Summary

MCM1 is an essential yeast DNA-binding protein that affects both minichromosome maintenance, in a manner suggesting that it has DNA replication initiation function, and gene expression. It activates α-specific genes together with MATα1, and represses α-specific genes together with MATα2. Alone, MCM1 can activate transcription. To determine whether different domains of the protein mediate these diverse functions, we constructed and analyzed several mcml mutants. The gene expression and minichromosome maintenance phenotypes of these mutants suggest that the role of MCM1 in DNA replication initiation may not involve transcriptional activation. However, both transcription and replication activities require only the 80-amino-acid fragment of MCM1 homologous to the DNA-binding domain of the serum response factor (SRF). This small fragment is also sufficient for cell viability and repression of α-specific genes. A polyacidic amino acid stretch immediately adjacent to the SRF homologous domain of MCM1 was found to be important for activation of α-specific genes in a cells. Mutants lacking the acidic stretch confer higher expression from an α-specific UAS in a cells in addition to lower expression in α cells, suggesting that negative regulation at this site occurs in a cells, in addition to the well-documented positive regulation in α cells.

[Key Words: Replication factor MCM1; transcription activation; replication initiation; regulation]

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1987, Huberman et al. 1987, 1988], the mcm1-1 phenotype suggests that MCM1 is important for replication initiation. MCM1 may affect ARS function by binding to the origin as part of the replication initiation complex or it may act indirectly by affecting the expression of another protein that acts at ARSs. MCM1 is essential for cell division, although it is required for replication initiation or for regulation of other essential genes is unknown.

Although MCM1 is a small protein with only 286 amino acids [Passmore et al. 1988], it may be similar to other eukaryotic transcription factors in having separate DNA-binding, transcription activation, and regulatory domains. If so, different domains may be important for its different activities. MCM1 is a member of a gene family including the serum response factor [SRF] [Norman et al. 1988], another yeast gene, ARG80 [ARG RI] [DuBois et al. 1987, Passmore et al. 1988], and several plant homeotic genes [Sommer et al. 1990; Yanofsky et al. 1990]. The proteins are all share homology in a region that was identified as the DNA-binding domain of SRF. The amino-terminal portion of MCM1 [amino acids 18-98] is 70% identical to the portion of SRF that was shown to be essential for DNA binding and dimerization [Norman et al. 1988]. The two proteins share similar DNA-binding specificity, each binding to the palindromic recognition sequence CC(A/T)6GG [Norman et al. 1988; Passmore et al. 1989]. Therefore, the homologous domain is likely to be the DNA-binding domain of MCM1 as well. Immediately following the SRF homologous domain of MCM1 are 20 amino acids, of which 19 are either aspartate or glutamate. This region may be an acidic activation sequence, although the acidic residues are much more highly clustered than acidic activation domains of other transcription factors such as GAL4 and GCN4 [Hope and Struhl 1986; Keegan et al. 1986]. The carboxy-terminal half of the protein is 50% glutamine and may behave like the glutamine-rich activation domains of Sp1 and AntP [Courey and Tjian 1988; Courey et al. 1989]. Although truncated genes that lack much of the polyglutamine domain are functional [Passmore et al. 1988, Jarvis et al. 1989], the effect of removing the entire polyglutamine domain has not been tested previously.

In this study we specifically alter or delete portions of MCM1 which, as described above, may be separate functional domains. We analyze the effect of these mutations on the known activities of MCM1 in vivo: transcription activation, minichromosome maintenance, and regulation of mating-type-specific genes. This mutant analysis should reveal whether different functions of MCM1 require different domains, and provide insight into how MCM1 affects replication initiation and transcription activation and how it may interact with cofactors to mediate gene regulation.

Results

The SRF-homologous domain of MCM1 is sufficient for viability.

The MCM1 mutant proteins constructed are missing the acidic or polyglutamine domains, have substitutions of portions of other transcription factors, or have insertions into the putative DNA-binding domain, as shown in Figure 1. We made mutations that alter the coding sequence with minimal changes in the transcript to avoid alterations that might affect transcript stability. The mcm1–ΔQ allele codes for a protein with no polyglutamine domain, mcm1–ΔDE removes only the acidic stretch, and mcm1–ΔDEQ codes for a protein lacking both acidic and polyglutamine domains. The mcm1–ΔN7,ΔDEQ mutant has an additional deletion of amino acids 2–17, leaving only the SRF-homologous domain of MCM1. The mcm1–SRF/DE and mcm1–gcn4/DE(Q) alleles were constructed to assay whether the acidic stretch could be replaced with a less acidic sequence or with a known acidic activating sequence. The portion of SRF immediately following the SRF/MCM1-homologous domain was inserted in place of the acidic stretch in mcm1–SRF/DE, resulting in 7/34 acidic amino acids, as compared to 20/23 acidic amino acids in MCM1. A portion of GCN4 characterized previously as an acidic activation domain [Hope et al. 1988] was substituted for the acidic stretch and part of the polyglutamine region of MCM1 in mcm1–gcn4/DE(Q) [see legend to Fig. 1]. The mcm1–Xho92 and mcm1–Bam92 alleles have four amino acids [either AlaArgAlaAla or ArgLeArgAla] inserted in-frame after amino acid 92, in the region of MCM1 homologous to SRF.

Because MCM1 is an essential gene, we tested whether yeast with each of the mutant genes alone is viable, using the plasmid shuffle assay [Boeke et al. 1987] in a strain with a chromosomal deletion of MCM1. We found that all of the mutants except mcm1–Xho92 and mcm1–Bam92 provide sufficient MCM1 function for viability. This result localizes the essential domain of MCM1 to the 80 amino acids homologous to SRF.

Because mcm1–Xho92 and mcm1–Bam92 are unable to rescue lethality of an mcm1 deletion, even in high copy, we tested whether these mutations affect the amount or the activity of the resulting mutant MCM1 protein. Immunoblot analysis of a wild-type strain containing these high-copy constructs shows that the mutant proteins are present at similar levels as wild-type MCM1 on a similar plasmid [Fig. 2], suggesting that these mutations do not affect the stability or expression of MCM1. Therefore, these mutations must affect MCM1 activity, perhaps by interfering with dimerization, by analogy to the role of the homologous region of SRF [Norman et al. 1988].

MCM1-dependent gene expression and minichromosome maintenance in mcm1 mutants are uncorrelated

Because MCM1 affects plasmid stability, we analyzed the phenotypes of the viable mutants by replacing the wild-type MCM1 gene on chromosome XIII with each of the mutated genes [as described in Materials and methods]. We used a haploid MATα strain, into which we similarly introduced the mcm1-1 point mutation. The
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Final result is a set of isogenic strains differing only in the MCM1 allele. Growth curves showed that each mutant has a doubling time of ~1.5 hr at 30°C, similar to wild type, and none of the mutants are heat or cold sensitive for growth (data not shown). Therefore, even when the mutant genes are present in single copy, no growth defect is observed.

The ability of each mutant MCM1 protein to activate transcription was monitored using the DSE1α-lacZ reporter gene shown in Figure 3, integrated in single copy on chromosome III. Expression of β-galactosidase is absolutely dependent on MCM1 (R. Elble, in prep.) and is not cell type specific. The level of β-galactosidase in wild-type a or α cells is 40-fold higher from the reporter containing the MCM1-binding site than from an otherwise identical construct lacking it. The mcm1 mutants have varying effects on expression from this promoter (Table 1). Removal of only the polyglutamine domain (mcm1-ΔQ) results in a decrease in β-galactosidase expression to 40% of wild-type levels, whereas removal of the acidic domain (mcm1-ΔDE) results in wild-type expression levels. Removal of both acidic and polyglutamine domains (mcm1-ΔDEQ) results in a further drop in β-galactosidase activity to 18% of wild-type levels. Additional removal of amino acids 2–17 has no effect; activity in mcm1-ΔN17DEQ is 20% wild type. The mcm1-1 mutant has the lowest expression of β-galactosidase at 6% wild-type levels, whereas the SRF substitution for the acidic domain has greater activity than wild-type MCM1 and the GCN4 substitution reduces activity to intermediate levels (22% of wild type). These results suggest that the acidic stretch is not required for transcription activation, because its deletion has no effect, whereas the polyglutamine domain may contribute to activation, because expression is reduced twofold in this mutant. The even lower activity in mutants lacking both domains may be due to instability of these truncated proteins, which will be addressed below.

Because the mutants vary widely in expression level from an MCM1-dependent promoter, we tested for minichromosome maintenance (Mcm), to determine whether the two phenotypes are equally affected in each mutant. We transformed each strain with YCpl01 (ARS1, CENS, LEU2) and calculated the loss rate of the minichromosome per cell per generation of nonselective growth. The loss rates are also shown in Table 1. Although the two smallest mutant proteins, MCM1-ΔDEQ and MCM1-ΔN17DEQ, confer a somewhat higher loss rate than wild-type (cf. 0.07 and 0.06 with 0.02), only mcm1-1 and mcm1-gcn4/DE(Q) have severe Mcm defects (loss rates of 0.30 and 0.21, respectively).

There is no correlation between the gene expression

Figure 1. Schematic of mutant proteins. For MCM1 sequence, see Passmore et al. [1988]. The numbers above the MCM1 sequence refer to the last amino acid in the boxed section. MCM1-ΔQ is altered after Gly118, with the ArgGlyArg non-native sequence added followed by a stop codon. MCM1-ΔDE has amino acids 98-120 replaced with ValAlaThr. MCM1-ΔDEQ introduces ValTyrSTOP after Pro97, whereas MCM1-ΔN17DEQ has an additional deletion of amino acids 2–17. MCM1-1 is a change of Pro97 to Leu. MCM1-SRF/DE introduces Val after Pro97, followed by amino acids 223–251 of SRF (Norman et al. 1988), and then AspSerThr and amino acids 121–286 of MCM1. MCM1-GCN4/DE(Q) inserts ValAspAlaPro after Pro97, followed by amino acids 85–150 of GCN4 (Hope et al. 1988) and amino acids 153–286 of MCM1. MCM1-Xho92 and MCM1-Bam92 are both four amino acid in-frame insertions after amino acid 92 of MCM1, as shown.
do not all merely affect MCM1 protein stability, because the two phenotypes show different severity in the same mutant. We confirmed this prediction by testing whether any of the mutations result in lower levels of the mutant protein. Immunoblot analysis of a wild-type strain in which the mutant proteins are expressed from high-copy 2-micron plasmids is shown in Figure 4. The MCM1-1 protein is present at greater than wild-type levels, and MCM1−ADE, MCM1−SRF/DE, and MCM1−GCN4/DE(Q) are present at slightly lower levels. MCM1−ΔQ appears less abundant, whereas MCM1−ΔDEQ is not detectable under these conditions. These small mutant proteins may be less abundant or may have different transfer properties than the larger mutants. MCM1−ΔN17DEQ was not tested because mcm1−ΔN17DEQ and mcm1−ΔDEQ have identical phenotypes, and our antiserum, which was generated against a fusion protein with the first 92 amino acids of MCM1, may not bind quantitatively to the smaller fragment. The apparently low level of MCM1−ΔDEQ protein is consistent with the previous result that mcm1−ΔDEQ has a more severe phenotype than mcm1−ΔQ, even though deletion of the acidic stretch alone, in mcm1−ΔDE, has no effect.

To distinguish whether the mutations primarily affect binding affinity or the activity of the protein once bound to DNA, we tested the effect of overproducing the mutant proteins on expression from DSE14-lacZ. DSE14 is a high-affinity binding site for MCM1. Overproduction of wild-type protein, by expression from a 2-micron vector, results in at least fivefold more MCM1 protein [Fig. 4] but only 1- to 1.7-fold higher levels of β-galactosidase from a DSE14-lacZ reporter [Passmore et al. 1989], suggesting that this binding site is saturated at single-copy gene dosage of MCM1. If transcription activation is reduced in a mutant because of reduced occupancy of the DSE14-binding site, due to either reduced DNA-binding affinity or lower protein level, overproduction of the mutant protein should result in increased β-galactosidase activity. Conversely, if the mutant protein is less active but binds well, β-galactosidase expression should remain low, regardless of overproduction of the mutant protein.

Three of the mutants tested show increased activity when overproduced on 2-micron plasmid [Table 2]. The largest increase occurs with overproduction of MCM1-1 protein in the mcm1-1 strain. Expression from DSE14 is increased over eightfold, nearing wild-type levels. This result is consistent with an observation by Keleher et al. [1988] that mcm1-1 mutant extracts bind the STE2 operator in DNA band-shift assays approximately fivefold less efficiently than wild-type extracts and suggests that the mcm1-1 mutation affects binding affinity. β-Galactosidase expression in the mcm1−ΔDEQ strain increases fourfold when MCM1−ΔDEQ is overproduced, reaching 58% of wild-type expression levels, consistent with the previous result that this truncation affects MCM1 protein level. Activity also increases threefold in the GCN4-substituted mutant with overproduction of that construct, to even higher levels than wild-type MCM1, suggesting that the insertion of the GCN4 acti-
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Non-cell-type-specific: DSE$_{14}$-lacZ

Hind III STE2-lacZ

TCGATTTCCTAATTAGGAAAAGCT

α-specific: MФα1PQ-lacZ

SalI HindIII HindIII STE2-lacZ

GTCGACGGAACACC'IWCCTAATTAGGACATGAAATrCCCAAGCTT

P Q

Figure 3. Tester genes for measuring MCM1-dependent transcription. The binding sites DSE$_{14}$ and MФα1PQ are described in Passmore et al. (1989), and STE2-lacZ is described in Smith (1986). MCM1-binding sites are shown in bold, and α1 or α2 recognition elements are underlined. All constructs are cloned into the SalI site downstream of LEU2 and integrated in single copy on chromosome III.

In contrast, the acidic stretch is not important for MCM1 function at the DSE$_{14}$ site by two criteria: First, mcm1$\Delta_{α}Q$ has wild-type or very near wild-type activity; second, overproduction of MCM1$\Delta_{α}DEQ$ or MCM1$\Delta_{α}Q$ yields the same activity from DSE$_{14}$ (Table 2), both give approximately half wild-type levels of β-galactosidase. The ability of the MCM1$\Delta_{α}DEQ$ and MCM1$\Delta_{α}N_{17}DEQ$ proteins to activate transcription equally well (Table 1) localizes the remaining transcriptional activation activity to the SRF-homologous domain of MCM1. These results suggest that the 80-amino-acid SRF-homologous domain of MCM1 is capable of transcription activation as well as DNA binding, whereas the polyglutamine domain also contributes to the full activity of the protein.

The effect of MCM1 mutant protein overproduction on gene expression showed that the same mutants that have a minichromosome maintenance defect are the ones that reduce DSE$_{14}$-binding site occupancy. If the minichromosome maintenance defect is also due to reduced DNA binding, then overproduction of the mutant protein should suppress this defect as well. Mutant strains containing YCp101 and either the high-copy vector YEp24 (2μ ARS, URA3) or YEp24 bearing the mutant mcm1 gene were tested for maintenance of the YCp101 plasmid. Cells were grown in synthetic complete (SC) medium lacking uracil to retain the YEp24 derivative while allowing loss of the YCp101 tester plasmid. The loss rates of YCp101 (Table 3) show that in all three Mcm-defective mutants tested, minichromosome maintenance is restored to near wild type when the mutant gene is present on the YEp24 plasmid. These results suggest that in each mutant, the Mcm defect is also due to reduced rather than altered function of the mutant protein.

The acidic domain is important for activation of α-specific genes in a cells

We anticipated that because many of these mutations affect expression from a non-cell-type-specific MCM1-
Table 1. MCM1-dependent transcription activation and McM phenotype of mcml mutants

| Allele              | Expression from DSE\textsubscript{14} (%) wild-type activity | Minichromosome maintenance loss rate of YCP101 |
|---------------------|-------------------------------------------------------------|------------------------------------------------|
| mcml\textsuperscript{+} | 100                                                         | 0.02                                           |
| mcml—AQ            | 40                                                          | 0.02                                           |
| mcml—AD\textsubscript{E} | 100                                                        | 0.02                                           |
| mcml—AD\textsubscript{EQ} | 18 (19)                                                    | 0.07 (0.08)                                   |
| mcml—AN\textsubscript{1},AD\textsubscript{EQ} | 20                                                         | 0.06                                           |
| mcml—1             | 6 (16)                                                      | 0.30 (0.23)                                   |
| mcml—SRF/DE        | 144                                                        | 0.03                                           |
| mcml—gcn4/DE(Q)    | 22                                                         | 0.21                                           |

Expression from the integrated DSE\textsubscript{14}—lacZ tester gene was measured by \(\beta\)-galactosidase activity assay of two separate cultures from each strain. Assays were done with 1 ml of each culture, grown in YPD at 30°C to OD\textsubscript{600} 1.0, as described in Guarante (1983). Activity varied by 10% or less between duplicate cultures. Results shown are from one experiment, normalized to percent wild-type activity. Similar experiments gave the same pattern of activity. Wild-type cells generally had 12 Miller units of \(\beta\)-galactosidase. Activities in parentheses are those of cells grown at 23°C. Minichromosome maintenance assays were done at 30°C, using YPD as the nonselective growth medium. Loss rates were calculated for two independent transformants from each strain; the average is shown here. Variations in loss rate within a strain were <20%. A similar experiment using YCP1 as the tester plasmid gave identical loss rates. Loss rates in parentheses are from cells grown at 23°C.

Independent promoters, they would also affect expression of \(\alpha\)-specific genes, which are activated by MCML1 in conjunction with MAT\(\alpha\)1. The two types of promoters might be affected differently in the different mutants because of involvement of this cofactor in \(\alpha\)-specific gene activation. In vitro-binding studies have shown that \(\alpha\)1 increases the binding affinity of MCML1 to the "PQ" sites of \(\alpha\)-specific genes (Passmore et al. 1989). Therefore, we tested the effect of each mutant on \(\alpha\)-specific expression using the same lacZ reporter gene as before, but with an \(\alpha\)-specific upstream sequence, as shown in Figure 3. This reporter, MF\textsubscript{a}1PQ—lacZ, contains a 60-mer oligonucleotide corresponding to the most proximal MCML1/\(\alpha\)1-binding site of the MF\(\textsubscript{a}1\) promoter (Inokuchi et al. 1989) in place of the DSE\textsubscript{14}. The reporter gene was again integrated into each mutant strain in single copy at the LEU2 locus on chromosome III. Expression from this \(\alpha\)-specific reporter gene was 17-fold higher in wild-type \(\alpha\) cells than in isogenic \(\alpha\) cells, demonstrating that it behaves as expected in the two cell types.

Results of \(\beta\)-galactosidase assays in the ATa mcml mutants [Fig. 5A] show that the mutants affect \(\alpha\)-specific expression differently than they do nonspecific expression. Expression from the two promoters is compared in Figure 5B. All mutants lacking the acidic stretch show greatly reduced activity from the \(\alpha\)-specific promoter compared to DSE\textsubscript{14}. In particular, mcml—AD\textsubscript{E}, which lacks only the acidic stretch, expresses the \(\alpha\)-specific construct poorly (13% wild type) while being a good activator of the same reporter with the DSE\textsubscript{14}-binding site instead (100% wild type). The mutants containing the acidic stretch, mcml—AD\textsubscript{Q} and mcml—1, activate transcription from the \(\alpha\)-specific promoter to the same level relative to wild type as they do the nonspecific promoter with DSE\textsubscript{14} (~50% for mcml—AD\textsubscript{Q} and 5% for mcml—1). These results suggest that in contrast to expression from the nonspecific promoter, the acidic stretch of MCML1 is important for expression from an \(\alpha\)-specific promoter.

MCM1 DNA-binding domain is sufficient for repression of \(\alpha\)-specific genes in \(\alpha\) cells

MCM1 has been implicated not only in activation of \(\alpha\)-specific genes but in repression of \(\alpha\)-specific genes in \(\alpha\) cells. The cooperative binding of MCML1 and MAT\(\alpha\)2 at the operators of \(\alpha\)-specific genes is believed to be responsible for their repression in \(\alpha\) cells. We tested whether these mcml mutants affect repression. We again studied expression from an integrated STE2—lacZ reporter gene, now containing 700 nucleotides upstream of STE2 [Fig. 3]. This region contains the MCML1/\(\alpha\)2 operator as well as UAS\textsubscript{s} and is sufficient for \(\alpha\)-specific expression (Smith 1986). Expression from this promoter is 80-fold higher in \(\alpha\) cells than in isogenic \(\alpha\) cells [Fig. 6].

Results of \(\beta\)-galactosidase assays in the ATa mcml mutant strains [Fig. 6] show that most of the mutants are able to repress the \(\alpha\)-specific gene. Only mcml—gcn4/DE(Q) shows substantial derepression, with 28% of wild-type ATa activity, whereas mcml—1 has a slight effect, with approximately threefold higher \(\beta\)-galactosidase levels than wild-type \(\alpha\) cells, consistent with the reduced DNA-binding affinity of the MCML1 protein. The derepression observed in the mcml—gcn4/DE(Q) mutant is most likely due to structural alterations that affect the ability of the hybrid protein to bind to DNA with the \(\alpha\)2 protein, because no portion of MCML1 is missing from this mutant that is important for DNA binding or corepression with \(\alpha\)2 (cf. with mcml—AD\textsubscript{EQ}).

The mcml—AD\textsubscript{E}, mcml—AD\textsubscript{Q}, and mcml—AD\textsubscript{EQ} mutants show no derepression of \(\alpha\)-specific genes, suggesting that neither the acidic nor the polyglutamine domain is required for their repression. However, if an mcml mutant were unable to activate transcription of STE2—lacZ in this genetic background, no conclusion could be reached concerning the ability of the mutant protein to repress transcription of \(\alpha\)-specific genes. Therefore, we tested expression of STE2—lacZ in isogenic ATa cells derived from two of the mcml mutants. We found no difference in \(\beta\)-galactosidase expression from this promoter in mcml—AD\textsubscript{E} or mcml—AD\textsubscript{EQ} \(\alpha\) strains compared to wild-type \(\alpha\) strains: Each strain expresses ~100 Miller units of \(\beta\)-galactosidase [Fig. 6]. The low level of \(\beta\)-galactosidase in the \(\alpha\) strains is therefore due to corepression with \(\alpha\)2, which must only require the amino-terminal portion of MCML1.

The dramatic effects of these mcml mutants on expression of mating-type-specific genes in \(\alpha\) cells should
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result in mating defective phenotypes. We assayed the ability of each of the MATα mcml mutant strains to mate with wild-type tester strains, as shown in Figure 7. The mating defects correspond well with the observed defects in mating-type-specific gene expression. Only the mcml-ΔQ and mcml-SRF/DE mutants are able to mate at all temperatures, consistent with the finding that only these mutants have at least 25% wild-type expression from the α-specific reporter gene. Only the mcml-gcn4/DE(Q) mutant mates with other α cells rather than with a cells, an a-like faker phenotype similar to that of mata1 mata2 double mutants [Strathem et al. 1981], which is consistent with the high level of α-specific gene expression and low level of α-specific gene expression in this mutant. Mating is improved and expression from MFα1PQ-lacZ increases 3- to 10-fold with overproduction of wild-type or mutant proteins in their respective strains. However, mcml-gcn4/DE(Q) becomes a weak bi-mater, rather than mating only with a cells, and expression from STE2-lacZ suggests that corepression with α2 is not restored with overproduction of the MCM1-GCN4/DE(Q) protein [data not shown].

The acidic domain reduces MCM1 activity at an α-specific UAS in α cells

The current model for α-specific gene regulation is that only positive regulation occurs, by cooperative binding of MCM1 and MATα1 in α cells and that binding of MCM1 alone to the upstream elements of these genes is too weak to allow their expression in α cells. However, MCM1 can bind to PQ elements of MFα1 and STE3 in vitro with high affinity in the absence of α1 [Tan et al. 1988; Passmore et al. 1989; Ammerer 1990]. Therefore, we wondered whether α-specific gene expression is regulated in a cells, as well as in α cells, in this case to decrease MCM1 binding or activity at PQ elements. If so, some of the MCM1 mutations may affect this regulation. We tested this hypothesis by assaying expression of the α-specific reporter gene, MFα1PQ-lacZ, in a wild-type strain, as shown in Table 2.

Table 2. Effect of MCM1 mutant protein overproduction on expression from DSE14-lacZ

| Allele          | Expression from DSE14 (% wild-type activity) | Fold difference |
|-----------------|--------------------------------------------|-----------------|
| MCM1 + YEp24   | 100                                        | 1.0             |
| + YEp24 MCM1   | 98                                         | 1.0             |
| mcml-ΔQ + YEp24| 46                                         | 1.3             |
| + YEp24 mcml-ΔQ| 59                                         | 1.3             |
| mcml-ΔDE + YEp24| 71                                        | 1.1             |
| + YEp24 mcml-ΔDE| 80                                        | 1.1             |
| mcml-ADEQ + YEp24| 15                                       | 3.9             |
| + YEp24 mcml-ADEQ| 58                                       | 3.9             |
| mcml-1-1 + YEp24| 10                                        | 8.4             |
| + YEp24 mcml-1-1| 84                                        | 8.4             |
| mcml-SRF/DE + YEp24| 82                                        | 1.2             |
| + YEp24 mcml-SRF/DE| 102                                       | 1.2             |
| mcml-gcn4/DE(Q) + YEp24| 60                                       | 3.2             |
| + YEp24 mcml-gcn4/DE(Q)| 193                                      | 3.2             |

Duplicate cultures of each strain were grown at 30°C in SC-uracil media to OD₆₀₀ 1.5, and β-galactosidase activity was assayed by using 1 ml of each culture as described [Guarante 1983]. Activity varied by 20% or less between the duplicates. The experiment was repeated yielding similar results to those shown.

Figure 4. Effect of the mcml mutations on the level of the resulting protein. Immunoblots using anti-β-gal-MCM1, are shown. Transformants of strain BJ2168 were grown in SC-uracil media to OD₆₀₀ 1.5, and crude extracts were loaded onto a 10% gel [A] or a 15% gel [B]. [A] Approximately 3 μg of total protein was loaded in each lane. A 2-min exposure is shown. The wild-type MCM1 protein from the chromosomal MCM1 gene runs as the ~40-kD band of approximately equal intensity in each lane. [B] Approximately 1.5 μg of total protein was loaded in each lane. A 5-min exposure is shown. Lanes 1 and 2 were loaded with the same extracts as in A. With overexposure, bands of the expected size of MCM1 (~40 kD) and MCM1-ADEQ (~11 kD) appear in the YEp24-mcml-ADEQ lane, with the smaller band having lower intensity than the band from the chromosomal MCM1 gene.
Table 3. Effect of MCM1 mutant protein overproduction on minichromosome stability

| Allele               | Loss rate of YCp101 |
|----------------------|---------------------|
| mcm1-ΔDEQ            |                     |
| + Yep24              | 0.25                |
| + Yep24 mcm1-ΔDEQ    | 0.04                |
| mcm1-1               |                     |
| + Yep24              | 0.38                |
| + Yep24 mcm1-1       | 0.03                |
| mcm1-gcn4/DE(Q)      |                     |
| + Yep24              | 0.16                |
| + Yep24 mcm1-gcn4/DE(Q) | 0.05            |

Minichromosome maintenance assays were done at 30°C in SC + leucine media, lacking uracil. Dilutions from initial and final cultures were plated on YPD and replicated to SC-uracil and SC/uracil/leucine. Loss rate of YCp101 was calculated by using the percentage of cells containing Yep24 (+ mcm1) that also contain YCp101. Loss rates shown are averages from testing two independent transformants, whose loss rates varied by <20%.

Discussion

Functional domains of MCM1

DNA-binding domain This study revealed that the three domains of MCM1 identified by similarity to other proteins do provide different functions, as summarized in Figure 9. However, the 80-amino-acid region homologous to SRF was found to be sufficient for most MCM1 functions, including cell viability, minichromosome maintenance, transcription activation from a non-cell-type-specific MCM1-dependent promoter, and corepression with α2 of α-specific genes. MCM1 is therefore unlike most previously studied eukaryotic transcription factors, which have separate DNA-binding and activation domains (for review, see Mitchell and Tjian 1989),
suggesting that transcriptional activation by MCM1 may occur by a different mechanism from that of most other transcription factors. Further experiments will be required to determine whether transcriptional activation requires an activity of MCM1 beyond DNA binding, such as making specific contacts with other proteins or possibly affecting the DNA or chromatin structure. The MCM1/α2/DNA complex may be similar to the ternary complex of SRF, p62TCF, and DNA, which was suggested to require only the DNA-binding domain of SRF (Schröter et al. 1990). It will be interesting to determine whether the cooperativity of DNA binding by MCM1 and α2 involves specific protein–protein interactions or whether it is mediated by interaction of each protein with the DNA.

In light of our finding that the DNA-binding domain of MCM1 is sufficient for its function, it is interesting that ARG80 [ARG RI], which has 70% identity to MCM1 in these 80 amino acids [Passmore et al. 1988], cannot substitute for MCM1. Even overproduction of ARG RI cannot rescue the lethality of an mcm1 deletion (R. Elble, unpubl.). ARG RI has not been demonstrated to bind DNA and has no apparent transcription activation ability [Qiu et al. 1990], suggesting that ARG RI and MCM1 may differ in amino acids that are important for DNA-binding affinity or transcriptional activation. Interestingly, the mcm1-1 point mutant at Pro97 has the most severe phenotype of all the viable mutants we examined, suggesting that Pro97 is important for MCM1 function. SRF also has a proline at the homologous position, whereas ARG RI does not.

Acidic domain The acidic domain of MCM1 is important for regulation of α-specific genes. All mutants lacking the acidic domain show low expression of an α-specific reporter gene in a cells, even if they have no defect in expressing a non-cell-type-specific gene. When a mutant protein lacking the acidic domain is overproduced in a cells, the α-specific reporter gene is expressed at an abnormally high level compared to the level with overproduction of MCM1 proteins containing an acidic domain. These results suggest that the acidic domain is important for both positive and negative regulation of MCM1 activity at α-specific promoters. Neither substitution of a portion of SRF or of an acidic activation domain of GCN4 could replace the function of the MCM1 acidic stretch in either cell type.

In α cells, the acidic stretch may be necessary to form a ternary complex of MCM1, α1, and the PQ DNA. Tan and Richmond (1990) showed that only the smallest pro-
normally not bound with protein in a cell, and that de-oL cells, it seems likely that regulation of MCM1-dependent transcriptional activation of α-specific genes in vitro-binding studies with the wild-type and mutant proteins. Two transformants of each plasmid into the MATα MCM1 strain with the integrated MFe1PQ–lacZ reporter gene were grown at 30°C in SC-uracil media to OD600 1.5. β-Galactosidase activity was assayed by using 1 ml of each culture as described (Guarante 1983) and is expressed as a percentage of the activity of an isogenic MATα strain containing YEpr24, tested simultaneously. Activity varied by 20% or less between the two transformants.

Although negative regulation at the α-specific UAS in a cells has not been reported previously, results of an earlier study support this hypothesis. Jarvis et al. (1989) found that activity from a reporter gene with the STE3 PQ element in a cells was half that from the same promoter with only the P element. When they overproduced MCM1, activity from the P element increased to 560 units, whereas activity from the PQ element increased only to 150 units. This result suggests that the Q element negatively modulates the ability of MCM1 to either bind or activate transcription at the neighboring P element in a cells. With these data and our observation that removal of the acidic stretch increases MCM1 activity at PQ in a cells, and by analogy with regulation in α cells, it seems likely that regulation of MCM1-dependent transcriptional activation of α-specific genes in a cells may also be mediated by protein(s) binding at the Q element. An alternative possibility is that the PQ site is normally not bound with protein in a cells and that deletion of the acidic stretch could increase MCM1-binding affinity to PQ in the absence of cofactors, resulting in a level of α-specific gene expression intermediate to that of wild-type a cells and the α1-induced level of α cells. In vitro-binding studies with the wild-type and MCM1–ΔE proteins should distinguish these possibilities.

Polyglutamine domain The polyglutamine domain was found to be dispensable for all MCM1 functions. However, mutants lacking the polyglutamine domain had at most 60% of wild-type MCM1 transcriptional activation activity, on both a nonspecific and α-specific promoter. The reduced activity at the nonspecific promoter was shown not to result from an effect on DNA-binding site occupancy. The polyglutamine domain therefore contributes to the transcription activity of MCM1. In addition, the polyglutamine domain may contribute to the stability of the MCM1 protein, because MCM1–ΔQ and MCM1–ΔEQ appear less abundant by Western blots. Unlike the polyglutamine domains of Sp1, which were shown by Courey and Tjian (1988) to be required for its transcription activation ability in Drosophila cells, the MCM1 polyglutamine domain contributes only weakly to its total activity. Polyglutamine domains are common among yeast proteins but have not yet been demonstrated to be important for any function. It is not clear, therefore, whether the MCM1 polyglutamine domain could be acting similarly to that of Sp1 in transcriptional activation.

Role of transcription factors in yeast DNA replication initiation

In yeast, both MCM1 and another transcription factor, ABFI, have been implicated in replication initiation. ABFI was identified as a factor that binds DNA within several ARSs (Buchman et al. 1988; Diffley and Stillman 1988), and ABFI-binding sites were shown to increase the efficiency of ARS function in a minichromosome maintenance assay (Walker et al. 1990). The identification of these transcription factors as potential DNA replication initiation factors in yeast suggests that chromosomal replication initiation may be similar to that of the eukaryotic viruses. Viral replication initiation is enhanced by the direct binding of mammalian transcription factors to the replication origin. This enhancement was shown recently to require only the DNA-binding domain of the transcription factor (Mermod et al. 1989; Verrijzer et al. 1990).

Although both transcription and replication activity of MCM1 are contained in an 80-amino-acid domain, the phenotypes of the mcm1 mutants described in this study

![Figure 8](image-url) Expression of an α-specific gene in an α strain overproducing MCM1 mutant proteins. Two transformants of each plasmid into the MATα MCM1 strain with the integrated MFe1PQ–lacZ reporter gene were grown at 30°C in SC-uracil media to OD600 1.5. β-Galactosidase activity was assayed by using 1 ml of each culture as described (Guarante 1983) and is expressed as a percentage of the activity of an isogenic MATα strain containing YEpr24, tested simultaneously. Activity varied by 20% or less between the two transformants.

![Figure 9](image-url) Functional domains of MCM1. A summary of the activities attributed to the different parts of the protein is shown. The boxed regions correspond to those in Fig. 1.
suggest that the role of MCM1 in replication initiation is probably not indirectly mediated through affecting transcription of another gene. Comparison of the phenotypes of those mutants with a minichromosome maintenance (Mcm) defect, mcml-1, mcml-gcn4/DE(Q), and mcml-∆DEQ, with those that are wild type for MCM (mcml-1-∆DE, mcml-1-ΔQ, and mcml-1-SRF/DE) shows that the only phenotype yet found that differentiates these two groups is DSE14 DNA-binding site occupancy but not with gene expression and DNA replication by binding to replication origins. Clearly, however, they do not rule out the possibilities that MCM1 acts indirectly to affect replication, perhaps in combination with cofactors that interact with it in ways unlike α1 or α2, or by affecting expression of a number of genes, or even that the total effect of mcml1 mutations on minichromosome maintenance may result from both direct and indirect activities. Further study of mcml1 mutants, combined with mutational analysis of the ARS elements will be important in resolving this issue.

Materials and methods

Strains

Escherichia coli strains used were DH5α [BRL] for routine cloning, GM2163 [hisD2 mcrB1 dam13::Tn9 dcm6] from New England Biolabs, for preparation of DNA that can be cut at the StuI site in MCM1, and C236 [mut ung thi relA] for oligonucleotide-directed mutagenesis. Yeast strains 8534-8C [MCM1 ARS1 CEN5 URA3] and CJ236 [dut ung thi relA] were used for mating tests; BJ2168 [MCM1 met4] and CJ236 [dut ung thi relA] were used for transient transformation with plasmids. Strains used were DHSe* (BRL) for routine cloning of the resultant diploid, were used for constructing and testing plasmids. Yeast strains made by transient transformation with pCJ105 [MATa leu2-3,112, ura3-521 and an isogenic MCM1 derivative YCp88-lexA-gcn4-D19 (Hope et al. 1988; from K. Struhl). The resulting construct was put back into frame by cutting with SaII, filling in, and religating. Each construct was verified to have the expected junction sequence by restriction mapping and double-stranded dye sequencing, using a primer from +185 to +200 of the gcn4 gene from pG3.5 (Hill et al. 1986) constructs containing a mutant gene were made by using the Sphi–KpnI wild-type fragment with the mutagenized fragment. Correct constructs were confirmed by restriction mapping, and for mcml1 and mcml1-ΔN27, DEQ by sequencing. YEp351 (Hill et al. 1986) constructs containing a mutant gene were made by using the Sphi–BamHI fragment from the Yip5 constructs. Construction of mcml1 mutants and tester plasmids

The mcml1-ΔQ and mcml1-1 alleles were derived from previously cloned mutations on Xhol–EcoRI fragments containing part of MCM1 in Yip5 (Passmore et al. 1989). mcml1–ΔQ was derived from Yip5 B453 [Maine 1984], which had a BamHI linker that mapped near the acidic stretch of MCM1. We sequenced through the linker insertion site by double-strand dye sequencing using primers within the MCM1 gene at +185 to +200 and +468 to +450 from the ATG. The deduced amino acid sequence, altered following Gly116 is shown in Figure 1.

The mcml1–DE, mcml1–DEQ, mcml1–SRF/DE, mcml1-gcn4/DE(Q), and mcml1–AN27, DEQ mutations are derived from the same oligonucleotide mutagenesis experiment. The Sphi–EcoRI fragment with part of MCM1 (Passmore et al. 1988) was cloned into KS− (Stratagene), which had the SaII site destroyed. Oligonucleotide mutagenesis was performed by using the Bio-Rad reagents and the mutagenic oligonucleotide 5′-GTCCTAAGCGCCCCCTTGCACTATCGAAACGC-3′, which replaces 60 nucleotides coding for the acid stretch with a SacI–HindIII site. mcml1–DE was derived by cutting with AccI, filling in the ends, and religating. mcml1–DEQ was derived by cutting with HindIII and cloning in an XbaI linker with stop codons in all three open reading frames, 5′-TACTAGTCGAGACTAGT-3′. mcml1–SRF/DE was derived by cutting with AccI, filling in the ends, and ligating in an 80-nucleotide HindIII fragment of the SRF gene from pG3.5 (Hill et al. 1988; from R. Treisman). mcml1–gcn4/DE(Q) was derived by cutting with SaII and KpnI and inserting a 195-nucleotide SaII–KpnI fragment from the Gcn4 derivative YCP88-lexA-gcn4-D19 (Hope et al. 1988; from K. Struhl). The resulting construct was put back into frame by cutting with SaII, filling in, and religating. Each construct was verified to have the expected junction sequence by restriction mapping and double-stranded dye sequencing, using a primer from +185 to +200 of MCM1. mcml1–AN27, DEQ was derived from mcml1–DEQ by oligonucleotide-mediated mutagenesis, using 5′-CACCCAGCAAAAATGAGAAGAAAAGTACGA-3′, which loops out amino acids 2–17. Transformants were screened by sequencing with a primer from −47 to −37 of the MCM1 gene.

All of the mutated fragments were subcloned into the yeast shuttle vectors Yip5 and YEp24 containing the entire MCM1 gene on a 3.4-kb Xhol–BamHI fragment, by replacing the 1.2-kb Sphi–Kpnl wild-type fragment with the mutagenized fragment. Correct constructs were confirmed by restriction mapping, and for mcml1-1 and mcml1–AN27, DEQ by sequencing. YEp351 (Hill et al. 1986) constructs containing a mutant gene were made by using the Sphi–BamHI fragment from the Yip5 constructs. mcml1–Xho92 and mcml1–Bam92 were made by cutting a YEp351 plasmid containing the wild-type MCM1 gene with StuI and ligating in either an Xhol or BamHI 12-nucleotide linker. DNA from resulting transformants was sequenced by using the +185 to +200 primer and determined to have only one linker inserted in each case.

Tester plasmids were derived from pcDH or pcDH–DSE14 (Passmore et al. 1989). DSE14-lacZ was made by cutting pcDH–DSE14 with BamHI and BglII and religating to remove the centromere so that the plasmid could be integrated into a yeast chromosome. The control plasmid with no MCM1-binding site was made by cutting pcDH with SaII and HindIII, filling in, and religating. MFlo1PO–lacZ was made by cloning MFlo1p60 (Passmore et al. 1989) into pcDH at the SaII and HindIII sites. STE2–lacZ was made by replacing the SaII–SacI fragment of pcDH with the corresponding fragment of pcD14 (Smith 1986). Centromeres were removed from all plasmids to allow integration, as described for DSE14–lacZ.

Yeast methods

Plasmid shuffle assays were done as described [Boeke et al. 1987]. In each case, strain C2/501 was transformed with a positive control plasmid [the vector containing MCM1], a negative control plasmid [the vector alone], and the vector containing the mutated mcml1 gene. Ability of the mutant to provide MCM1...
function was analyzed by growth on 5-fluoro-orotic acid (5-FOA), which selects for cells that have lost YCp501. The experimental sample and positive and negative controls were streaked on the same 5-FOA plate to ensure that growth reflected the ability of the mutant gene to complement the mcm1 deletion, rather than variation in 5-FOA concentration.

The MCM1 gene was replaced with each of the mutant alleles, using a two-step method. YIp5 containing the mutated mcm1 gene was digested with Sphi to target plasmid integration to the MCM1 locus. Transformants of 8534-8C were selected on SC–uracil media, and recombinants retaining only one copy of the MCM1 gene were selected on 5-FOA [Boeke et al. 1984]. These isolates were tested for retention of the wild-type or mutant allele by genomic Southern blots, except for mcm1-1. Yeast genomic DNA was digested with appropriate restriction enzymes to differentiate mutant from wild type [mcm1–ΔQ has a BamHI site, mcm1–ΔDE has an NruI site, mcm1–ΔDEQ and mcm1–AN–ΔEQ have an XbaI site, mcm1–SRF/DE has a Sall site, and mcm1–gcn4/DE(Q) has a PvuI site at the mutation]. DNA was separated on agarose gels, blotted to nylon membranes, and probed with random-prime-labeled fragments of lacZ. Transformants with only the expected 13-kb band, lacking a plasmid-sized fragment, were chosen for β-galactosidase assays.

β-Galactosidase activity was measured according to Gurant (1983). Units were calculated as [(1000 × OD_{600})/time (min) × vol (ml) × OD_{600}] [Miller 1972]. Cultures were grown to the same OD_{600} [ranging from 1–1.5 in different sets of assays], and activity was normalized to the activity of the wild-type strain, measured simultaneously. Cultures were grown in either YPD or SC media, as stated in the table footnotes. Minichromosome maintenance assays were done as follows: A colony grown on selective media was suspended in 0.2 ml of water. A 0.1-ml aliquot was used to inoculate 5 ml of nonselective media [either YPD or SC–uracil], and cultures were grown with aeration until saturated (~10 generations). Dilutions of the initial suspension were plated on YPD plates, and colonies were counted to determine the initial concentration of viable cells; these plates were then replica-plated to SC–leucine to determine the initial percentage of plasmid-bearing cells. Final samples were treated similarly. The number of generations of nonselective growth [n] was calculated as logfinal concentration/initial concentration/log2. The loss rate per cell per generation was calculated as 1 – (final % plasmid-bearing cells/initial % plasmid-bearing cells)^n. Each assay set was done simultaneously to eliminate variations due to temperature or media. Loss rates were found to be twofold higher in synthetic compared to YPD media for all strains except for mcm1-1 and mcm1–gcn4/DE(Q).

Antiserum preparation and immunological techniques

Antiserum was generated against a β-gal–MCM1 fusion protein containing only the amino-terminal 92 amino acids of MCM1. A 0.3-kb BamHI–Stul fragment from pETMCM1(1–188) [Passmore et al. 1989] was inserted into pUR278 [Röther and Müller-Hill 1983]. E. coli strain DH5α containing this plasmid was induced with IPTG to express the β-gal–MCM1 fusion protein. Cells were lysed by boiling in SDS sample buffer [Laemmli 1970], and total protein was separated on preparative 6% acrylamide SDS-PAGE. The β-gal–MCM1 fusion protein was cut out, electroeluted, and used to immunize rabbits. The rabbits were boosted twice, and antiserum was collected 2 weeks after the final boost.

Immunoblots were performed by standard methods. Yeast extracts were made by pelleting log phase yeast [OD_{600} 1.5] and resuspending in SDS sample buffer with glass beads, followed by repeated boiling and vortexing. Protein concentration in the extract was estimated by using the Bio-Rad assay on diluted extracts, with protein standards to which we added similar amounts of sample buffer. Proteins were separated by SDS-PAGE on 10% or 15% acrylamide gels and transferred to nitrocellulose by using a semidyf blotter [Hofer] and the three-buffer system [Kyhse-Anderson 1984], adding SDS to 0.1% to all buffers. Nonfat dry milk (5%) was used to block the membranes, and primary antibody was diluted 1 : 1000 and detected by using horseradish peroxidase-conjugated goat anti-rabbit IgG (BRL, 1 : 6000). Visualization was by enhanced chemiluminescence [Amersham].

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