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The DNA binding and oligomerization domain of MCM1 is sufficient for its interaction with other regulatory proteins

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The *MCM1* gene encodes an essential DNA binding protein that, in cooperation with the transactivators α1 and STE12 and the repressor α2, confers mating specificity to haploid yeast cells. We show that the amino-terminal third of the MCM1 protein is sufficient for the physical interaction with these factors. A strain expressing just 98 amino acids encompassing the oligomerization and DNA binding domains of *MCM1* is viable and mating competent. This motif exhibits considerable similarity to a domain of the mammalian transcription factor SRF. A 98 amino acid hybrid gene coding for the MCM1 DNA binding domain and SRF dimerization domain is sufficient for viability but not for the expression of mating type specific genes. *In vitro* binding studies suggest that a region of ~50 amino acids of MCM1 is essential for providing contacts with α1, α2 and STE12.

Key words: mating-specific genes/MCM1/SRF/yeast

Introduction

The regulation of many developmental programmes can be attributed to specific protein-protein interactions between different transcription factors. The best studied examples concern proteins that contain special sequence motifs responsible for the formation of heterodimers between different members of the same family of DNA binding proteins. Classical cases are proteins containing the leucine zipper motif or helix-loop-helix proteins (Vinson *et al.*, 1989; Murre *et al.*, 1989). The combinations of different subunits into a heterodimer allows considerable flexibility in DNA recognition, activation and repression (Jones, 1990). In this work, we investigate a different class of DNA binding proteins that seem to form mostly homodimers. The protein dimers show fairly promiscuous interactions with additional, by structure completely unrelated DNA binding proteins. It is the formation of these larger, oligomeric complexes that correlates with the properties of the target promoter. The homodimeric core proteins are evolutionarily widely conserved. Proteins belonging to this class are intimately involved in the early response of mammalian cells to growth factors, with serum response factor (SRF) as the prototype (Norman *et al.*, 1988; for review see Treisman, 1990). In plants a closely related family is necessary for flower development, and its members include gene products such as AGAMOUS of *Arabidopsis thaliana* and DEFICIENS A of *Antirrhinum majus* (Ma *et al.*, 1991; Sommer *et al.*, 1990). In budding yeast, two such transcription factors, MCM1 and ARG80, have been identified (Passmore *et al.*, 1988; Dubois *et al.*, 1987). Although they appear closely related by sequence, their functions are not interchangeable under normal conditions and are exerted in different biological processes.

In yeast, the *MCM1* gene is central to the regulation of cell type specific transcription (Sprague, 1990; for review see Dolan and Fields, 1991). The protein is part of a molecular switch that determines which of the two sets of genes that establish mating specificity are activated. The positions of the switch (**a**-specific genes on, α-specific genes off and vice versa) depend primarily on the presence or absence of two DNA binding proteins called α1 and α2 (Strathern *et al.*, 1981). The establishment of an α-specific cell type requires the physical interaction of MCM1 with these proteins (Bender and Sprague, 1987; Keleher *et al.*, 1988). Thereby, α1 serves as a co-activator for α-specific genes, α2 as a co-repressor of **a**-specific genes. Differences in the MCM1 recognition site and its immediate sequence context are also important. **a**-specific promoters usually contain an extended palindromic motif (called P-box; Bender and Sprague, 1987) flanked by specific sequences that are contact sites for the α2 protein. α-specific elements consist of a slightly degenerate P-box for which one flanking region has been replaced by another consensus sequence called the Q-box (Bender and Sprague, 1987; Jarvis *et al.*, 1988). Two hypotheses have been advanced that explain α1 dependence of these elements. In the first, the emphasis has been placed on differences in DNA affinity between MCM1 and α1-MCM1 complexes leading to differential occupation of α-specific sites *in vivo* (Bender and Sprague, 1987). In the second, α1-dependent conformational changes of MCM1 have been regarded as crucial for activation of α-specific promoters (Tan and Richmond, 1990). High level expression of both **a**- and α-specific genes requires an additional DNA binding factor encoded by the *STE12* gene (Fields *et al.*, 1988; Dolan *et al.*, 1989; Errede and Ammerer, 1989). Like α1 and α2, STE12 is able to cooperate directly with MCM1 for DNA binding even if this cooperation has only been demonstrated in the context of **a**-specific promoters (Errede and Ammerer, 1989; M.Primig and G.Ammerer, unpublished data). Figure 1 summarizes those DNA-MCM1 complexes for which the formation of a ternary complex has been correlated with a cell type specific activity. Although MCM1 is able to interact with all three proteins, α1, α2 and STE12, they appear to be unrelated by primary sequence comparison to either MCM1 or one another.

In addition to its role in cell type determination, *MCM1* has an essential role for cell growth. *MCM1's* vital functions have not been identified yet but may be related to DNA replication (Maine *et al.*, 1984). Recent evidence also

suggests that *MCM1* is involved in cell cycle regulated activation of *SWI5*, a gene whose transcription is restricted to a window from S-phase to mitosis. In this case, cooperative binding with an as yet genetically unidentified protein is essential for full activation (D.Lydall *et al.*, in press). Therefore, factors other than those involved in cell type determination exist which increase their binding affinity by interaction with MCM1.

The main aim of this work has been to identify those regions of MCM1 that are essential for the cooperation with its cell type specific partners. Basically, the MCM1 protein can be divided into four distinct domains (Passmore *et al.*, 1988; Ammerer, 1990). An amino-terminal basic region (1–47) is followed by a more hydrophobic area (48–97). This is followed by a stretch of negatively charged amino acids (98–120) and a 166 amino acid long tail (121–276) which contains several runs of glutamine residues. Both C-terminal domains have been considered as potential activation regions either of the acidic type such as found in GCN4 (Hope *et al.*, 1988) or glutamine rich type as claimed for the mammalian transcription factor Sp1 (Courey and Tjian, 1988). The region between amino acids 17 and 98 shows extensive conservation between SRF and MCM1. For both proteins this region is essential for DNA binding and oligomerization (Norman *et al.*, 1988; Ammerer, 1990). It has been proposed that serum stimulated activation of the c-*fos* gene requires the cooperation of SRF with another DNA binding protein called $p62^{TCF}$ (Shaw *et al.*, 1989). The regions of SRF that are important for recruitment of $p62^{TCF}$ seem to overlap with its DNA binding and dimerization domains (Schröter *et al.*, 1990). In this paper we report very similar findings regarding MCM1's interaction with its cell type specific regulators.

Results

Cells expressing a 98 amino acid domain of MCM1 are viable and mating competent

Previous experiments indicated that extended regions of the MCM1 carboxy-terminal sequences were dispensible for its biological function (Jarvis *et al.*, 1989; Passmore *et al.*, 1989). At the same time it was known that a carboxy-terminal deletion product up to amino acid 92 lost its ability to dimerize and bind DNA or to support the essential functions of MCM1 (Ammerer, 1990; Jarvis *et al.*, 1989). For the studies described here we constructed a gene encoding 98 amino-terminal amino acids of MCM1. This region encompasses all sequences of MCM1 that exhibit similarity with SRF. For SRF the same domain is sufficient for DNA binding (Norman *et al.*, 1988; Schröter *et al.*, 1990). The truncated *MCM1* gene (henceforth referred to as *mcm1-98*) was fused to the *Schizosaccharomyces pombe ADH* promoter (*SpADH*) and integrated at one *URA3* locus of a diploid strain heterozygous for wild-type *MCM1* and an *mcm1* deletion (see Figure 2, Table I and Materials and methods). This strain allowed us to obtain haploid cells that contained the *SpADH–mcm1-98* fusion either in the wild-type background or in combination with the *mcm1* deletion. Both strains are fully viable (under all temperature conditions) without any notable differences to wild-type cells.

Comparing the mating behaviour of an isogenic set of **a** and α cells, we found that the mating specificity is fully maintained in strains expressing the *mcm1-98* product, although quantitative assays indicated a 2-fold reduction in mating competence for cells that survive with the *mcm1-98* gene. However, neither **a** nor α *mcm1-98* cells show an increase for inappropriate mating events with partners of the same mating type (Figure 3). The amount of pheromone secreted by the different cells was determined by a halo plate assay. While the amount of **a**-factor secreted remained unaffected by the truncated *MCM1* allele (data not shown), the amount of α-factor was reduced ~3- to 5-fold in *mcm1-98* and *mcm1-98/MCM1* strains (Figure 3D) revealing an interfering effect of the truncated protein. Compared with the reduction of halo size observed in *matα1* or *ste12* strains the defect caused by *mcm1-98* seems insignificant (data not shown). These results suggest that one can expect only minor, *mcm1-98* related effects on the expression pattern of cell type specific genes.

To provide more evidence, we isolated RNA from the different strains and probed for the presence of mRNAs corresponding to the α-specific receptor gene (*STE3*), the α-specific pheromone gene (*MFα1*), the **a**-specific receptor gene (*STE2*) and the **a**-specific pheromone translocator gene (*STE6*). We found that the α-specific RNA levels are somewhat decreased in *mcm1-98* cells although this reduction does not reflect any severe impairment of *MFα1* or *STE3* transcription (Figure 4). For example, a strain expressing the highly α-specific *mcm1-1* allele (a change of proline 97 to leucine) reveals a much stronger defect concerning the transcription of α-specific genes (Passmore *et al.*, 1988). Compared with *mcm1-98*, a much more pronounced defect is also obtained with the truncated version of *mcm1-1* (*mcm1-98/97L*; data not shown). In cell types where mating-specific genes are normally inactive or repressed, an *mcm1-98*-dependent increase in transcription was not observed. We conclude that the cell type specific regulation of pheromone and receptor genes is not severely

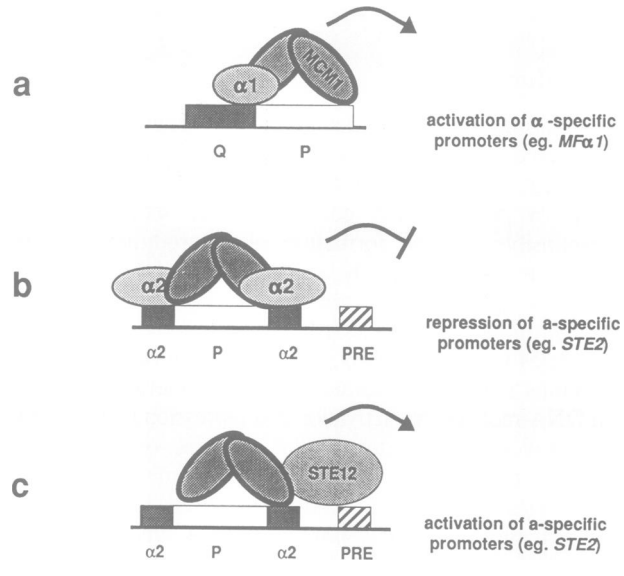

Fig. 1. DNA–protein complexes with cell type specific promoter elements. (**a**) shows the prototypical α-specific P-Q box. The formation of an α1–MCM1 complex is a precondition for transcriptional activation by this *cis*-acting element. (**b**) and (**c**) indicate a UAS element derived from the **a**-specific STE2 promoter. Cooperation between α2 and MCM1 is necessary for repression. Binding of a STE12–MCM1 complex correlates with high levels of transcriptional activation.

compromised by the carboxy-terminal truncation in the *mcm1-98* gene.

STE12 and α2 interact with mcm1-98

The *in vivo* data suggest that cooperation of mcm1-98 with α1, α2 and STE12 proteins should still occur. To test this prediction we performed *in vitro* DNA binding experiments. Crude extracts were prepared from *MCM1*, *MCM1/mcm1-98* and *mcm1-98* strains (Table I). They were assayed for DNA binding by gel retardation with a 47 bp fragment from the *STE2* promoter (Ammerer, 1990). This **a**-specific UAS contains the α2 operator sequence in addition to one PRE

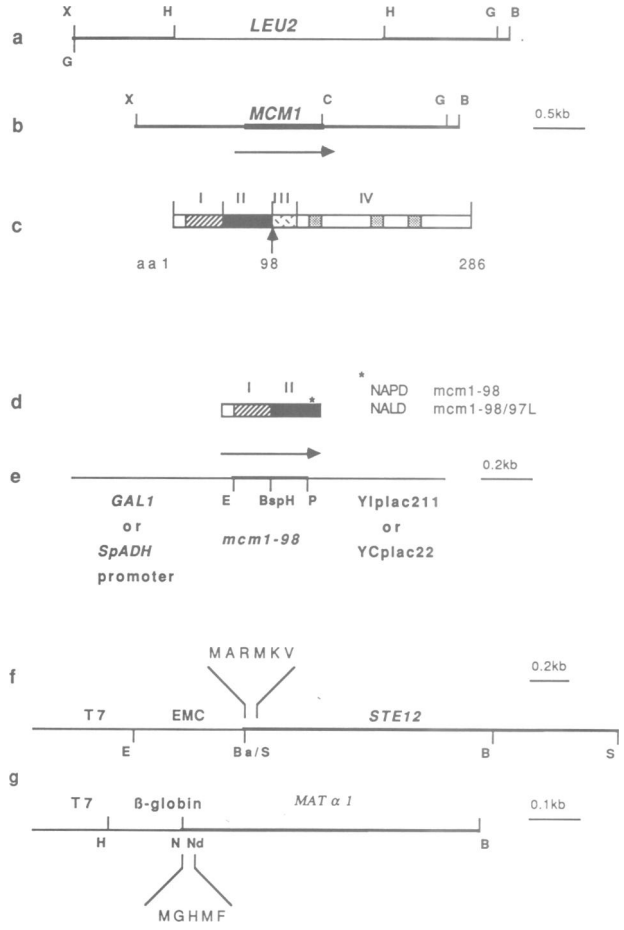

Fig. 2. Plasmids used for strain constructions and *in vitro* expression. (**a**) Diagram of the *mcm1Δ::LEU2* construct (plasmid pGA1748). The part containing the *LEU2* gene is indicated by a thin line. (**b**) The 3200 bp *Xho*I fragment containing the coding region of *MCM1* (thick line) that also indicates the area replaced by the *LEU2* gene. Direction of transcription is indicated by an arrow. (**c**) The domain structure of the *MCM1* translation product. I and II contain the two domains necessary for DNA binding and dimerization (the hatched area indicates the basic region, the filled area the more hydrophobic region). Domain III represents the stretch of negatively charged amino acids. The dotted squares in domain IV mark the runs of glutamines. (**d**) Diagram of the *mcm1-98* translation product. The star indicates the sequence of the last four amino acids encoded by the wild-type and the mcm1-1 alleles. (**e**) represents the gene fusions between the *GAL1* promoter or the *SpADH* promoter and the coding region of the *MCM1* minimal domain. (**f**) and (**g**) Templates for *in vitro* transcription of *STE12* and *MATα1*, respectively (plasmids pMP101 and pMP120). The amino-terminal sequences of the translation products are indicated. The following restriction sites are shown: *Bam*HI (B), *Bgl*II (G), *Bsp*HI (BspH), *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), *Nco*I (N), *Nde*I (Nd), *Pst*I (P), *Sac*I (S), *Xho*I (X) and a fusion between a *Bal*I and a *Sty*I site (Ba/S).

(pheromone response element), the recognition site for the STE12 protein (Errede and Ammerer, 1989). As can be seen in Figure 5A and C, the overall amount of DNA binding activity appears to be comparable in all three extracts. Although the experiment in Figure 5A was done with excess of protein, similar experiments with probe excess indicated that the mcm1-98 extract is ~2- to 3-fold more active than the wild-type extract (data not shown). Additionally, in samples from strains expressing both forms of the protein, we observed a complex that is probably due to the formation of heterodimers between the full length and the truncated form of MCM1. Other complexes are consistent with the interpretation that they represent an α2–MCM1 or a STE12–MCM1 complex (Errede and Ammerer, 1989; Ammerer, 1990). For example, the signal proposed to constitute the mcm1-98–α2 complex is only visible in extracts from α cells but not **a** cells. The addition of *Escherichia coli*-produced α2 protein to the extracts will result in an overall increase in binding activity as well as the enhancement of a slower migrating complex (Figure 5B). Similarly, the addition of *in vitro* synthesized STE12 identifies the presumed STE12–mcm1-98 complex (Figure 8A, lane2; data not shown). As predicted, in all cases the ability to form the respective α2 and STE12 complexes is independent of the size of the *MCM1* product.

mcm1-98 is sufficient for recruitment of α1

Since it has been suggested that sequences outside the minimal binding domain are necessary to promote α1–MCM1 interaction (Tan and Richmond, 1990) and/or transcriptional cooperation (Christ and Tye, 1991) we considered *in vitro* binding experiments with the α1 protein especially important. The crude yeast lysates described above were analysed by gel retardation assays using an α-specific element from the *MFα1* promoter (*MFα1*-B, Ammerer, 1990). As seen in Figure 5C (lanes 12, 14 and 17) this element binds full length MCM1 only weakly, leading to a single complex (M) in extracts derived from **a** cells (lane 17). Even with α cell extracts the intensity of the signal does not increase substantially although one can now distinguish two closely spaced bands (M and M/α1; lanes 12 and 14). This observation is consistent with a report by Bender and Sprague (1987) which implied that endogenous α1 concentrations are normally insufficient to be detected by a gel retardation assay. However, as seen in lanes 13, 15 and 16, the addition of *E.coli*-produced α1 protein (Tan *et al.*, 1988) increases MCM1–DNA complex formation considerably. In contrast to these results, extracts from *mcm1-98* cells will lead to a strong signal already in the absence of exogenous α1 (m; lanes 17 and 18). In this case, however, addition of α1 produces a new, more slowly migrating complex (m/α1; lanes 16 and 19). As α1 cannot bind specifically to DNA on its own (Tan *et al.*, 1988), a straightforward interpretation for this new signal is that it constitutes a ternary complex between α1, mcm1-98 and DNA. Support for this interpretation comes from the observation that a complex of similar size can also be reconstituted with *in vitro* synthesized mcm1-98 and α1 (data not shown). From comparison between the wild-type and the truncated protein, it seems as if slightly less α1–MCM1 complex is formed from extracts containing the small product (Figure 5C, lanes 13 and 19), suggesting a lower affinity of mcm1-98 for α1 compared with the affinity of the wild-

Table I. Genotypes of yeast strains

Yeast strain	Genotype	Source
W303-1A	*MAT***a**, *leu2, trp1, ura3, his3, ade2, can1*	R.Rothstein
W303-1B	*MATα*, same as above	R.Rothstein
W303	*MAT***a**/*MATα* same as above	R.Rothstein
GA117	*MAT***a**/*MCM1*/*mcm1::LEU2*	W303 Tfn
SL2	*MAT***a**/*MATα*, *MCM1*/*mcm1::LEU2*, *GAL1-mcm1-98::URA3*/*ura3*	GA117 Tfn
SL3	*MAT***a**/*MATα*,*MCM1*/*mcm1::LEU2*, *ADH-mcm1-98::URA3*/*ura3*	GA117 Tfn
SL3-2A	*MATα*,*mcm1::LEU2*, *ADH-mcm1-98::URA3*	SL3
SL3-2B	*MAT***a**, *MCM1*, *ADH-mcm1-98::URA3*	SL3
SL3-10B	*MAT***a**, *mcm1::LEU2*, *ADH-mcm1-98::URA3*	SL3
SL3-10C	*MATα*, *MCM1*, *ADH-mcm1-98::URA3*	SL3
SL2-2B	*MAT***a**, *mcm1::LEU2*, *GAL1-mcm1-98::URA3*	SL2
SL-2A	*MATα*, *mcm1::LEU2*, *GAL1-mcm1-98::URA3*	SL2
MP151	*MATα*, *mcm1::LEU2*, *GAL1-mcm1-98::URA3* [*YCp*, *TRP1*, *ADH-mcm1-98*]	SL2-2A Tfn
MP152	*MATα*, *mcm1::LEU2*, *GAL1-mcm1-98::URA3* [*YCp*, *TRP1*, *ADH-mcm1-47/SRF*]	SL2-2A Tfn
MP153	*MATα*, *mcm1::LEU2*, *GAL1-mcm1-98::URA3* [*YCp*, *TRP1*, *ADH-mcm1-98/97L*]	SL2-2A Tfn
MP157	*MAT***a**, *mcm1::LEU2*, *GAL1-mcm1-98::URA3* [*YCp*, *TRP1*, *ADH-mcm1-47/SRF*]	SL2-2B Tfn
R757	*MATα*, *sst2*, *met1*, *his6*, *can1*, *cyh2*, *rme1*, *gal2*	R.K.Chan
K2149	*MAT***a**, *HMLa*, *HMRa*, *ho-βgal*, *bar1:: HISG*, *leu2*, *trp1*, *ura3*, *ade2*, *can1*, *met*, *his3*	K.Nasmyth
K217	*MATα*, *his1*	K.Nasmyth
K216	*MAT***a**, *his1*	K.Nasmyth
RM9-2D	*MATα*, *mcm1-1*, *leu2*, *his4*	B.Tye
RM9-2C	*MAT***a**, *mcm1-1*, *leu2*, *his4*	B.Tye

All strains in the first column are isogenic with W303-1A

type product. The difference becomes more apparent using limiting amounts of α1 produced in an *in vitro* translation system (Figure 5D). Addition of the same amount of α1 produces more trimeric complex with MCM1 than with mcm1-98. The MCM1/mcm1-98 heterodimer seems to exhibit an intermediate behaviour. In the absence of α1, it binds more efficiently to the *MF*α1-B element than the MCM1 dimer but still less well than the *mcm1-98* product. Addition of α1 still leads to a considerable increase in binding activity (Figure 5C, lanes 14 to 17; M/m and M/m/α1).

mcm1-98 and α1 bind cooperatively in vivo

Since mcm1-98 has slightly different *in vitro* DNA binding properties compared with MCM1, we wished to address the following questions by means of *in vivo* footprint analysis. Has the increased affinity of the *mcm1-98* product for the α-specific element any consequences for the occupancy of such a binding site *in vivo*? If not, can cooperation between α1 and mcm1-98 still be observed at *in vivo* concentrations of the proteins? **a** and α cells carrying the wild-type or the *mcm1-98* gene were treated with DMS, their DNA was isolated and cleaved at methylated Gs. We analysed the cleavage products by linear amplification reactions with *Taq* polymerase (Saluz and Jost, 1989) using a primer hybridizing within the *MF*α1 promoter. The DMS induced cleavage pattern at the *MF*α1-B element reveals distinct differences between **a** *MCM1* and α *MCM1* cells, with the **a**-specific pattern indistinguishable from the one generated with unoccupied, *in vitro* modified DNA (Figure 6, lanes 1 and 3; H.Winkler and G.Ammerer, in preparation). By analysing DNA from *mcm1-1* cells, we have shown in independent experiments that the generation of the α-specific pattern is in fact due to MCM1 occupation. An identical footprint pattern can be generated *in vitro*, using highly purified fractions of MCM1 protein (H.Winkler and G.Ammerer, in preparation). The results obtained for *mcm1-98* strains were virtually identical to those observed with wild-type cells (Figure 6, lanes 4–7). The pattern diagnostic for MCM1

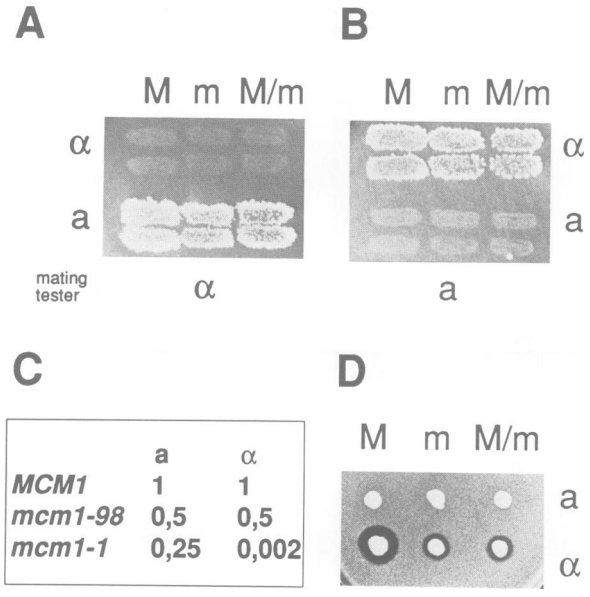

Fig. 3. Mating and pheromone production assays of strains expressing *MCM1* or the *mcm1-98* truncation. (**A**) and (**B**) patch mating assays, (**C**) quantitative mating assays and (**D**) α-factor assay. In the patch assay, streaks of the tested strains were replica plated onto a lawn of mating tester cells on minimal medium. As all haploid cells are unable to grow under these conditions, the formation of growing colonies within a patch indicates successful mating events (Sprague, 1991). (M) indicates a strain with the MCM1 allele, (m) with the mcm1-98 allele, (M/m) indicates a strain expressing both alleles. The mating type for the upper two colony streaks is α, for the lower two streaks it is **a**. An α mating type strain (K217) was used as tester in (A) and an **a** mating type strain (K216) in (B). (C) represents the results of a quantitative mating assay (Hartwell, 1980). The mating efficiencies are the number of diploids formed with *MCM1* strains related to those for an *mcm1-98* or *mcm1-1* strain. The *MCM1* and *mcm1-98* strains are isogenic but the *mcm1-1* strains have a different genetic background. Assays were done with logarithmically growing cells at 30°C. (D) shows a halo assay (Sprague, 1990) using the growth inhibition of an **a** *bar1* strain (K2149) as indicator. The α strains are spotted in the bottom row, the isogenic **a** strains in the top row.

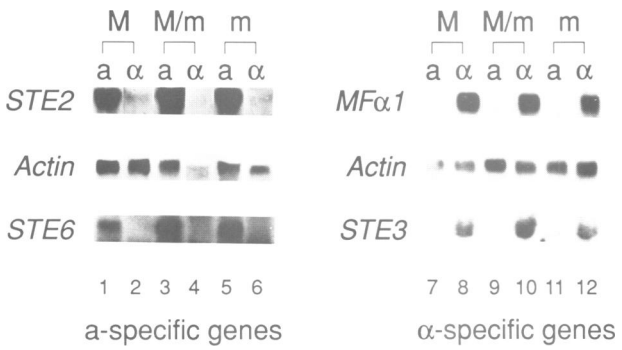

Fig. 4. Transcription of genes involved in pheromone and receptor synthesis. Northern blot analysis with RNA isolated from **a** cells (odd numbered lanes) and α cells (even numbered lanes). The strains differ only in their expression of *MCM1* and *URA3* but are otherwise isogenic. Lanes 1, 2, 7 and 8 have RNA from wild-type *MCM1* (M), lanes 3, 4, 9 and 10 have RNA from a strain heterozygous for *MCM1* and *mcm1-98* (M/m), lanes 5, 6, 11 and 12 have RNA from a *mcm1-98* strain (m). The probes used for hybridization are indicated. The actin probe served as internal control for the *STE2* and *MFα1* transcripts. The *STE6* and *STE3* RNAs were measured in an independent experiment.

binding appears only in α cells. Therefore, mcm1-98 alone still does not bind under *in vivo* conditions to the *MFα1*-B element. It is equally clear that the mcm1-98 protein still requires α1 to be able to bind at this particular promoter element. The data strongly support our proposition that the main structures required for recruitment of α1 are located within those domains of MCM1 that show homology with the mammalian transcription factor SRF.

An MCM1 – SRF hybrid is functional for cell viability

MCM1 and SRF are 70% identical across 81 amino acids that seem essential for dimerization and DNA recognition (Norman *et al.*, 1988). Indeed, under certain conditions overlapping binding specificities between the SREs and P-boxes for both factors have been observed (Hayes *et al.*, 1987; Passmore *et al.*, 1989). Nevertheless, we found that SRF as produced *in vitro* in a rabbit reticulocyte system is not able to bind as efficiently as MCM1 to the *STE2* operator or any of the binding sites derived from the *MFα1* promoter (data not shown). Assuming that the amino acids most likely to determine DNA sequence specificity are located within the N-terminal basic portion of the two proteins, we constructed a 98 amino acid hybrid that contained 47 amino acids of MCM1 followed by 51 amino acids of SRF (Figure 7A). In effect, this created an *mcm1* mutant with 17 amino acid changes across the dimerization domain. The hybrid gene was tested for its ability to complement the lethal effects of an *mcm1* deletion. To do this, strains were constructed whose sole functional *MCM1* copy was regulated by the *GAL1* promoter integrated at the *URA3* locus (Figure 1; Table I). These strains were transformed with a plasmid containing the *SpADH* promoter fused to the *mcm1-47/SRF* hybrid. Unlike control strains, transformants containing this plasmid are now able to survive on glucose containing media, although such cells grow slightly more slowly than the wild-type controls. The result suggests that the fusion protein is able to perform the essential functions of MCM1, albeit with somewhat reduced efficiency. We have not determined yet whether the slight growth defect is due to increased plasmid loss (equivalent to the original mcm phenotype; Maine *et al.*, 1984) or suboptimal provision for other essential functions.

The MCM1 – SRF hybrid protein is unable to interact with cell type specific factors

Both **a** and α cells are clearly able to survive with the altered MCM1 protein. However, are such cells still competent to conjugate? We found that *mcm1-47–SRF* cells are completely sterile in both mating types. As measured by quantitative mating assays the formation of diploids is at least six orders of magnitude below wild-type cells and certainly much lower than α *mcm1-1* mutants (data not shown; Christ and Tye, 1991). Since the same result was obtained with **a** and α cells it seems likely that the mating defect is the consequence of a general failure to activate and regulate **a** and α-specific functions rather than the result of inappropriate expression of both sets at the same time. This interpretation was fully supported by analysis of cell type specific transcripts and by *in vitro* binding studies. Probing for *STE2, STE3* and *MFα1* RNAs, we found that they are virtually absent in strains expressing the mcm1-47–SRF hybrid (Figure 7B and C). As shown in Figure 8, extracts from strains expressing the hybrid gene contain binding activity for the *STE2* operator as well as for the *MFα1*-B fragment. The complex runs slightly more slowly than the equivalent mcm1-98 complex, possibly due to changes in its conformation or to differences in the charge of the proteins. Nevertheless, the binding activities per amount of protein are quite similar, indicating that the hybrid protein is able to dimerize and bind to *MCM1* recognition sequences. The protein is neither able to cooperate with *in vitro* produced STE12 nor can such a complex be detected in bandshift assays with the crude extract itself (Figure 8A). Similarly, the interaction with α1 cannot be observed although extracts from a control strain are proficient in α1 recruitment (Figure 8C). Testing for the cooperation with α2 protein, we find that a potential complex was formed, albeit with highly reduced efficiency (Figure 8B). These data are fully consistent with the results obtained by Northern analysis. As a consequence, we propose that the amino acid changes introduced by the SRF moiety must encompass residues that are normally involved in specific contacts with the proteins that mediate the cell type specific functions of MCM1.

Discussion

In this work, we defined domains of the MCM1 protein that are necessary for its cooperative binding with other regulatory proteins. Surprisingly, we found that the 98 amino-terminal amino acids of MCM1 are sufficient for its biological function including its role in the expression and regulation of cell type specific pheromone and receptor genes. One can easily understand these results based on our demonstration that the direct physical interactions between MCM1 and its regulatory partners are maintained by the truncated protein. Our work also confirms and extends an independent study by Christ and Tye (1991) that leads to similar conclusions. These authors investigated the effects of MCM1 variants on the mating competence. Additionally, they measured the effects on transcriptional activation using a variety of MCM1 binding sites linked to a reporter gene. Despite several agreements between the two studies there also exist some discrepancies. Christ and Tye's results imply

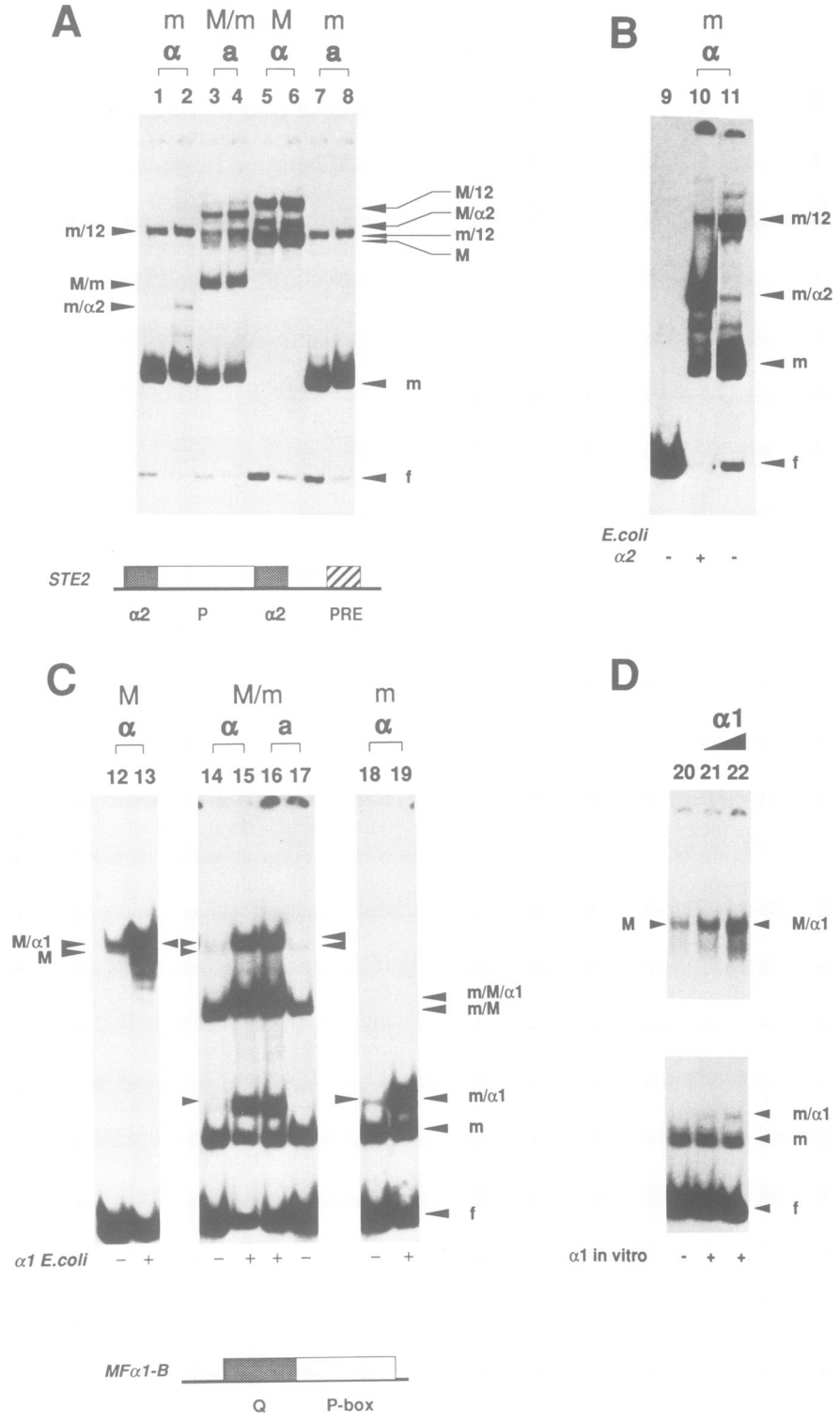

Fig. 5. The *mcm1-98* product interacts with STE12, α2 and α1. Gel retardation assays were done with *MCM1* (M), *mcm1-98* (m) cell extracts or extracts containing both forms of the MCM1 protein (M/m). The mating type of the strains from which extracts were prepared is specified underneath. (**A**) and (**B**) show experiments with the *STE2* operator probe, (**C**) and (**D**) with the *MF*α1-B probe. The presumed MCM1 (M), mcm1-98 (m), STE12 (12), α1 and α2 composition of each complex is indicated next to an arrow. (f) marks the position of the free probe. In the odd numbered lanes of (A) the DNA was incubated with 10 μg protein, in the even numbered lanes with 20 μg. The same amount was used in (B) lane 11. Lane 9 constitutes the control without extract. *E.coli*-produced and purified α2 was added to the extract in lane 10. In (C), purified α1 protein (produced in *E.coli*, Tan *et al.*, 1988) was added to the extracts in lanes 13, 15, 16 and 19. The α1-containing complexes visible in the other lanes are derived from endogenous protein. (D) shows the effect of *in vitro* produced α1 on complex formation with MCM1 (upper) and mcm1-98 extracts (lower). The data from parallel but independent reactions are superimposed. One μl of reticulocyte lysate was added to the extracts with twice the amount of α1 in lane 22 compared with lane 21.

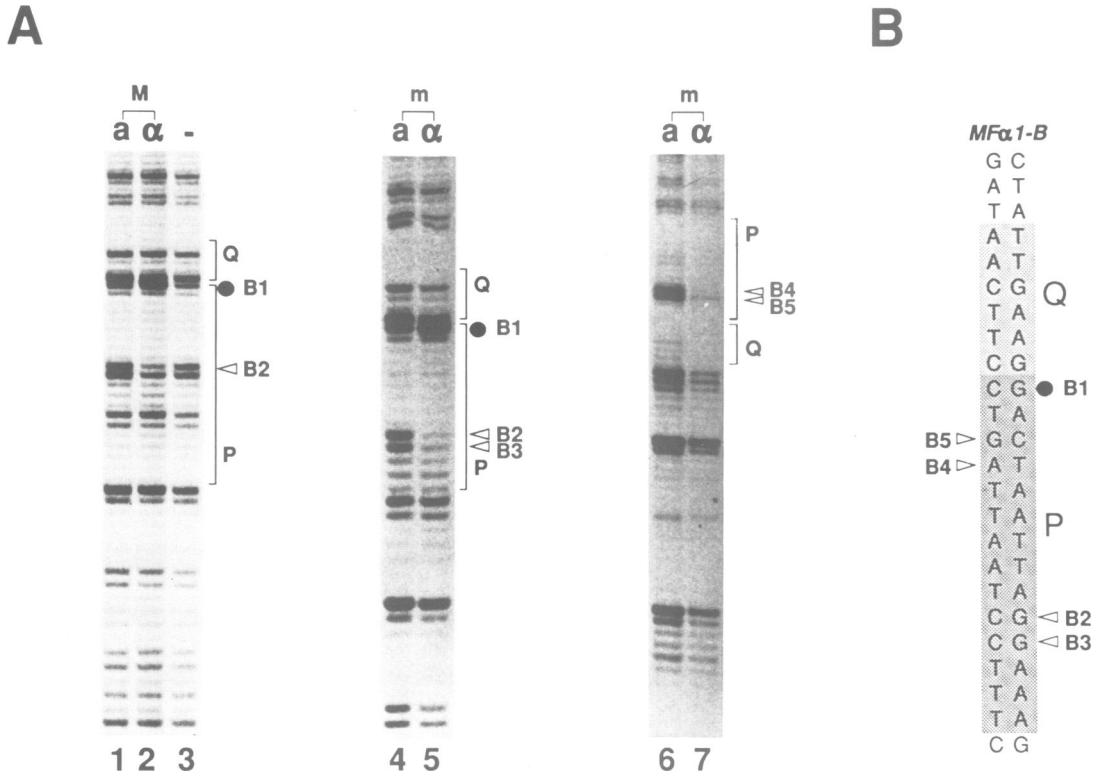

Fig. 6. *In vivo* footprint analysis. (**A**) shows the DMS induced cleavage pattern of the *MFα1* promoter between the positions −385 to −335 for the coding strand (lanes 1–5) and the positions −320 to −375 for the non-coding strand (lanes 6 and 7). Lanes 1 and 2 show the patterns found in *MCM1* strains. They are compared with *in vitro* modified DNA (lane 3). Lanes 4–7 contain DNA from *mcm1-98* cells. A summary of the differences between **a** and α cells is presented in (B). A filled circle indicates an enhancement of DMS sensitivity, an open arrowhead indicates increased protection. The DNA sequence encompasses the P-Q element of *MFα1*-B.

that the acidic domain is considerably more important for α-specific activation functions than observed by us. Compared with our measurements, they also report a more severe defect for α-specific mating. To resolve these apparent differences one has to take into account that α- but not **a**-specific promoters are quite sensitive to the expression levels of MCM1 (Passmore *et al.*, 1989; Christ and Tye, 1991). Christ and Tye obtained *mcm1-98* expression by direct gene replacements at the *MCM1* locus, while we made use of a heterologous promoter fusion integrated at a different chromosomal site. mcm1-98 could, therefore, have been overproduced in our cells leading to suppression of an otherwise more severe defect. Nevertheless, it is equally likely that expression of the truncated gene could lead to decreased RNA or protein levels due to different stabilities. Indeed, measurements of MCM1 protein concentration by Christ and Tye seemed to indicate that the truncated protein is present in lower amounts compared with the wild-type product (see Figure 4B in Christ and Tye, 1991). Lacking specific antibodies against N-terminal sequences of MCM1 we have just compared binding activity with the *STE2* promoter as a measure of MCM1 present in the cells. We estimate that our *mcm1-98* construct enhances overall binding activity at the most 2- to 3-fold. The combined differences could easily explain the less severe effects in our strains.

From our observation that α1 interacts better with variants containing the acidic stretch of MCM1 (Figure 5D), one may be tempted to use loss of α1 interaction as the main explanation for the α-specific defect *in vivo*. However, our *in vivo* footprint data would argue against this interpretation. It seems that even under *in vivo* conditions, mcm1-98 still binds to an element of the *MFα1* promoter in a highly α1-dependent manner. We have shown for another α-specific element of the *MFα1* promoter that wild-type MCM1 binding as such is not sufficient for activation (H.Winkler and G.Ammerer, in preparation). Therefore, we prefer the interpretation that the acidic domain is not so important for recruitment of α1 by MCM1 (which enables MCM1 to form a complex at the promoter) but more for their cooperative interaction with other components of the transcriptional machinery (see also Tan and Richmond, 1990).

What is the function of the MCM1 C-terminal domains?

If the deletion of the C-terminal domains has only a minor effect on mating and no effect with regard to viability why are they maintained by the yeast cell? Perhaps two of our observations are relevant to this question. First, the deletion of the negatively charged region results in increased binding of the *MCM1* product at α-specific elements *in vitro*. This is not only true for *mcm1-98* (Figure 5C) but also for an internal deletion of the acidic domain that leaves the polyglutamine stretches intact [similar to the one described by Christ and Tye (1991) as *mcm1*-ΔDE; data not shown]. Second, the lack of an acidic domain has a noticeable effect on the transcriptional activation of the α-pheromone promoter. The carboxy-terminal domains (but especially the acidic domain) may therefore serve a role in enhancing the

discrimination of **a** and α-specific elements by MCM1. The acidic domain lowers the affinity for α-specific motifs in the absence of α1 while at the same time increasing the activation by the dimeric protein complex. We believe that such a modulatory effect is quite important in the natural environment. Although our quantitative mating assays have shown that the ability to mate as such is hardly affected, these assays may not reflect the constraints naturally encountered by a yeast cell. Indeed, Jackson and Hartwell (1990) have shown that the amount of pheromone produced by a cell is the most critical parameter for partner selection. Therefore, in a competitive environment, the difference between higher and lower pheromone synthesis may have more severe consequences for the mating success than is implied by our assays.

It has been found for several developmentally important transcription factors that surprisingly large parts of their sequence can be deleted, essentially without destroying their biological function (Davis *et al.*, 1990; Gibson *et al.*, 1990). This fact leads to the question of whether these additional sequences are just the consequence of a historical accident or whether they are nevertheless maintained under selective pressures. We suggest that many of these seemingly superfluous sequences will enhance discriminatory mechanisms thereby increasing the fidelity of certain regulatory processes. The consequence of even gross protein alterations may be subtle and not always obvious under normal experimental conditions.

MCM1 is necessary for a-specific activation

The importance of MCM1 in the activation of **a**-specific genes has been somewhat disputed. A clear correlation of MCM1 binding and activation has only been shown for an isolated fragment of the *STE2* promoter. A mutant binding site that cannot bind MCM1 completely abolishes the ability of this fragment to activate a heterologous promoter (Ammerer, 1990). However, a conclusive study has never been reported on the complete promoter. With respect to the *BAR1* promoter, a substantial defect is caused by the deletion of the MCM1 binding site (Kronstadt *et al.*, 1987), but *BAR1* is not essential for the mating process. This is in contrast to *STE6*, an **a**-specific function involved in the transport of the pheromone. Deletion analysis of the promoter implied the existence of redundant activation elements that are independent of the MCM1 binding site (Wilson and Herskowitz, 1986). Additionally, all viable *mcm1* mutations described so far exert an effect only on α-specific transcription (and thereby mating) but left **a**-specific activation mainly intact (Passmore *et al.*, 1987; Christ and Tye, 1991). One of these mutants, *mcm1-1* exhibits reduced DNA binding affinity *in vitro* (mentioned in Keleher *et al.*, 1988; M.Primig, unpublished observation). This result suggests that either the defect is enhanced and of more severe consequence for α-specific elements or that in general MCM1 is not important for the transcriptional activation of **a**-specific functions. The pronounced effect of the mcm1-47−SRF fusion on the ability to mate as an **a** cell now provides more direct evidence that the latter is not the case. As a consequence, we propose that the inability of mcm1-47−SRF to interact with STE12 is responsible for the **a** mating defect although we cannot yet exclude the possibility that mcm1-47−SRF has attained the properties of a repressor (independent of α2) with long range effects on neighbouring UAS sequences.

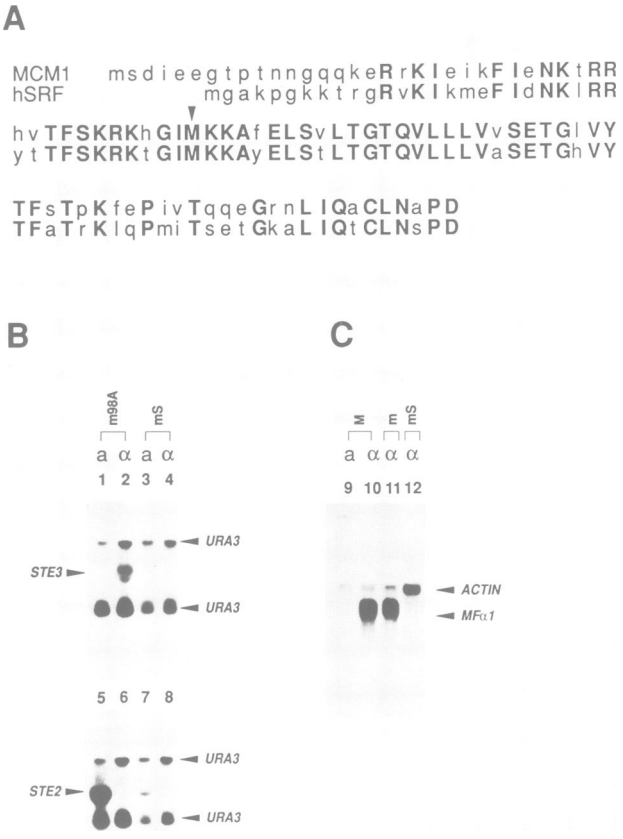

Fig. 7. A MCM1−SRF hybrid is defective in cell type specific transcription. (**A**) shows an amino acid sequence alignment of the DNA binding and dimerization domains of MCM1 and SRF. The mcm1-47−SRF fusion was constructed using a *Bsp*HI restriction site common to both genes. Its position is indicated by an arrow over the encoded methionine. Amino acids identical in the MCM1 and SRF core sequences are written in capital letters. (**B**) shows a Northern analysis of strains transformed with either plasmid pGA1835 (coding for *mcm1-98*) or plasmid pISE3 containing the mcm1-47−SRF fusion. The RNA was probed with a fragment covering the *URA3* gene (lanes 1−8), the *STE3* gene (lanes 1−4) and the *STE2* gene (lanes 5−8). The larger *URA3* transcript is a consequence of the *ura3-52* allele, the smaller transcript comes from the integrated fusion vector. (**C**) shows the expression level of the *MF*α1 RNA in strains with different *MCM1* alleles: lane 9 is from an **a** strain (SL2-2B) transformed with a plasmid containing wild-type MCM1. The strain was grown on glucose. All other lanes are from transformants of the isogenic α strain (SL2-2A). Lane 10, *MCM1*; lane 11, *mcm1-98*; lane 12, *mcm1-47−SRF*.

Specificity of MCM1 in protein−protein interactions

The hybrid between MCM1 and SRF is not able to activate any of the cell type specific promoters tested. This distinct defect is certainly not due to the lack of binding specificity. The hybrid gene can rescue a cell from the lethality caused by an *mcm1* deletion. Additionally, the binding activity observed *in vitro* is comparable with wild-type MCM1 and appears to be higher than that seen with the *mcm1-1* product (data not shown). The most likely cause of the difference in activation is the inability of the fusion product to provide an interactive surface for the cell type specific factors. This view is fully supported by our *in vitro* binding data. In addition, we suggest that the region between amino acids 48 and 92 is the most likely one involved in the interaction with the auxiliary factors. This prediction has recently been verified by Mueller and Nordheim who find that an exchange of just four amino acids of SRF with MCM1 derived residues is sufficient to support cooperative binding of the SRF

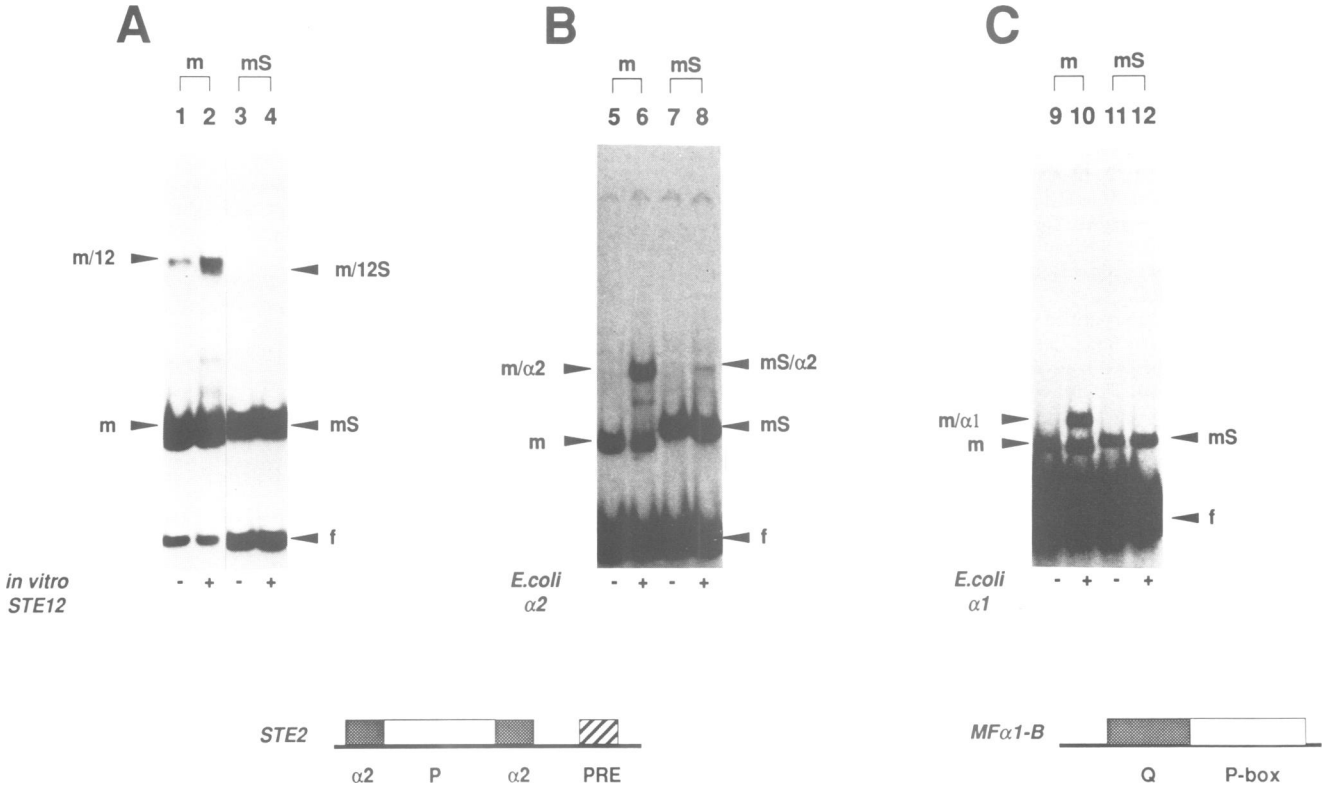

Fig. 8. mcm1-47−SRF is defective in its interaction with STE12, α2 and α1. The probes used in these bandshift assays are a 47 bp fragment of the *STE2* promoter (**A**) and (**B**) and a fragment containing the *MF*α1-B sequence (**C**). Extracts were prepared from strains expressing mcm1-98 or mcm1-47−SRF from a plasmid. Bandshifts with mcm1-98 extracts (m) are shown in lanes 1, 2, 5, 6, 9 and 10, with mcm1-47−SRF extracts (m/S) in lanes 3, 4, 7, 8, 11 and 12. *In vitro* synthesized STE12 was added in lanes 2 and 4 (A), α2 purified from *E.coli* was added in lanes 6 and 8, α1 generated from *E.coli* was added in lanes 10 and 12 (C).

minimal domain with STE12 (see accompanying paper, Mueller and Nordheim, 1991). It will be interesting to see whether a similar result can be obtained for α1 and α2 and to what extent the contact sites with the three different proteins will require the same amino acid residues.

Materials and methods

Plasmids

We constructed a *mcm1*Δ::*LEU2* allele using 5′ and 3′ flanking regions derived from ExoIII/S1 generated truncations of the original 3.2 kb *Xho*I−*Bam*HI fragment containing the *MCM1* gene (Ammerer, 1990). In the pIC19R derived plasmid pGA1748, a 2200 bp fragment containing the *LEU2* gene replaces the complete *MCM1* coding region (nucleotide positions −47 to 867 from the initiator ATG; Figure 2a). The *mcm1-98* allele was synthesized by polymerase chain reaction (PCR). The following oligonucleotides were used as primers: TTGGTGAATTCAATATTATA-GCCACCCAGCAAAAATG and GAGGACTGCAGTCAATCAGGGG-CGTTAAGACAGGCC. The *mcm1-98/97L* was similarly obtained but using the oligonucleotide GAGGACTGCAGTCAATCAAGGGCGTAAGAC-AGGCCTG as non-coding strand primer. The *mcm1-98* PCR product was subcloned as an *Eco*RI−*Pst*I fragment into pUC9 to give plasmid pGA1798. The same *Eco*RI−*Pst*I fragment was combined with an 800 bp *Hin*dIII−*Eco*RI fragment containing the *GAL1−10* promoter in YIplac211 (Johnston and Davies, 1984; Gietz and Sugino, 1988) to give the plasmid pSL1 for galactose inducible expression of mcm1-98 in yeast (Figure 2e). The plasmid pSL2 is analogous to pSL1 except that it was constructed using a 700 bp *Hin*dIII−*Eco*RI fragment of the *S.pombe ADH* promoter (Russell and Nurse, 1986) instead of GAL1. Plasmid pGA1835 contained the *SpADH−mcm1-98* fusion subcloned in YCplac22. The *mcm1-98/97L* expressing plasmid (pGA1836) was constructed in a similar way using the PCR product cut with *Eco*RI and *Pst*I. A plasmid suitable for *in vitro* expression of the minimal domain of SRF was kindly provided by R.Treisman. This plasmid contains the T7 promoter and the untranslated leader of the β-globin gene on a *Hin*dIII−*Nco*I fragment followed by a 269 bp SRF coding region (positions 755−1024; Norman *et al.*, 1988) as a *Nco*I−*Bam*HI fragment. The minimal domains of SRF and MCM1 were combined using a unique *Bsp*HI site that is common to both genes. The fusion was cloned behind the *SpADH* promoter in the yeast vector YCplac22 to give plasmid pISE3. The *MAT*α1 gene was subcloned behind the T7 promoter/β-globin leader as a PCR generated fragment cut with *Nco*I and *Pst*I (to give the plasmid pMP92). We used the oligonucleotides GACGC-CATGGACATATGTTTACTTCGAAGCCTG and GATAAGGATCCT-CCGCCACGACCACACTC as primers. The construct leads to a *MAT*α1 product with three additional amino-terminal amino acids that do not interfere with α1 function (Figure 2g). The plasmid for *in vitro* transcription of *STE12* (pMP101) was a derivate of plasmid pE5LVPO containing the T7 promoter linked to the EMC untranslated leader (Parks *et al.*, 1986). The *STE12* coding sequence was linked to the EMC leader using a blunted *Sty*I end located upstream of the initiator ATG of *STE12*. For *in vitro* transcription the plasmid was cut at a *Sac*I site close to the termination codon of *STE12*. The transcript leads to an *in vitro* translation product with an amino-terminal extension of three amino acids and a carboxy-terminal truncation of 20 amino acids. Both changes are irrelevant for STE12 DNA binding activity.

Yeast strains, mating assays, pheromone sensitivity assays

All strains used for binding assays and Northern blots were isogenic to strain W303-1A (source R.Rothstein). The *MCM1* gene was disrupted by transforming a diploid strain with plasmid pGA1748 (linearized with *Bgl*II). The gene fusions carried on plasmids pSL1 and pSL2 were integrated at the *URA3* locus using either *Apa*I or partially *Nco*I digested plasmid DNA. Other W303-1A strains (Table I) were obtained by sporulating and dissecting the appropriate diploid parent or by standard yeast transformation procedures. Yeast strains were generally manipulated as described in Sherman *et al.* (1986). The quantitative mating assays were performed as described by Hartwell (1980). Pheromone sensitivity assays (Sprague, 1990) were done on standard YEPD plates and incubated at 30°C.

In vitro transcription and translation systems, yeast cell extracts, E.coli protein

RNA was synthesized as published by van der Werf *et al.* (1986) with a few modifications. 5 μg of linearized template DNA were incubated with 400 units of T7 RNA polymerase (generously provided by Martin Nicklin) at 37°C for 1 h in a 200 μl reaction [1× buffer was 18 mM Na_2HPO_4,

2 mM NaH_2PO_4, 10 mM DTT, 50 mM NaCl, 4 mM rNTPs, 8 mM spermidine 3HCl, 8 mM $MgCl_2$ and 40 units RNasin]. After addition of EDTA to a final concentration of 12 mM, the reaction mix was heated to 65°C for 5 min and chilled on ice. The RNA was extracted with saturated phenol and chloroform and precipitated with LiCl (final concentration of 2.5 M). RNA was added to a rabbit reticulocyte lysate system (Promega L4210) at a concentration of 10–20 μg/ml. Standard reactions were incubated for 1 h at 30°C. Immediately after completion of the translation reaction the lysates were used for DNA binding experiments.

Yeast extracts were prepared from logarithmically growing cells according to Pfeifer *et al.* (1987). The protein concentration as determined by Bio-Rad protein assay was adjusted to 5 or 10 μg/μl. Extracts were aliquoted and stored at −80°C.

Chromatographically purified fractions of α1 and α2 proteins were generously supplied by Song Tan and Tim Richmond (ETH, Zürich). The proteins were synthesized in *E.coli* and purified as described (Tan *et al.*, 1988; Ammerer, 1990). The percentage of active protein was not determined. For binding experiments, the appropriate dilution of concentrated fractions was determined empirically.

DNA binding assays

Radioactive probes were generated by labelling gel purified restriction fragments with Klenow DNA polymerase. Protein–DNA binding assays were done in 10 μl of binding buffer (20 mM Tris pH 8.0, 40 mM NaCl, 4 mM $MgCl_2$, 1 mM DTT, 5% glycerol). Between 0.2 and 3 μl of lysate containing the desired factor and/or 10 μg of a crude protein extract were incubated with 0.5 ng of ^{32}P-labelled DNA and 1 μg of poly d(I-C) for 10 min at room temperature. The samples were loaded onto a 5% 19:1 polyacrylamide:bisacrylamide gel in 0.5×TBE (Ammerer, 1990).

Northern analysis

Total RNA was isolated from yeast cells and separated electrophoretically as described (Price *et al.*, 1991). The RNA was transferred onto GeneScreen NEF-972 (DuPont) filters as recommended by the supplier. The following probes were labelled by random priming and used for hybridization: an 1100 bp *Hin*dIII fragment containing the *URA3* gene (YIp5), a 1600 bp *Hin*dIII fragment of the *STE2* gene (pGA1820), a 1400 bp *Hin*dIII fragment of the *STE3* gene (pGA1625), a 3400 bp *Xba*I–*Hin*dIII fragment of the *STE6* gene (pAH21), a 600 bp fragment encompassing just the coding region of *MF*α1 (pGA201) and a 3500 bp *Bam*HI–*Eco*RI fragment encoding the *ACT1* gene (pAH301).

DMS protection assays

In vivo footprinting assays followed the procedure described by Saluz and Jost (1989) with minor modifications. 600 ml cultures where grown and harvested at an OD_{600} of 2.0–3.0. Cells were resuspended in 1.5 ml YEPD and 5 μl DMS were added. After incubation for 5 min at room temperature, the reaction was stopped by addition of 40 ml of chilled TNEβ (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 40 mM NaCl, 100 mM β-mercaptoethanol). Genomic DNA was then isolated and was resuspended in a final vol of 150 μl TE. 50 μl aliquots were cut with *Hin*fI and subsequently cleaved with piperidine.

DNA synthesis reactions were primed with the following end labelled oligonucleotides: GACATCCCGTTCTCTTTGGTAATCTGC to obtain coding strand information and AGATGCATCTTTTCACATGGTCCACTC for the opposite strand. Thirty rounds of primer extension were carried out in a thermal cycler using *Taq* polymerase (8 U) and 800 pg of end-labelled oligonucleotide in 100 μl reactions, where denaturation was at 94°C, 1 min, annealing at 64°C, 2 min and primer extension at 72°C for 3 min. After primer extension, the DNA was precipitated by addition of 0.5% CTAB (hexadecyltrimethylammonium bromide). The DNA was further purified by two ethanol precipitations and was finally resuspended in 5 μl 0.1 M NaOH, 2 mM EDTA. After the addition of 5 μl loading buffer (8 M urea, 0.03% xylene cyanol, 0.03% bromophenol blue) 2–5 μl of the samples were analysed on 8% polyacrylamide gels containing 8 M urea. The dried gels were autoradiographed overnight at −80°C.

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