results, after the long exposure of cells to this temperature, preventing the display of the more obvious changes observed in this thesis.

There is evidence that Cbf1p is not required for transcription of the *MET16* gene (Kent *et al.*, 1994); evidence has also been published indicating its absolute requirement (Kuras and Thomas, 1995). However, although Kuras *et al.* (1995) show that the concentration of mRNA of a derepressed culture decreases when methionine is added and

this does not occur in *cbf1* cells; they do not show what the basal level is in repressed *CBF1* cells (it is detectable and shown in Chapter 5) and whether this same level could persist under repressing conditions or become increased upon derepression in *cbf1* cells. Therefore, although the Cbf1p requirement for *MET16* transcription is still controversial, the work presented in this thesis suggests that it is essential for the chromatin remodelling to occur under nutrient starvation. Taken together these data suggest that the chromatin remodelling and transcriptional activity may also be separable events at the *MET16* locus. In favor of the independence of these two events is the fact that YAG214(Cbf1p_{E231A}) can sustain methionine independent growth but is unable to reorganise chromatin at this locus when cells are starved or induced to sporulate.

It is puzzling that Cbf1p binds to loci with a diversity of levels of transcription activity during meiosis (Chu *et al.*, 1998). It locates to promoters where it seems to interact with components of the specific and general transcription machinery, there are also Cbf1p binding sites within the coding region of some loci (Kent *et al.*, 1999). In general, the level of transcription activity at loci containing Cbf1p binding sites does not correlate with the chromatin reorganisation at these sites during starvation. These different situations, among which modification of chromatin structure can be clearly observed in starved cells, suggest that the Cbf1p dependent reorganisation of chromatin may be required for a process other than transcription.

6.3 Cbf1p-dependent Chromatin Remodelling and Meiosis Competence

In Chapters 3 and 4 it is shown that in cells undergoing meiosis a Cbf1p dependent reorganisation of chromatin occurs at and around Cbf1p binding sites. In Chapter 4 it is also shown that *cbf1* cells are unable to enter meiosis, specifically they are unable to enter pre-meiotic DNA replication. It has been recently shown that, at the promoter of the gene coding for the regulator of meiosis specific gene expression Ime2, the levels of bound acetylated histone H3 are increased concomitant with an increase in transcription during meiosis; in a *gcn5* mutant these two events do not occur and cells are unable to enter pre-meiotic DNA replication (Burgess *et al.*, 1999). Only one meiosis specific gene, the *UME1* locus (*unscheduled meiotic gene expression*) contains a potential Cbf1p-binding site (N. A. Kent, personal communication) but this locus codes for a repressor of transcription of meiotic specific genes; *ume1* mutants activate transcription of meiotic-specific genes during the mitotic cell cycle (Strich *et al.*, 1989) and the levels of the protein product of this gene, Wtm3 (*WD repeat-containing transcription modulators*) are not altered during meiosis

(Pemberton and Blobel, 1997). Therefore it is unlikely that Cbflp has an effect on meiosis through the modification of expression levels of this gene. This suggests that what renders *cbfl* cells unable to enter meiosis might be their inability to remodel chromatin structure at multiple sites rather than its possible effect on a single gene specific for the process.

Although during derepression of *MET16* there is a change in the chromatin structure at this locus, the MNase cleavage pattern generated under these conditions is similar to only one of the various patterns observed at the same locus during meiosis. One possibility is that the modification of the chromatin structure is part of the reorganisation of the genome prior to the entry into meiosis, since the absence of methionine is one of the conditions that triggers meiosis (Freese *et al.*, 1984). Two signals may be reaching this locus simultaneously, one through Cbflp and one through the specific factors Met4 and Met30 (Kuras *et al.*, 1996; Thomas *et al.*, 1995). Since the intracellular concentration of AdoMet is determinant for the decision to enter meiotic development, Cbflp may have been recruited to *MET* promoters to couple starvation signal with chromatin reorganisation.

The inability of *cbfl* mutants to sporulate may not be due to its possible effect on transcription at *MET* genes but due to inability to reorganise chromatin at multiple Cbflp binding sites. Indeed, some methionine auxotrophs have been shown to sporulate (Freese *et al.*, 1984; Wejksnora and Haber, 1974) and addition of 1mM methionine to cells in spoulation medium actually inhibits meiosis (Freese *et al.*, 1984). Moreover, the YAG214 mutant (Cbflp_{E231A}) can sustain methionine independent growth (Kent *et al.*, 1994), indicating that the transcription of the sulfur metabolism pathway genes containing Cbflp binding sites does not become impaired, but cells are both unable to reorganise chromatin and to enter meiosis (Chapter 5).

In Chapter 5 it is also shown that the effect of nutrient conditions on Cbflp dependent chromatin remodelling paralells the effect of such conditions on initiation and inhibition of meiosis. Conditions triggering chromatin reorganisation at Cbflp binding sites promote the entry into meiosis; conditions which do not modify chromatin structure inhibit meiosis. These data also suggest that the chromatin reorganisation itself plays an important role required to enter meiosis and independent of its possible effect on transcription. Figure 38 shows a model of the effect of various nutrients on the Cbflp dependent chromatin remodelling and meiosis.

It has been suggested that Cbflp may participate in holding homologous chromosomes during recombination (Kent *et al.*, 1994) and it has been found that a protein complex containing Cbflp is able to bind a Holliday junction (Nissom, 1998); the

reorganisation of chromatin observed in this work could be related to meiotic recombination. During meiosis double-stranded DNA breaks (DSB's) occur at recombination hot spots and other recombination sites. These DSB's have been found to be associated to DNaseI hypersensitive sites. Using *pho4* and *pho80* mutants which maintain two different chromatin structures at *PHO5* it has been shown that the distribution of DSB's at this region during meiosis is dependent on the pre-existing chromatin structure; this has lead to the hypothesis that the initiation of recombination events may be determined by the structural features of chromatin established before the initiation of meiosis (Wu and Lichten, 1994). The Cbf1p-dependent remodelling of chromatin observed during starvation of cells may be contributing to establish a chromatin structure, required prior to the initiation of the meiotic S phase and compatible with the formation of DSB's at multiple sites.

Starvation not always leads to meiosis in yeast. Under certain conditions, deprivation for some nutrients leads to filamentous invasive growth in haploid cells or to pseudohyphal growth in diploid cells. Cbf1p seems to be also involved in these pathways. I had disrupted the *cbf1* locus in wild type cells competent for invasive and pseudohyphal growth. Paul Nutten, in this laboratory, has shown that these *cbf1::URA3* cells become unable to sustain these types of growth under the same conditions which stimulate them in wild type cells (personal communication).

Entry into meiosis, pseudohyphal growth and invasive growth have in common that they are all stimulated by some kind of nutrient starvation. The impairment to enter these processes in cells with disruptions in the gene coding for Cbf1p suggest that this factor may be a component of a system sensing nutrient availability. These also suggest that Cbf1p may be part of a response to less favorable nutrient conditions, which involves the reorganisation of chromatin structure in order to enter such development processes leading to various different types of cells.

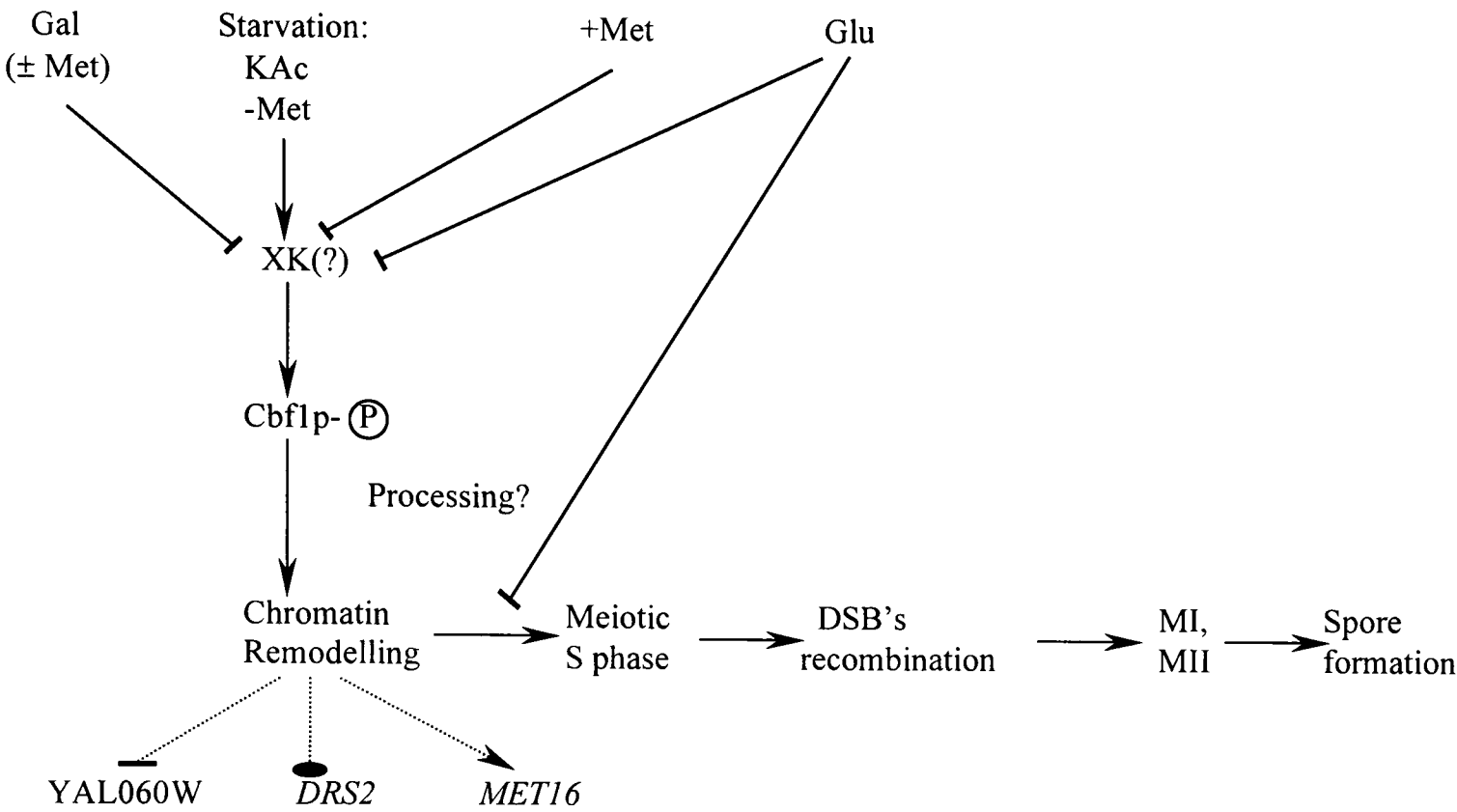


FIGURE 38. Model. The activity of an as yet unidentified kinase (XK) functions as a sensor of nutrient conditions. The activity of this kinase is inhibited in the presence of carbon sources such as glucose and galactose and by the presence of methionine in the growth medium. In the presence of a non-fermentable carbon source (KAc) and/or the absence of methionine it becomes activated. XK then phosphorylates Cbf1p, which in turn may be processed, and this leads to interaction with complexes that reorganise chromatin at multiple Cbf1p-binding sites. Once chromatin has been remodelled and the inhibition of further stages by other compounds relieved, the meiotic S phase initiates. The loci at which chromatin remodelling occurs may be derepressed (as *MET16*) or not altered at all during the early phase of meiosis (as *DRS2*) or even repressed at later stages (as *YAL060W*).

6.4 Concluding Remarks and Future Prospects

Cbflp has been described as a determinant of specific chromatin structures at its binding sites (Kent *et al.*, 1994) and as a tethering factor for transcriptional activators at some *MET* genes (Kuras *et al.*, 1997). The data presented in this thesis show that the DNA binding factor Cbflp plays a more active role in the genome dynamics, since it is involved in the reorganisation of chromatin at multiple sites under starvation conditions. Further studies using small probes and restriction enzymes (Almer and Horz, 1986) will be required to map more precisely the position of the protein complexes (presumably nucleosomes) which are changing positions during these transitions. Here it is also shown that the DNA binding activity of Cbflp and a negative charge at its residue 226 are required for the chromatin reorganisation to occur. The analysis of the ability of additional Cbflp mutants to display the chromatin transitions will be useful in defining the relevance of other domains in this new role of Cbflp.

Although at some loci the Cbflp-dependent chromatin reorganisation is concomitant with transcription, the relationship between these two processes at loci containing Cbflp binding sites requires further clarification. This could be achieved by using mutants on the components of transcription factors specific for particular loci, by mutating *cis* elements required for transcription at one or more loci containing Cbflp binding sites and then analysing whether any of those have an effect on the chromatin reorganisation triggered by starvation. It has been puzzling that the effect of deletion of *cbfl* on meiosis is greater than that expected from its effect on mitosis; one of the reasons could be that during meiosis an additional function, involving the N-terminal domain of Cbflp, is required to reorganise chromatin. As shown in this work, the ability to reorganise chromatin at Cbflp binding sites correlates with ability to enter meiosis. This can be further investigated using strains with mutations in components of protein complexes involved in chromatin remodelling and analysing how they affect both the Cbflp dependent chromatin remodelling at its binding sites and their ability to enter meiosis. A further set of conditions triggering meiosis could also be used.

In summary the results presented in this thesis show that Cbflp is involved in the regulation of chromatin reorganisation at multiple binding sites during starvation. Such results are compatible with a model in which Cbflp plays a role as part of a system sensing the nutrient availability and transforming a starvation signal into a reorganisation of chromatin through the yeast genome.

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