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Multiple Phosphorylated Forms of the *Saccharomyces cerevisiae* Mcm1 Protein Include an Isoform Induced in Response to High Salt Concentrations

MIN-HAO KUO,[1,2] ELIZABETH T. NADEAU,[1] AND ELIZABETH J. GRAYHACK[1]*

Department of Biochemistry and Biophysics, School of Medicine, University of Rochester, Rochester, New York 14642,[1] *and Department of Biology, River Campus, University of Rochester, Rochester, New York 14627*[2]

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The *Saccharomyces cerevisiae* Mcm1 protein is an essential multifunctional transcription factor which is highly homologous to human serum response factor. Mcm1 protein acts on a large number of distinctly regulated genes: haploid cell-type-specific genes, G_2-cell-cycle-regulated genes, pheromone-induced genes, arginine metabolic genes, and genes important for cell wall and cell membrane function. We show here that Mcm1 protein is phosphorylated in vivo. Several (more than eight) isoforms of Mcm1 protein, resolved by isoelectric focusing, are present in vivo; two major phosphorylation sites lie in the N-terminal 17 amino acids immediately adjacent to the conserved MADS box DNA-binding domain. The implications of multiple species of Mcm1, particularly the notion that a unique Mcm1 isoform could be required for regulation of a specific set of Mcm1's target genes, are discussed. We also show here that Mcm1 plays an important role in the response to stress caused by NaCl. G. Yu, R. J. Deschenes, and J. S. Fassler (J. Biol. Chem. 270:8739–8743, 1995) showed that Mcm1 function is affected by mutations in the *SLN1* gene, a signal transduction component implicated in the response to osmotic stress. We find that *mcm1* mutations can confer either reduced or enhanced survival on high-salt medium; deletion of the N terminus or mutation in the primary phosphorylation site results in impaired growth on high-salt medium. Furthermore, Mcm1 protein is a target of a signal transduction system responsive to osmotic stress: a new isoform of Mcm1 is induced by NaCl or KCl; this result establishes that Mcm1 itself is regulated.

The *Saccharomyces cerevisiae MCM1* gene encodes an essential DNA-binding protein that is apparently multifunctional. Mutations in *MCM1* affect autonomously replicating sequence-specific minichromosome maintenance, recombination, chromosome segregation, and haploid cell-type-specific gene expression (17, 18, 33, 35, 46, 67). In addition, potential target genes of Mcm1 protein encode proteins involved in cell cycle regulation (G_1 and G_2), cell wall and membrane integrity, cellular metabolism, and stress tolerances (41, 43, 52). Based on the diversity of this group of genes, we suggested that Mcm1 protein acts as a global regulator that could play a role in cell cycle control (41). The essential involvement of Mcm1 protein in G_2 control of the cell cycle was demonstrated recently: depletion of Mcm1, which is required for expression of G_2-cell-cycle-regulated genes *SWI5* (43) and *CLB2* (45), results in cell cycle arrest at G_2 and defects in transcription of many G_2-cell-cycle-specific genes (2).

The primary biochemical functions of Mcm1 protein are its sequence-specific DNA-binding activity and interactions with gene-specific cofactors that also recognize specific DNA sequences. An 80-amino-acid DNA-binding domain of Mcm1, which is 75% identical to the human serum response factor (SRF), is essential as well as minimally sufficient for known Mcm1 functions; this domain is located in the N-terminal portion of Mcm1 protein (residues 18 to 97) (9, 13, 71). In this essential domain the N-terminal 56 amino acids, called the MADS box, are highly conserved in a family of proteins, most of which are transcription factors involved in cellular development and differentiation (see Fig. 1) (reviewed in reference 79). These amino acids are involved in sequence-specific DNA binding and dimerization (10, 61, 69, 77, 90). The C-terminal 24 amino acids of the domain are involved in protein-protein interactions, both within the dimer (in addition to MADS box interactions) and with other proteins, such as gene-specific cofactors and possibly the transcription apparatus (9, 10, 57, 69). Mcm1 protein also contains a 17-amino-acid N-terminal extension, as well as an acidic stretch and a glutamine-rich domain in the carboxyl two-thirds of the protein. Although these domains are dispensable for viability (9, 13, 71), at least one of them, the acidic domain, has been implicated in the modulation of a specific Mcm1 function, α-cell-type-specific gene expression (13).

Mcm1 protein recruits specific cofactors to different types of genes at which it acts. The type of regulation imposed depends on the cofactor: differences in both the agents that modulate a response and the effect of the regulators on basal transcription are observed. With the α-cell-type-specific α1 protein and STE12 protein, Mcm1 activates transcription of α-cell-type-specific genes (3, 4, 33, 66, 67, 82). With α2 protein, Mcm1 represses expression of **a**-cell-type-specific genes (17, 35). The interaction of α2 protein with global repressor proteins results in the positioning of phased nucleosomes on the promoter (11, 14, 28, 36, 39, 59, 78, 88). With Ste12 protein, Mcm1 protein activates pheromone-responsive genes (10, 19, 32, 38, 57). Ste12 protein contains a transcriptional activation domain and is phosphorylated after pheromone treatment (38, 81). With Sff, Mcm1 protein is required for expression of the cell cycle-regulated gene *SWI5*. The combination of Mcm1 and Sff repositions the initiation site of transcription of *SWI5* (43) and also likely regulates expression of several other genes expressed in the G_2 phase of the cell cycle (2, 45).

* Corresponding author. Mailing address: Department of Biochemistry and Biophysics, Box 712, School of Medicine and Dentistry, University of Rochester, Rochester, NY 14642. Phone: (716) 275-2765. Fax: (716) 271-2683. E-mail: grayhack@medinfo.rochester.edu.

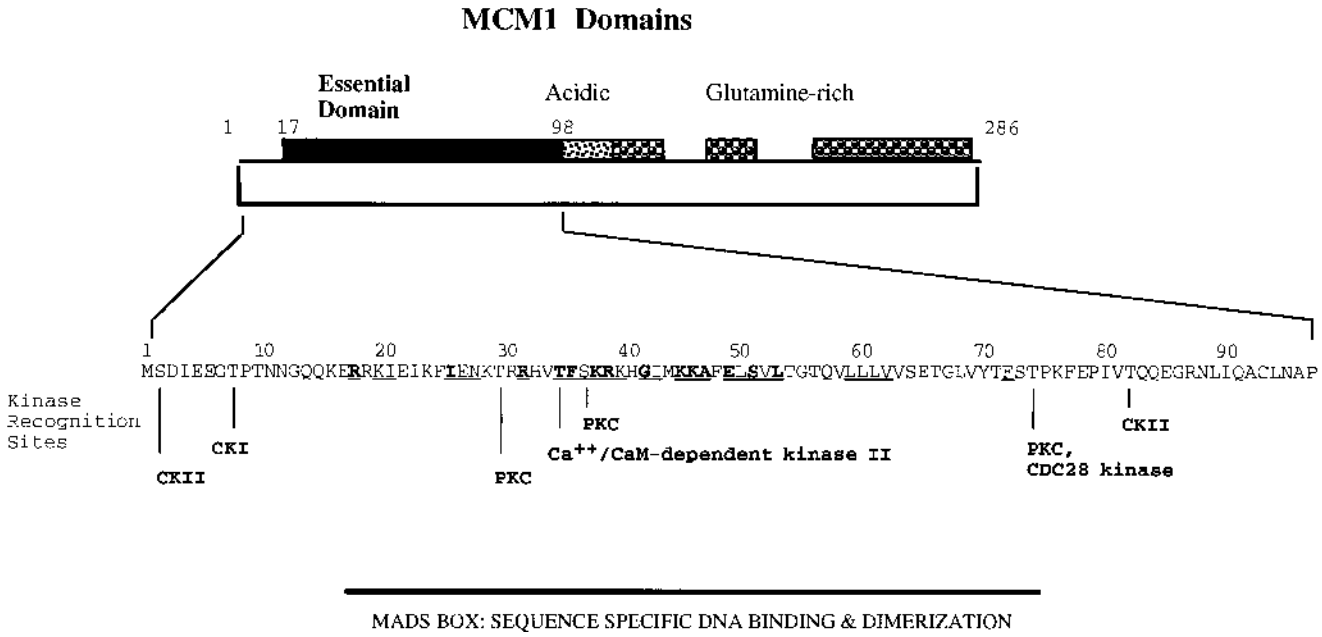


FIG. 1. Diagram of Mcm1 protein structure. The domains of the full-length Mcm1 protein are indicated. The amino acid sequence and potential protein phosphorylation sites of the first 98 amino acids of the Mcm1 protein, including the 80-amino-acid essential domain, are shown. Invariant (bold and underlined) and highly conserved (underlined) residues of the MADS box are indicated (79). CKI, casein kinase I; CKII, casein kinase II; PKC, protein kinase C. The 56 amino acids designated as the MADS box are indicated.

Mcm1 protein might act at diverse sets of genes solely by recruiting appropriate cofactors to cognate DNA elements, an explanation that suffices for regulation of the cell-type-specific genes and pheromone-responsive genes. Alternatively, the activity of Mcm1 itself might be altered by posttranslational modification in such a way that its transcriptional regulatory activity with specific cofactors is modulated. Cell-cycle-specific expression of *CLB2* and *SWI5* is likely to require either posttranslational modification of one of the two regulators (Mcm1 and Sff) or an additional cell cycle-regulated factor since, in vivo, both Mcm1 and the cofactor, which has been suggested to be identical to Sff, appear to occupy the *CLB2* promoter throughout the cell cycle (2).

There is circumstantial evidence that Mcm1 activity itself may be regulated. First, transcription activation by Mcm1 protein is affected by *sln1* (93) and *spt13*/*gal11* (92) mutations, although neither mutation influences the level of Mcm1 protein. Sln1 protein is a yeast homolog of the prokaryotic two-component signal transduction systems (56, 65) and has been implicated in the HOG1 osmosensing response modulated by microtubule-associated protein (MAP) kinases (44). Spt13/Gal11 protein is a component of the general transcription apparatus which affects function of a number of transcription activators (37). Second, the minichromosome maintenance function of Mcm1 protein can be modulated posttranscriptionally in response to the flux of glycolysis (12).

We considered that Mcm1 protein might be phosphorylated for two additional reasons. First, protein kinase consensus recognition sites for protein kinase C, casein kinase I, casein kinase II, Ca^{2+}/calmodulin (CaM)-dependent protein kinase II, and $p34^{CDC28}$ kinase are found in the Mcm1 protein sequence, in the first 98 amino acids (68) (Fig. 1); some of these putative recognition sites (T30, T35, and S37) are conserved among MADS box proteins. Second, terminal phenotypes of *mcm1* mutants are similar to those resulting from various protein kinase pathway defects. For example, cells depleted of Mcm1 protein have enlarged mother cells with multiple, elongated buds (2), a morphology similar to that of casein kinase I, casein kinase II, and protein phosphatase 2A mutants (27, 63, 73, 74, 86). In addition, an *mcm1* allele causes hyperrecombination and other DNA metabolism defects (18), a phenotype observed in protein kinase C mutants (31).

We show here that Mcm1 protein is phosphorylated in vivo. Two major phosphorylation sites map to the 17 amino acids immediately N-terminal to the MADS box. Mcm1 is also likely to be phosphorylated less efficiently at several additional positions outside the amino-terminal domain. Several (more than eight) isoforms of Mcm1 are resolved by acidic isoelectric focusing (IEF): the heterogeneity of the isoforms is not eliminated either by deletion of the N-terminal domain or by mutation of both phosphorylation sites in the N terminus; by contrast, only two major isoforms of Mcm1 are seen after phosphatase treatment. Thus, a protein which has been considered an entity may be several different molecules. We suggest that individual isoforms of Mcm1 could participate in different regulatory loops and thus act as independently regulated molecules.

Since the Sln1 protein, which affects Mcm1 function (93), is implicated in the response to osmotic stress mediated by the HOG pathway (44, 56), we explored the effect of mutations in *mcm1* on growth on NaCl, KCl, and LiCl. Cells bearing an *mcm1* allele with the N-terminal domain deleted or with a mutation at the primary phosphorylation site are sensitive to salt stress; they survive poorly on 1.4 M NaCl compared to their isogenic parents with wild-type *MCM1*. Surprisingly, a yeast cell with an *mcm1* allele with a mutation in an invariant threonine residue in the essential MADS box domain exhibits better growth on high-salt medium. The simplest interpretation of these results is that Mcm1 regulates a gene or genes that are important for survival in high salt concentrations. Finally, we show that Mcm1 itself is likely to be a target of a signal transduction system responsive to high salt concentra-

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source
EJ66	*MAT***a** *trp1 leu2-3,112 ura3-52 his4*	K. Tatchell
EJ72	**a**/α *MCM1/MCM1 trp1/trp1 leu2-3,112/leu2-3,112 ura3-52/ura3-52 his4/his4*	K. Tatchell
EN115	**a**/α *mcm1::TRP1/MCM1 trp1/trp1 leu2-3,112/leu2-3,112 ura3-52/ura3-52 his4/his4*	This work
MK301	*MAT***a** *MCM1-HA trp1 leu2-3,112 ura3-52 his4*	This work
MK302	*MAT***a** *MCM1T30A trp1 leu2-3,112 ura3-52 his4*	This work
MK303	*MAT***a** *MCM1T35A trp1 leu2-3,112 ura3-52 his4*	This work
MK306	*MAT***a** *MCM1(Δ2-17)HA trp1 leu2-3,112 ura3-52 his4*	This work
MK308	*MAT***a** *MCM1(Δ2-17)/T35A-HA trp1 leu2-3,112 ura3-52 his4*	This work
MK724	*MAT***a** *mcm1::TRP1 trp1 leu2-3,112 ura3-52 his4* [YCplac33-*MCM1(1-98)HA URA3*]	This work
MK542	*MAT***a** *mcm1::TRP1 trp1 leu2-3,112 ura3-52 his4* (YCplac33-*MCM1-HA URA3*)	This work
MK206	*MAT***a** *mcm1::TRP1 trp1 leu2-3,112 ura3-52 his4* [YCplac111-*MCM1Δ(2-17)HA LEU2*]	This work
EN1092	*MAT***a** *mcm1::TRP1 rp1 leu2-3,112 ura3-52 his4* (YCplac111-*MCM1S2A-HA LEU2*)	This work
EN1076	*MAT***a** *mcm1::TRP1 rp1 leu2-3,112 ura3-52 his4* (YCplac111-*MCM1T8A-HA LEU2*)	This work
EN1082	*MAT***a** *mcm1::TRP1 rp1 leu2-3,112 ura3-52 his4* (YCplac111-*MCM1T10A-HA LEU2*)	This work
EN1088	*MAT***a** *mcm1::TRP1 rp1 leu2-3,112 ura3-52 his4* (YCplac111-*MCM1T8A, T10A-HA LEU2*)	This work
EN948	*MAT***a** *mcm1::TRP1 rp1 leu2-3,112 ura3-52 his4* (YCplac111-*MCM1S2A,T8A-HA LEU2*)	This work
JF819	*MAT***a** *his4-917 lys2-128δ leu2-1 ura3-52*	J. Fassler
JHRY20-2Ca	*MAT***a** *leu2-3,112 his3 Δ200 ura3-52 pep4::URA3*	T. Stevens

tions: a new Mcm1 isoform is induced by treatment with high levels of either NaCl or KCl for 90 min.

MATERIALS AND METHODS

Plasmid DNAs, yeast strains, and Mcm1 protein synthesis in vitro. Yeast strains used in this study and their relevant genotypes are listed in Table 1.

To construct the *MCM1-HA* fusion gene, *Sac*II and *Xba*I sites (underlined below) were introduced immediately upstream of the stop codon of the *MCM1* gene by PCR with the primer BN2 (CCAGGCCTGTCTTAACGCC), which hybridizes to the *MCM1* open reading frame, and the primer MK12 (GGAAA TCGATAAGATTATCTAGATATCCGCGGTATTGGCCTTG), which also contains the native *Cla*I site 3′ to the stop codon. An *Eco*RI-*Cla*I fragment from this PCR was substituted for the *Eco*RI-*Cla*I sequence in pSP73-*MCM1*, which contains the 3-kb *Sph*I-*Bam*HI fragment encompassing the entire *MCM1* gene as well as upstream and downstream regulatory sequences. The trimeric HA sequence, isolated as a *Sac*II-*Xba*I fragment from pBluescript II SK^+-$(HA)_3$ DNA (constructed by Tyers et al. [85]), was inserted in-frame at the C terminus of *MCM1* to create pSP73-*MCM1-HA*. To construct the truncated *MCM1* fusion, *MCM1(1-98)HA*, a *Sac*II site was introduced immediately C terminal to amino acid 98 by PCR with primers MK13 (ATTCCGCGGTCAGGGGCGTTAAG) and BN1 (TCGATTCAGTTATAGGG); a *Nar*I-*Sac*II fragment from this PCR was introduced into pSP73-*MCM1-HA* to make pSP73-*MCM1(1-98)HA*. Yeast vectors were derived from YCplac33, YEplac195, or YCplac111 (23) by insertion of the *Sph*I-*Bgl*II fragments with the *MCM1-HA* or *MCM1(1-98)HA* fusion genes. To obtain plasmids in which the *Nar*I, *Kpn*I, and *Eco*RI sites within the *MCM1* gene were unique, vector sequences between the *Sma*I and *Spe*I sites were removed to construct YCplac111Mcm1-HAΔSS.

To create a strain in which plasmid-borne, epitope-tagged *MCM1-HA* was the sole source of Mcm1 protein, one copy of the *MCM1* gene in an isogenic EJ72 diploid (80) was replaced with the *TRP1* gene, removing the essential 80 amino acids of *MCM1* to create EN115 (**a**/α *mcm1::TRP1*/*MCM1 leu2*$^-$/*leu2*$^-$ *ura3-52*/*ura3-52 his4*$^-$/*his4*$^-$). EN115, transformed with YCplac33-*MCM1-HA* or YCplac33-*MCM1(1-98)HA* (both have the URA3 gene), was sporulated. All Trp^+ spores were Ura^+, indicating that the chromosomal deletion of the *MCM1* gene was complemented by the plasmid copy of *MCM1-HA* or *MCM1(1-98)HA*. Two Ura^+ Trp^+ spores, MK542, with full-length *MCM1-HA*, and MK724, with *MCM1(1-98)HA*, were used for most experiments. To create MK206, in which the sole copy of *MCM1* is a plasmid-borne copy of *MCM1(Δ2-17)HA*, MK724, transformed with YCplac111-*MCM1(Δ2-17)HA* (a *LEU2 CEN*/*ARS* vector), was grown on 5-fluoroorotic acid (5-FOA) to select against YCplac33-*MCM1(1-98)HA* (7); Ura^- Leu^+ colonies were selected. Strain manipulations were checked by Southern analysis of DNA insertions and Western analysis of Mcm1-HA protein size.

In vitro synthesis of the Mcm1-HA fusion protein was carried out as described previously (24), except that a mixture of [^{35}S]methionine and ^{3}H-amino acids (Leu, Lys, Phe, Pro, and Tyr) (Amersham) was used for cyanogen bromide mapping. The *Nar*I-*Cla*I fragments from *MCM1-HA* and *MCM1(1-98)HA* were introduced into p*MCM1*-A, in which the ATG is adjacent to a T7 promoter (24).

Mutagenesis of Mcm1 protein. Site-specific mutations (40), introduced into *MCM1* with the oligodeoxyribonucleotides CAGCAAAAATGGCAGATATCG AAGAAGGT (S2A, *Eco*RV site), CAGACATCGAAGAGGT(A/G)CACCT (A/G)CTAATAATGGGCAACAG (T8A, T10A, *Apa*L1 site), ATGGCGGCG CGCTTTATTCTC (T30A, *Bss*HII site), TTTGGAAAATGCCACGTGGCGC CTTG (T35A, *Pml*I site), CTTCCTTTTCGCGAATGTCACATG (S37A, *Nru*I site), and TCTATCTTTCTTCTCCATTTTTGCTGGGTG [(Δ2-17)] (13) were subcloned into YCplac111-*MCM1-HAΔSS*.

Integration of mutated *mcm1* genes at the *MCM1* chromosomal locus. A one-step procedure to integrate a single copy of the *MCM1* gene with the HA epitope tag and the specified mutation in its normal chromosomal locus without duplication or addition of selectable markers was developed. MK724 [relevant genotype, *mcm1::TRP1* YCplac33-*MCM1(1-98)HA CEN*/*ARS URA3 trp1*$^-$ *ura3-52*] was transformed with *MCM1-HA* DNA fragments containing the desired mutations. Integrants were selected based on their ability to lose the plasmid-borne copy of *MCM1(1-98)HA* and the *URA3* gene and thus to grow on 5-FOA (7). Furthermore, integration of *MCM1-HA* DNA at the *MCM1* locus should result in concurrent loss of the *TRP1* gene at this site and thus a Trp^- phenotype. In particular, 3.3-kb *Sph*I-*Apa*I *MCM1-HA* fragments were gel purified (from ≈20 μg of plasmid DNA) and used to transform competent MK724 cells (76). Transformed cells, suspended in 5 ml of yeast extract-peptone-dextrose (YPD), were grown for 16 h at 30°C to allow loss of the *MCM1(1-98)HA URA3* plasmid, concentrated by centrifugation, washed with water, and plated on 5-FOA plates. 5-FOA-resistant colonies were picked after 3 to 14 days at 30°C and patched to a second 5-FOA plate before replica plating to synthetic dextrose without uracil (SD-Ura) and synthetic dextrose without tryptophan (SD-Trp) to screen for Ura^- Trp^- clones. The presence of full-length Mcm1-HA [or Mcm1(Δ2-17)HA] protein and the absence of Mcm1(1-98)HA protein was confirmed by Western analysis with the 12CA5 antibody against the HA epitope. None of the tested mutants showed a significant difference in the quantity of Mcm1 protein (data not shown). The position of the integration and the presence of appropriate mutant sequences were confirmed by restriction analysis of genomic or PCR-amplified genomic DNA. Typically 60% of the Ura^- cells were also Trp^-, and essentially all Ura^- Trp^- cells carried the appropriate *MCM1-HA* alleles at the *MCM1* locus.

Immunochemical assays, extracts, and labelling. To label yeast cells with [^{32}P]orthophosphate (NEN), log-phase cells were grown in YPD for three generations (~0.5 unit of optical density at 600 nm [OD_{600} unit]/ml), pelleted, washed with water, resuspended at 0.5 OD_{600} unit/ml in prewarmed low-phosphate medium (62), and grown at 30°C for 3 h, then labelled for 30 min with $H_3{}^{32}PO_4$ (8,500 to 9,120 Ci/mmol; NEN) at 100 μCi/OD_{600} unit. The crude extract was prepared as described below. Immunoprecipitation reactions were carried out with 0.2 μg of 12CA5 antibody per 500 μg of protein in 200 μl of phosphate-buffered saline: the mixture was gently shaken at 4°C for 1 h and collected on 2 μl of 10% protein A-positive *Staphylococcus aureus* fixed cells (Boehringer Mannheim) by mixing at 4°C for 1 h. Collection, washes, and buffers were as described (85) except that the final pellet was resuspended and incubated for 15 min at 37°C in 50 μl of 10 mM Tris-HCl (pH 7.9)–10 mM $MgCl_2$–50 mM NaCl–1 mM dithiothreitol (DTT) containing 0.1 to 0.5 μg of RNase A, to remove contaminating RNA. To phosphatase treat the immunoprecipitate, 20-μl reaction mixtures (50 mM Tris-HCl [pH 8.5]–0.2 mM EDTA) with and without 100 mM NaP_i (pH 7) containing 20 U of calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim) were incubated for 15 min at 37°C, and the reactions were terminated with 5 μl of 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading dye. Proteins were resolved by SDS-PAGE [10, 15, and 10 to 20% linear gradient gels for Mcm1-HA, Mcm1(1-98)HA, and both, respectively].

To prepare yeast extracts, yeast cells in log phase were harvested and washed in ice-cold water, and cells (30 OD_{600} units) were suspended in 100 μl of

extraction buffer (50 mM Tris-Cl [pH 7.5]–1 mM EDTA–1% SDS). After the addition of 0.4 g of acid-washed glass beads (450 to 500 μm), the cells were vortexed vigorously in two 90-s pulses with intermittent chilling, boiled for 3 min, and cooled. After the addition of 900 μl of cold 1% Triton X-100 in phosphate-buffered saline, the mixture was vortexed for 30 s and spun to remove the glass beads. After removal of insoluble material by centrifugation (15 min at 16,000 × *g*), protein concentration was measured with a Bio-Rad protein assay kit. Western analysis was performed on SDS-PAGE and IEF gels (20 to 50 μg of protein per lane) that were transferred to nitrocellulose, probed with 12CA5 antibody (Boehringer Mannheim) and then with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Bio-Rad), and visualized with an ECL kit (Amersham).

Peptide mapping. Prior to peptide mapping, labelled, immunoprecipitated, and gel-purified Mcm1-HA was excised and eluted from the gel directly (8). Cyanogen bromide cleavage reactions with purified labelled proteins in 140 μl of 70% formic acid were started by addition of 70 μl of 300-mg/ml CNBr in 70% formic acid; the mixture was incubated at room temperature for 90 min, and the reactions were terminated by lyophilization. The dried pellet was washed with 200 μl of water, lyophilized, and dissolved in Tricine-SDS gel buffer (20 μl), with 1 μl of 2 M Tris-HCl (pH 8.8) added to neutralize acid suspensions (75). Peptides were resolved on a 16.5% acrylamide (acrylamide/bis ratio, 16:1) Tricine-SDS gel (0.7 by 12 by 25 cm) (75). Proteolytic peptide mapping was performed as described elsewhere (8, 48, 58, 91), except that 250 μm of cellulose thin-layer chromatography plates (Analtech, Newark, Del.) were used. Peptides were resolved by electrophoresis in a pH 1.9 buffer (88% formic acid/glacial acetic acid/water ratio, 100:10:890) at 1,000 V for 25 min (Pharmacia FBP3000 apparatus) in the first dimension and by ascending chromatography in the second dimension (*n*-butanol/pyridine/glacial acetic acid/water ratio, 750:500:150:600); development was continued for 6 h after the buffer front reached the plate top.

Salt sensitivity/tolerance assays. Yeast cells, in log-phase growth in YPD, were diluted to 10^5 cells/ml and preconditioned by incubation for 1 h in liquid YPD containing 0.7 M NaCl as described elsewhere (6, 42) and spotted (5 μl containing 500 cells) on YPD plates containing 0, 0.7, or 1.4 M NaCl or 0.4 M LiCl or 1 M KCl. Growth at 30°C was monitored for 14 days.

IEF electrophoresis. Vertical slab gel IEF was performed essentially as described elsewhere (16, 49), except that a mixture of Pharmalytes, pH 2.5 to 5 and pH 4 to 6.5 (1:4 or 3:2), was used. Pharmalytes (2%, in the same ratio as in the gel) were added to whole-cell extracts (40 to 50 μg) in 90% VSIEF buffer; samples were brought to 40 μl with IEF loading buffer, containing 9.5 M urea, 2% Pharmalytes, 5.4% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 5% β-mercaptoethanol, and 0.1% SDS. Whole-cell extracts from log-phase cells were grown, washed, and lysed as described above, except that cells were suspended in 100 μl of 1% SDS instead of extraction buffer; the lysate was extracted with 900 μl of VSIEF buffer (9.5 M urea, 5.4% CHAPS, 5.5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate) (49) at room temperature, instead of 1% Triton. After removal of glass beads, these extracts were clarified by centrifugation at room temperature for 5 min.

For phosphatase treatment, soluble yeast extracts were prepared from JHRY20-2Ca (from Tom Stevens) (relevant genotype, Δ*pep4::URA3 leu2*$^-$) transformed with YCPlac111 *CEN LEU2*-based plasmids carrying either *MCM1-HA* or *MCM1(Δ2-17)HA*. Cells, grown in selective media overnight, were grown for three generations in YPD to an OD_{600} of ≈0.3, centrifuged, washed with cold L buffer [0.2 M Tris-HCl (pH 8.0), 0.39 M $(NH_4)_2SO_4$, 20% glycerol, 1 mM EDTA], and frozen in L buffer at 300 OD units/ml. Cells thawed in buffer CE [130 mM Tris-HCl (pH 8.0), 0.79 M $(NH_4)_2SO_4$, 13% glycerol, 5.66 mM EDTA, 5 mM DTT, 1% Triton X-100, 0.25 M sodium phosphate (pH 7.0), 0.1 mM sodium orthovanadate, 10 nM okadaic acid, 35 mM β-glycerophosphate, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml] were lysed with glass beads in a mini-bead beater (50). The crude extract was separated from the glass beads by puncturing the bottom of the tube, followed by washing of the beads with 1/2 volume of buffer W (25 mM sodium phosphate [pH 7.0], 5 mM DTT, 5 mM EDTA, 10% glycerol) and addition of phenylmethylsulfonyl fluoride (2 mM), and was centrifuged for 10 min in an Eppendorf centrifuge; then the supernatant was passed through a dried Sephadex G-25 column equilibrated with buffer T (30 mM Tris-Cl [pH 8.0], 2 mM EDTA, 2 mM DTT, 5% glycerol, 0.1 mM sodium orthovanadate, 35 mM β-glycerophosphate, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml). The column flowthrough, with 10 nM okadaic acid added, was frozen on dry ice and kept at −70°C.

Potato acid phosphatase (Boehringer Mannheim) was spun out of $(NH_4)_2SO_4$ suspension, resuspended in buffer P, containing 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.0), 100 μg of bovine serum albumin per ml, 5% glycerol, 50 mM NaCl, and 2 mM DTT, and passed through a Sephadex G-25 spin column in buffer P (without bovine serum albumin). Phosphatase reactions were performed at 37°C for 15 min in a solution containing 12 mM PIPES (pH 6.0), 10 mM sodium acetate (pH 5.4), 9 mM Tris-HCl (pH 8.0), 0.6 mM EDTA, 0.8 mM DTT, 2% glycerol, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml with either 130 mM NaCl or 25 mM NaP_i (pH 7.4), and 100 mM Na_2MoO_4 (81). Samples (40 μg of proteins, 10 μl), diluted to 40 μl with 1.2× IEF loading buffer (except for 1× urea) and with solid urea added to 9.5 M, were analyzed on vertical IEF gels and immunoblotted.

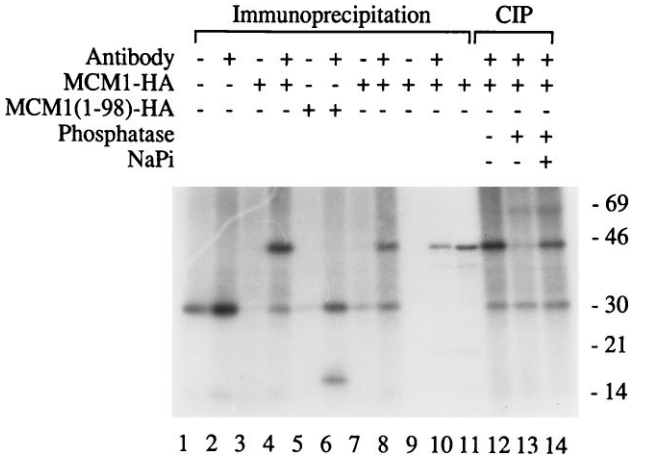


FIG. 2. Mcm1 is phosphorylated in the N-terminal 98 amino acids. Immunoprecipitation and dephosphorylation of Mcm1 proteins after in vivo labelling with [^{32}P]orthophosphate are shown. Lanes 1 through 8, immunoprecipitation from ^{32}P-labelled extracts with and without Mcm1-HA or Mcm1(1-98)HA, as indicated; lanes 9 and 10, in vitro ^{35}S-labelled Mcm1-HA immunoprecipitated after premixing with unlabelled yeast extract; lane 11, input, untreated ^{35}S-Mcm1-HA. Lanes 12 through 14 show phosphatase treatment of the immunoprecipitate shown in lane 4, as follows: mock treatment without CIP (lane 12) and treatment with 18 U of CIP (lane 13) and with 18 U of CIP and 100 mM NaP_i as an inhibitor for phosphatase action (lane 14). Molecular mass markers (in kilodaltons) are shown on the right.

RESULTS

Both Mcm1-HA and Mcm1(1-98)HA are phosphorylated in vivo. To see if Mcm1 is phosphorylated in vivo, we immunoprecipitated HA epitope-tagged Mcm1 protein from yeast cells labelled with [^{32}P]orthophosphate and resolved the precipitates with SDS-PAGE. Based on the immunoprecipitation results shown in Fig. 2, we conclude that Mcm1 protein is phosphorylated. In extracts made from yeast carrying the *MCM1-HA* gene, a ^{32}P-labelled protein migrating at 45 kDa was precipitated by the antibody (lanes 4 and 8). This protein is likely to be Mcm1-HA for two reasons. First, the ^{32}P-labelled 45-kDa band was seen only when both the antibody (compare lanes 4 and 8 with lanes 3 and 7) and the *MCM1-HA*-containing extracts (compare lanes 4 and 8 with lane 2) were used. Second, this ^{32}P-labelled band comigrates with authentic Mcm1-HA protein synthesized in vitro (compare lanes 4 and 8 with lanes 10 and 11).

To learn if the phosphorylation occurs in or near the essential DNA-binding domain (i.e., residues 18 to 98) (see Fig. 1) of Mcm1 protein, we ^{32}P-labelled cells bearing a truncated derivative of Mcm1 protein, Mcm1(1-98)HA, in which the nonessential C-terminal two-thirds of the molecule has been deleted (9, 13, 71). As seen in Fig. 2, lane 6, an 18-kDa ^{32}P-labelled band was specifically precipitated by anti-HA antibody from the yeast extract carrying the *MCM1(1-98)HA* gene. The predicted molecular weight of this protein (16,000) and its molecular size on the SDS gel (18 kDa) correspond well. Mcm1(1-98)HA appears to be modified in vivo comparably to full-length Mcm1 protein, since the ^{32}P labelling of the full-length and truncated Mcm1 proteins, as well as their concentrations (see, for example, Fig. 3D), is similar.

To ascertain if the labelling of Mcm1 by ^{32}P is due to phosphorylation, the immunoprecipitated materials were treated with CIP. When immunoprecipitated Mcm1-HA was treated with CIP, the radioactivity associated with Mcm1-HA was significantly reduced; moreover, when the phosphatase was inhibited by a high concentration of sodium phosphate, the label

was efficiently retained (Fig. 2, lanes 12 to 14). The same result was also obtained with Mcm1(1-98)HA (data not shown). Therefore, Mcm1 protein is phosphorylated, and Mcm1(1-98)HA, which lacks the nonessential C terminus, is comparably modified.

Phosphoamino acid analysis confirmed that Mcm1 is phosphorylated. Both phosphoserine and phosphothreonine in similar amounts were released from ^{32}P-labelled Mcm1-HA and Mcm1(1-98)HA; phosphotyrosine was not detected (data not shown). Thus it is likely that Mcm1 is phosphorylated at two or more positions.

Phosphorylation of Mcm1 protein occurs mainly within the first 17 amino acids. Since there are 15 serine and threonine residues in Mcm1(1-98) (Fig. 3A), we first mapped the major phosphorylation sites using cyanogen bromide, which cleaves at the single internal methionine (Met44) of Mcm1(1-98)HA. CNBr cleavage of the ^{3}H-labelled, in vitro-synthesized Mcm1(1-98)HA produced two well-resolved peptides of nearly the predicted molecular mass (Fig. 3B, lanes 5 and 6): a 6.6-kDa peptide, from amino acids S2 to M44 (predicted size, 5.1 kDa), and a 9.5-kDa band, corresponding to K45 to R142 (predicted size, 10.9 kDa). As shown in Fig. 3B (lanes 2 and 4), a single ^{32}P-labelled species was produced from the in vivo-labelled Mcm1 proteins. This peptide corresponds to S2 to M44. First, it comigrated with the lower CNBr cleavage product from Mcm1(1-98)HA synthesized in vitro (Fig. 3B, lane 6). Second, the same size peptide is derived from both full-length Mcm1-HA and Mcm1(1-98)HA (Fig. 3B, lanes 2 and 4, respectively). Only the peptide from amino acids 2 to 44 is common to both the full-length and the truncated Mcm1 proteins (Fig. 3A). Thus, the major phosphorylation sites of Mcm1 protein reside in the first 44 amino acids.

A significant loss of radioactivity occurred during CNBr cleavage and processing of both phosphorylated Mcm1 derivatives; this was particularly true of the full-length Mcm1-HA (Fig. 3B; compare lane 2 with lane 1). This was reproducibly observed. It is possible that multiple, minor phosphorylation sites are present which are responsible for the apparent loss of total radioactivity. However, in several longer exposures, we were unable to detect another specific band(s) from the rest of the protein.

Within the first 44 amino acids of Mcm1 protein, there are six serine and threonine residues: S2, T8, T10, T30, T35, and S37 (Fig. 3A). Amino acids S2, T8, and T10 reside in a region that is dispensable for the cell viability and cell-type functions of Mcm1 protein (13). S2 and T8 are putative casein kinase II and casein kinase I consensus recognition sites, respectively. Amino acids T30, T35, and S37 lie in the most conserved domain of the MADS box, involved in DNA binding. Among these, T35 and surrounding basic amino acids are invariant residues found in all MADS box protein thus far. T30 and S37 are highly conserved in that 85% of MADS box proteins maintain at least one serine or threonine in these two positions (our unpublished data; see reference 79 for the alignment). Because of the nearby basic residues which are also conserved, these three serines and threonine are potential targets for protein kinase C (T30, T35, S37) and Ca^{2+}/CaM-dependent protein kinase II (T35) (68).

To further identify the positions of phosphorylation, we therefore deleted the entire block of sequence from S2 to E17, producing Mcm1(Δ2-17), and individually mutagenized T30, T35, and S37 into alanine. Phosphorylation of these Mcm1 mutants was analyzed by in vivo labelling and immunoprecipitation. Somewhat surprisingly, cells carrying *MCM1-HA* bearing point mutations at T30, T35, or S37 as the sole source of Mcm1 were viable, indicating that alanine substitutions for these amino acids are sufficient for function of a MADS box protein. None of these mutations caused significant changes in the gross level of phosphorylation, as measured with ^{32}P labelling in vivo (data not shown).

The major phosphorylation site(s) is likely to reside between amino acids 2 and 17, because we were unable to detect a ^{32}P-labelled derivative of Mcm1(Δ2-17)HA (Fig. 3C, lanes 3 and 4). We attempted to label Mcm1(Δ2-17)HA carried on a *CEN* plasmid in two related backgrounds: a wild-type chromosomal copy of *MCM1* and a chromosomal *mcm1::TRP1*, with a plasmid-borne copy of *MCM1(1-98)HA*. In each experiment, we were unable to detect a ^{32}P-labelled, immunoprecipitated band corresponding to Mcm1(Δ2-17)HA (data not shown). To control conditions for labelling, extraction, and immunoprecipitation but set up a condition in which Mcm1(Δ2-17)HA was the only source of Mcm1 protein in one cell, we labelled a mixture of two strains, one of which (MK724) carried *MCM1(1-98)HA* and the other of which (MK206) carried *MCM1(Δ2-17)HA* as the sole source of Mcm1 protein. Under these conditions, only Mcm1(1-98)HA was labelled; no ^{32}P-labelled Mcm1(Δ2-17)HA was detectable (Fig. 3C, lanes 3 and 4). Separately, but simultaneously, a strain lacking an HA-tagged *MCM1* gene and a strain with wild-type *MCM1-HA* were labelled and analyzed (Fig. 3C, lanes 1 and 2). ^{32}P-labelled Mcm1-HA was easily detectable (Fig. 3C, lane 2). Failure to label Mcm1(Δ2-17)HA is not caused by reduced levels of the protein since, under the conditions of labelling, the amount of Mcm1(Δ2-17)HA protein is similar to the level of wild-type Mcm1-HA protein, as demonstrated by Western analysis (Fig. 3D, lanes 2, 3, and 4). Furthermore, the samples in Fig. 3C, lanes 3 and 4 are derived from independent isolates of the Δ2-17 mutagenesis procedure, both of which are sufficient as the sole source of MCM1 (data not shown).

The simplest explanation for the considerable reduction of ^{32}P-labelling in Mcm1(Δ2-17) is that the major phosphorylation targets, namely S2, T8, and/or T10, reside within this area. Alternatively, considering that a relatively large area has been deleted from Mcm1 protein to create the Mcm1(Δ2-17)HA mutant, it is possible that a conformational change resulting from the deletion diminishes phosphorylation elsewhere.

To confirm that the major phosphorylation sites lie between amino acids 2 and 17, we verified that the major tryptic phosphopeptides were cleaved by Glu-C protease, as expected for the tryptic peptide containing the first 17 amino acids; other potential tryptic phosphopeptides derived from the first 44 amino acids are not expected to be substrates for Glu-C protease (see Fig. 3A for the predicted trypsin and Glu-C cleavage sites). ^{32}P-labelled Mcm1(1-98)HA was subjected to two-dimensional peptide mapping after cleavage with trypsin (Fig. 4A) or with trypsin and Glu-C protease (Fig. 4B). The major tryptic phosphopeptides are clearly cleaved by Glu-C. Two major tryptic phosphopeptides are seen in Fig. 4A; both major spots disappear in the trypsin–Glu-C double digestion and are replaced with one major and two minor species at different positions (Fig. 4B), indicating that the phosphotryptic peptides are substrates for Glu-C endopeptidase. Furthermore, as predicted, if the phosphorylation sites lie at the amino terminus, migration of the major spots is not significantly altered if trypsin digestion is followed by digestion with chymotrypsin (data not shown). The existence of two major tryptic phosphopeptides (Fig. 4A), both of which are substrates for Glu-C protease and thus likely derived from the N terminus, may be due to differences in the number or identity of phosphoamino acids in these spots; this heterogeneity is consistent with our finding that both S2 and T8 are phosphorylation sites (see below). Several minor tryptic phosphopeptides, some of which are not

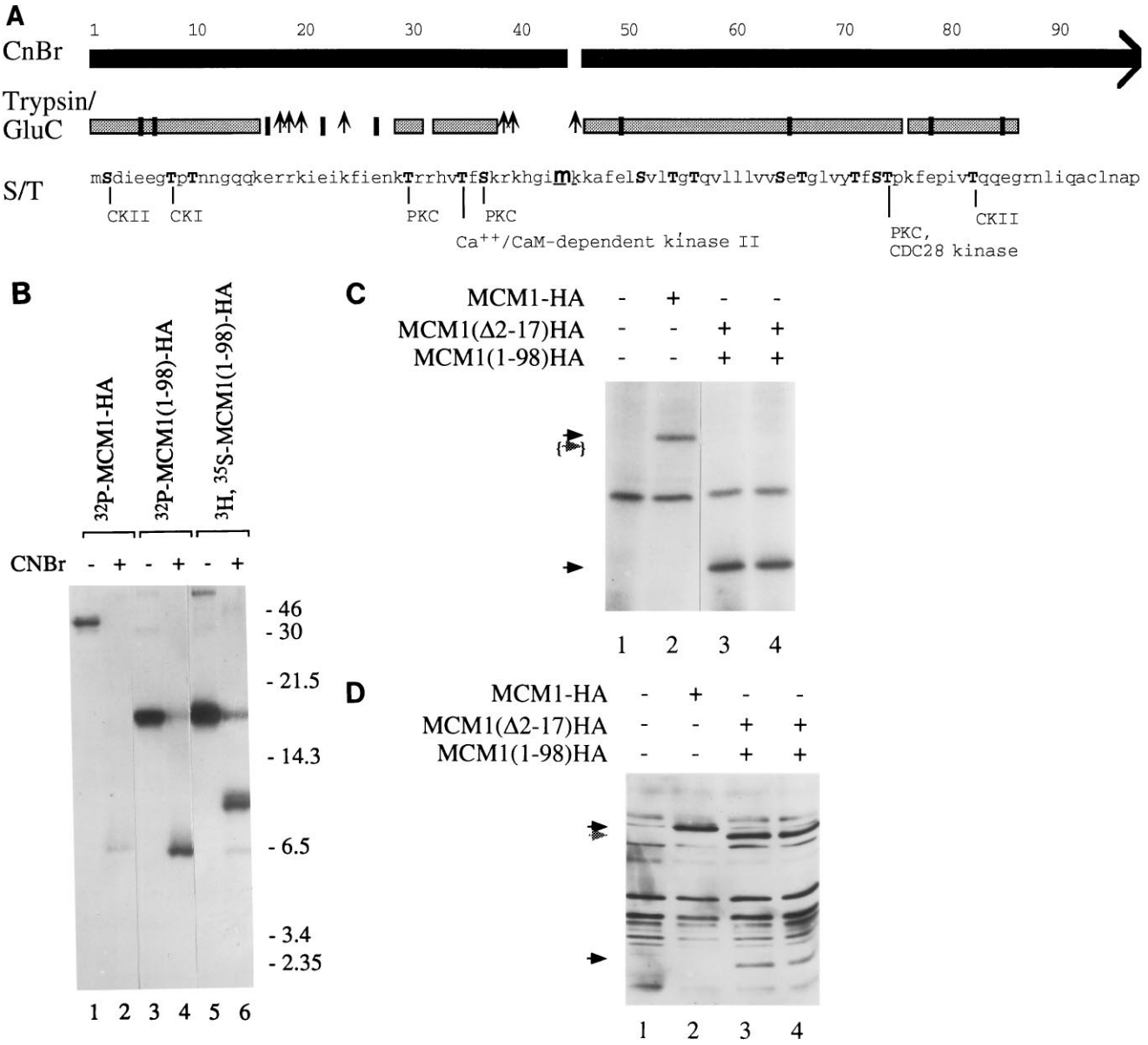


FIG. 3. Mapping major phosphorylation sites to the first 17 amino acids. (A) Diagram of amino acids 1 to 98, showing the predicted CNBr cleavage products (black boxes) as well as proteolytic peptides. CNBr cleavage at Met 44 (bold underlined "m") generates two peptides in Mcm1(1-98)HA (amino acids 2 to 44 and 45 to 142 [the C terminus of the HA epitope]), while the homologous peptide from Mcm1-HA is expected to contain amino acids 45 to 121. Predicted trypsin peptides containing serine or threonine are shown as shaded boxes. Additional sites of trypsin cleavage are indicated by arrows. Predicted sites of Glu-C cleavage are indicated by vertical bars. The sequence of Mcm1 from amino acids 1 to 98 is indicated with all serines and threonines in bold capitals; putative recognition sites are indicated as well. (B) Cyanogen bromide cleavage of Mcm1-HA and Mcm1(1-98)HA. Immunoprecipitated ^{32}P-labelled Mcm1-HA (lanes 1 and 2) and Mcm1(1-98)HA (lanes 3 and 4), as well as ^{35}S- and ^{3}H-double-labelled Mcm1(1-98)HA (lanes 5 and 6), were incubated in 70% formic acid with or without CNBr, as indicated, and resolved on an SDS-Tricine gel. Molecular mass markers (in kilodaltons) are shown on the right. (C) Immunoprecipitation of ^{32}P-labelled wild-type and mutant Mcm1 proteins. Early-log-phase cells were dispensed into two tubes for parallel treatment with radioactive orphophosphate or nonradioactive phosphoric acid. Cells were EJ66 (relevant genotype, no *MCM1-HA*) (lane 1), MK542 (relevant genotype, *MCM1-HA*) (lane 2), and mixed cultures of MK724 [relevant genotype, *MCM1(1-98)HA*] and MK206 [relevant genotype, *MCM1(Δ2-17)HA*] (lanes 3 and 4). The positions of full-length Mcm1-HA and Mcm1(1-98)HA are indicated by the upper and lower black arrows, respectively, while the expected position of Mcm1(Δ2-17)HA is indicated by a shaded arrow surrounded by braces. The position of Mcm1(Δ2-17)HA in lanes 3 and 4 of panel D is indicated by the shaded arrow. Two independent MK206 transformants, derived from different isolates of the Δ2-17 mutant, were assayed in lanes 3 and 4. In this experiment, the approximate ratio of MK206 to MK724 cells was 2:1, as assayed by counting the number of colonies from the mock-labelled mixed culture grown on SD-Ura (only MK724 could grow), SD-Leu (only MK206 could grow), or YPD plates (for total viable cells) (data not shown). This ratio corresponds with the protein band intensities in lanes 3 and 4 of panel D. (D) Western analysis of the same cells as those shown in panel C, except that these cells were mock labelled with nonradioactive H_3PO_4.

digested by Glu-C protease, could arise from other parts of Mcm1 which are phosphorylated inefficiently. Thus, both the failure to label Mcm1(Δ2-17) and the cleavage of major tryptic phosphopeptides with Glu-C provide support for the idea that major phosphorylation sites lie within the first 17 amino acids.

Multiple isoforms, predominantly due to different phosphorylation states, are detected with IEF. To learn if most or few of the Mcm1-HA molecules are phosphorylated and to learn how many species of Mcm1-HA are present in the cell, we resolved the isoforms of Mcm1-HA and Mcm1(Δ2-17)HA

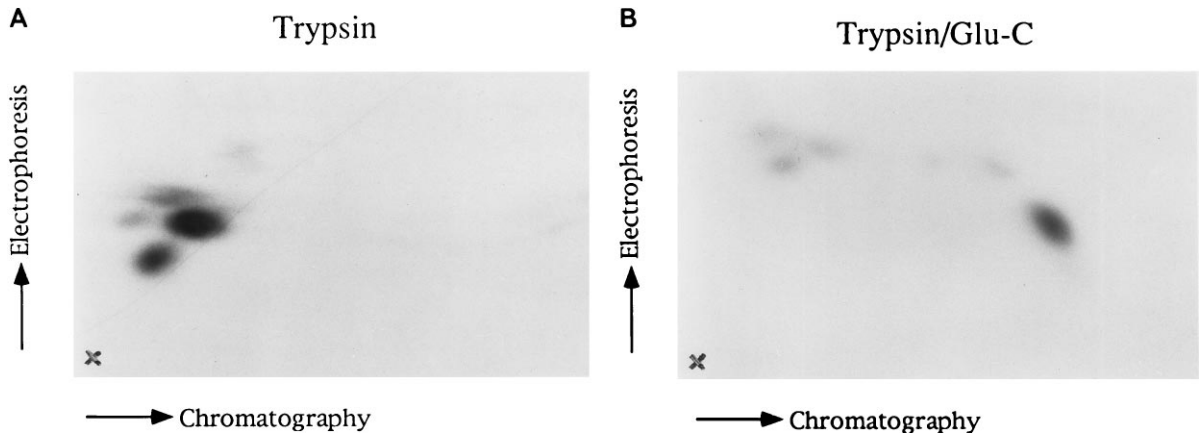


FIG. 4. Two-dimensional mapping of ^{32}P-Mcm1(1-98)HA after digestion with trypsin (A) or with trypsin and Glu-C protease (B). The first dimension was electrophoresis at pH 1.9, and the second dimension was ascending chromatography. Origins are marked "X."

on one-dimensional IEF gels in the acidic range. As shown in Fig. 5A (lane 2), full-length Mcm1-HA is separated into multiple bands by one-dimensional IEF, suggesting that the protein is modified at multiple positions. The major isoform (dark line) migrates between two other fairly abundant species, with some minor forms migrating to more acidic and basic positions. All the isoforms of wild-type Mcm1-HA migrate at 45 kDa in a second-dimension SDS-PAGE (data not shown), indicating that they most likely differ from each other by modifications. Mcm1(Δ2-17)HA is resolved into a similarly complex pattern, although the two most basic isoforms (which are near the bottom of the figure) are predominant (Fig. 5A, lane 3). The overall pI's of isoforms of Mcm1(Δ2-17)HA are shifted to a more basic spectrum (bottom of the gel) (compare lane 3 to lane 2), as expected from its calculated isoelectric point of 4.95, compared to 4.75 for Mcm1-HA.

We detected a surprisingly complex pattern of Mcm1-HA isoforms (Fig. 5A, lane 2). To learn if these multiple bands represent phosphorylated species, we examined the effect of phosphatase treatment on the mobility of Mcm1-HA in IEF gels. First, if most of the Mcm1-HA molecules carry one or more phosphates, we expect the protein as a whole to migrate to a more basic region of the gel after phosphatase treatment. If, on the other hand, only the acidic isoforms (upper part of the gel) are phosphorylated, then after phosphatase treatment, these acidic isoforms will be lost and will now migrate with the major isoforms. Second, if the heterogeneity of the isoforms is due to multiple phosphorylation of Mcm1, with some species bearing one phosphate and the more acidic isoforms bearing several phosphates, then we expect a reduced number of isoforms after phosphatase treatment.

As shown in Fig. 5B (compare lanes 2 and 4), acid phosphatase treatment results in a quantitative shift of the Mcm1-HA to a more basic isoelectric point (lane 4). The bands labelled "a" through "d" are the predominant species in the extract, while the band labelled "e" is the primary product after phosphatase treatment (compare lanes 2 and 4). Thus, since the major species is shifted to a more basic isoelectric point by phosphatase treatment, we conclude that most of the Mcm1-HA molecules carry at least one phosphate. Furthermore, at least some isoforms likely differ from each other by phosphorylation because the number of isoforms is decreased by phosphatase treatment. The collapse of the isoforms is likely due to phosphatase activity because the effects of acid phosphatase are partially inhibited by addition of phosphate and molybdenum (Fig. 5B, lane 5). The effect of phosphatase on the number of isoforms can be seen more clearly by using a dose curve of CIP and a darker exposure to reveal the full set of isoforms (Fig. 5C). At low doses of CIP, the isoforms behave as expected for a group in which all members bear a single phosphate: the pattern of isoforms is simply shifted to a more basic position (Fig. 5C; compare lanes 2 and 5 to lane 1). High doses of CIP collapse the isoforms to two fully dephosphorylated forms (Fig. 5C; compare lane 6 to lanes 5 and 1). Thus, most species differ by phosphorylation, but there may be an additional modification of Mcm1 to account for two final products after CIP treatment.

Major phosphorylation sites map at S2 and T8, but phosphorylation at these sites does not account for the full spectrum of isoforms. Based on the results of the in vivo labelling and mapping, we expected the major phosphorylation site(s) to map within amino acids 2 to 17. Treatment of Mcm1(Δ2-17)HA with acid phosphatase confirms the expectation that the majority of this protein is not phosphorylated. Acid phosphatase treatment has no effect on migration of the two major species, marked as bands e and f in Fig. 5D (compare lanes 2 and 4); likewise CIP treatment does not affect their migration (data not shown). Thus, it is demonstrated that these isoforms, constituting the majority of the Mcm1(Δ2-17)HA molecules, are not phosphorylated, because their pI is unchanged by the same phosphatase treatments that cause a mobility shift of most full-length Mcm1-HA. Four minor acidic isoforms of Mcm1(Δ2-17)HA (labelled "a" through "d") migrate as expected for singly phosphorylated derivatives of Mcm1(Δ2-17)HA; some of these isoforms decrease with phosphatase treatment (Fig. 5D, lanes 2 and 4). As expected, phosphatase inhibitors block this reaction (Fig. 5D, lane 5). Thus, it seems likely that some minor isoforms of Mcm1(Δ2-17)HA are phosphorylated, indicating that minor phosphorylation sites may map outside the N-terminal domain.

To precisely identify the phosphorylation sites in the N-terminal domain, we examined the isoforms of Mcm1 mutant proteins with alanine substitutions at the single serine (S2) and the two threonines (T8 and T10) in the N-terminal domain. As shown in Fig. 5E, mutations at both S2 and T8 change the Mcm1 isoforms; the simplest interpretation of our results is that Mcm1 is phosphorylated nearly quantitatively on S2 and less efficiently on T8. The mutation S2A has a dramatic effect on the isoform pattern: the S2A Mcm1 isoforms move to a more basic position relative to wild-type Mcm1 [Fig. 5E; com-

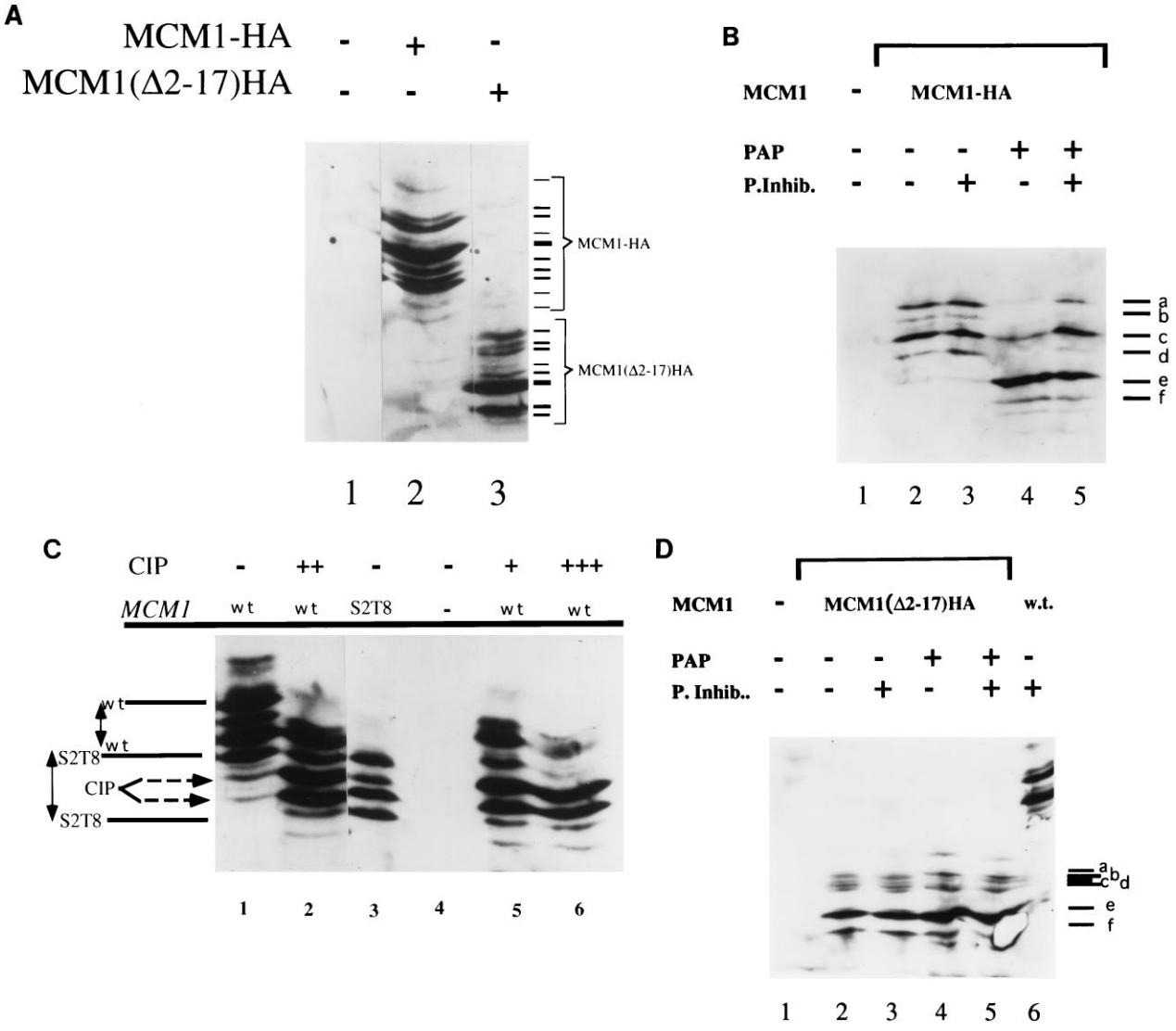


FIG. 5. Analysis of isoforms and several mutant forms of Mcm1-HA by IEF gel electrophoresis. (A) Whole-cell extracts with SDS and VSIEF buffer from strains JF819 (relevant genotype, no *MCM1-HA*) (lane 1), JF819 (YCplac111-*MCM1-HA*) (lane 2), and JF819 [YCplac111-*MCM(Δ2-17)HA*] (lane 3). (B) Whole-cell extracts were treated with potato acid phosphatase (PAP) as indicated. Samples received either NaCl or phosphatase inhibitors (phosphate and molybdenum) (P. Inhib.) as indicated. Samples were from MK724 [*MCM1(1-98)HA*], VSIEF extract (lane 1), or JHRY20-2Ca (YCplac111-*MCM1-HA*), soluble extracts (lanes 2 through 5). (C) Whole-cell soluble extracts of JHRY20-2Ca (YCplac111-*MCM1-HA*) were untreated (lane 1) or treated with 1, 0.2, or 4 U of CIP (lanes 2, 5, and 6, respectively). Lane 3, VSIEF extract of EN948 [*mcm1::TRP1* (YCplac111-*MCM1S2A,T8A-HA*)]; lane 4, whole-cell extract of MK724 [*mcm1::TRP1* (YCplac111-*MCM1(1-98)HA*)]. (D) Whole-cell extracts of JHRY20-2Ca [YCplac111-*MCM1(Δ2-17)HA*] were treated with potato acid phosphatase as indicated. Samples were from MK724 [*MCM1(1-98)HA*], VSIEF extract (lane 1); JHRY20-2Ca [*MCM1(Δ2-17)HA LEU2 CEN*], soluble extracts (lanes 2 to 5); and JHRY20-2Ca [*MCM1-HA LEU2 CEN*], soluble extracts (lane 6). (E) VSIEF whole-cell extracts from *mcm1::TRP1* strains bearing the designated *MCM1* alleles on YCplac111 vectors: wild-type *MCM1-HA* (w.t.) (lanes 1 and 8), *mcm1S2A-HA* (S2) (lanes 2 and 7), *mcm1T8A-HA* (T8) (lane 3), *mcm1T10A-HA* (T10) (lane 4), *mcm1T8A*, *T10A-HA* (T8 T10) (lane 5), and *mcm1S2A,T8A-HA* (S2 T8) (lane 6). Extracts shown in lanes 2 and 6 were derived from two isolates of the S2A mutant that were further subcloned and transformed into yeast independently.

pare lanes 2 and 7 (two independent isolates of S2A) to lanes 1 and 8]. Since almost all Mcm1 molecules are shifted to a more basic pI by the S2A mutation, it seems likely that S2 is normally phosphorylated with nearly 100% efficiency. Furthermore, S2 is likely to be a phosphorylation site (rather than to influence phosphorylation at another site, that is, to behave as part of a kinase recognition domain) because S2 is the only serine in the phosphorylated domain, and thus phosphorylation on S2 is required to account for the labelled phosphoserine in the phosphoamino acid analysis (data not shown). We note that S2A bands i and j (Fig. 5E, lanes 2 and 6) move to the same position as fully dephosphorylated Mcm1 (Fig. 5C, lanes 3 and 6). A major S2 band, labelled "h," migrates to a more acidic position than the final CIP products and thus is likely to be phosphorylated. A fourth S2 band, labelled "k," migrates at a more basic pI than the final CIP products and could lack N-terminal acetylation, which would make it more basic than an acetylated, dephosphorylated form.

The mutation T8A clearly results in loss of an acidic (phosphorylated) isoform, shown as band c (Fig. 5E; compare lanes 3 with lanes 1 and 8); three independent isolates of T8A all fail to produce band c (data not shown). The effect of the T8A mutation in causing loss of an acidic isoform (that is, a phosphoform) can also be seen in the context of an S2A mutation: in this case the acidic isoform migrating at position e in the S2A Mcm1-HA (Fig. 5E, lane 7) is absent in the S2A/T8A double mutant (lane 6). The T10A mutation causes a partial reduction in band c (Fig. 5E; compare lane 4 with lanes 1 and 5). We think that T8 is more likely to be the phosphorylation site itself while T10 might influence the efficiency of phosphorylation at T8. This interpretation is based on two observations. (i) The T10A mutation causes only a partial reduction in band c, while the T8A mutation causes quantitative loss of this band. (ii) The phenotype of the double mutant T8A/T10A is no more severe than that of T8A alone, consistent with the idea that T10 has no further effect once T8 is mutated. Furthermore, T8 matches a consensus casein kinase I site. Thus, the simplest interpretation of the data is that the T8 site is phosphorylated on ≈10 to 30% of the Mcm1 molecules (based on the estimated loss of acidic isoforms caused by T8A) and the efficiency of phosphorylation at T8 is influenced by T10.

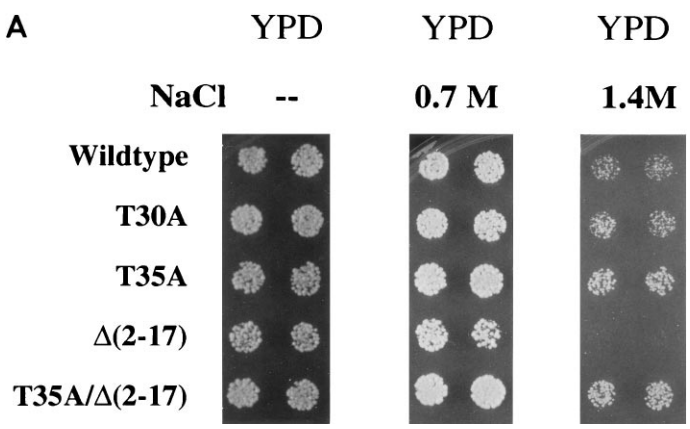
In addition to the quantitative phosphorylation at S2 and partial phosphorylation at T8, we think Mcm1 is phosphorylated incompletely at additional sites that lie outside the N-terminal region. Both the N-terminal deletion and the point mutations still produce isoforms that migrate like phosphorylated species. Mcm1(Δ2-17)HA exhibits several minor phosphorylated forms, that is, acidic isoforms, some of which are sensitive to phosphatase (Fig. 5D). Likewise, the double mutant S2A/T8A produces an isoform (band h in Fig. 5E) that migrates like a phosphorylated isoform, that is, at a more acidic pI than fully dephosphorylated Mcm1-HA (see Fig. 5C). Furthermore, the loss of the major multiply phosphorylated isoform caused by the T8A and T8A/T10A mutations reveals additional multiply phosphorylated Mcm1 molecules (band b in Fig. 5E) that are presumably modified at both S2 and additional residues. Although the modifications outside the region of amino acids 2 to 17 may be quantitatively minor, they may be central to Mcm1 function.

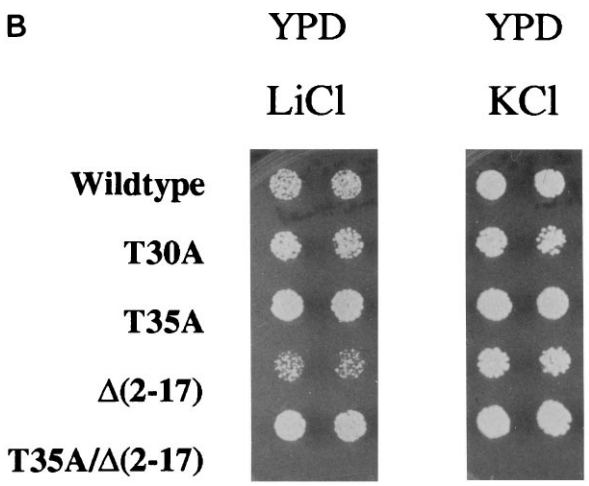
Mcm1 is implicated in the response to salt stress: mutations in the N terminus result in salt sensitivity, while a mutation in the MADS box results in better growth on high-salt medium. To begin to learn if phosphorylation of the amino terminus of Mcm1 protein is important for its function, we looked for a phenotype caused by deletion of S2 to E17. A set of isogenic strains bearing integrated copies of Mcm1 derivatives, including Mcm1(Δ2-17)HA, were compared. We examined the response to salt stress for three reasons. First, S2 and T8 are potentially casein kinase II and casein kinase I substrates, respectively. Members of each of these kinase families have been implicated in the response to salt stress: deletion of regulatory subunits of casein kinase II results in hypersensitivity to low concentrations of salt (5), and overproduction of one form of casein kinase I results in enhanced salt tolerance (73). Second, Mcm1 activity is affected by mutations in the *SLN1* gene (93), which is also implicated as a regulator of the HOG1 MAP kinase pathway, which is involved in response to osmotic stress (44). Third, we previously found Mcm1 binding sites upstream of genes implicated in cell wall and membrane function (41). Changes in the cell surface as well as internal changes in osmolarity likely participate in the response to salt stress (20, 25).

We found that deletion of amino acids 2 to 17, a region previously thought to be functionally dispensable (13), rendered cells sensitive to salt stress (Fig. 6). The *mcm1(Δ2-17)HA* mutation results in a defect in growth on high-salt medium after pretreatment of cells with intermediate concentrations of salt. Growth of a set of isogenic strains bearing stably integrated single copies of *MCM1-HA* or *mcm1(Δ2-17)HA* on media with and without NaCl was compared after pretreatment for 1 h with 0.7 M NaCl in liquid YPD medium. Strains bearing the *mcm1(Δ2-17)HA* mutation are selectively defective in growth on 1.4 M NaCl (Fig. 6A). The inability to grow on 1.4 M NaCl cannot be due to loss of viability during exposure to 0.7 M NaCl, because wild-type and *mcm1(Δ2-17)HA* strains grew comparably on YPD plates without NaCl after the pretreatment (Fig. 6A). It is clear that *mcm1(Δ2-17)HA* does not cause sensitivity to 0.7 M NaCl, because similar growth of wild-type and *mcm1(Δ2-17)HA* cells is observed on plates with YPD and 0.7 M NaCl (Fig. 6A). We observe qualitatively similar results if the cells are plated directly on high-salt plates without the adaptation in 0.7 M NaCl.

To learn if and how other mutations in Mcm1 affected growth on high-salt medium, we examined growth of isogenic strains bearing mutations in the MADS box of *MCM1* as the sole integrated copy of *MCM1*. Mutations at two positions, T30A and T35A, were investigated since neither mutant exhibited obvious defects in growth or mating, but T35 is an invariant amino acid in all MADS box proteins and T30 is a semiconserved amino acid. Cells bearing T30A grow exactly like wild-type cells on 1.4 M NaCl after treatment with 0.7 M NaCl (Fig. 6A). Surprisingly, cells bearing the T35A mutation grow slightly faster on plates containing 1.4 M NaCl, exhibiting visible growth on 1.4 M NaCl plates at least 24 h earlier than wild-type cells (Fig. 6A). This resistance to high salt concentrations caused by *MCM1T35A* can also be observed by comparing the percent survival of wild-type and T35A cells plated directly onto high-NaCl (1.2 M) medium or a medium with 0.4 M LiCl (data not shown). Furthermore, strains carrying the double mutation Δ(2-17)/T35A grow well in the presence of 1.4 M NaCl (Fig. 6A). These proteins are present at similar concentrations in vivo (data not shown), so suppression is not likely to be due to overcoming the effects of limiting Mcm1 concentrations.

NaCl inhibits cell growth for two reasons: (i) it causes osmotic stress that has to be counterbalanced by production and retention of glycerol internally, and (ii) it is toxic to the cell and has to be removed. To begin to probe the role of Mcm1 in the cellular response to stress, we examined the growth of these mutants on 1 M KCl (which imposes high osmostress without the toxicity of sodium) and on 0.4 M LiCl (which has higher cellular toxicity but produces less osmotic stress). The *mcm1*





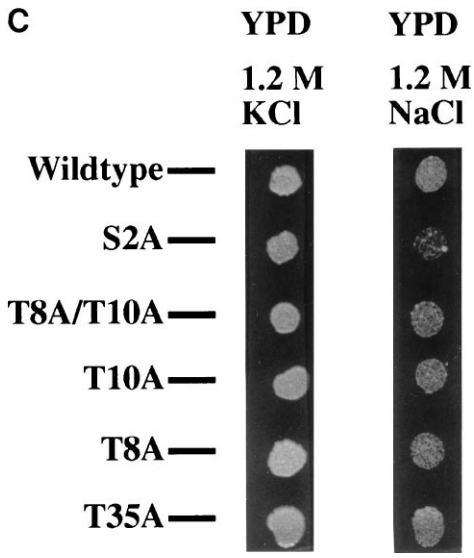


FIG. 6. Altered responses to salt stress conferred by specific *MCM1* mutations. (A) Growth on YPD is compared to growth on YPD containing 0.7 M or 1.4 M NaCl. Cells were conditioned in liquid YPD medium containing 0.7 M NaCl for 1 h prior to the inoculation. Plates were incubated at 30°C and were monitored daily. The cells were photographed after 24 h of growth on YPD, 63 h of growth on YPD with 0.7 M NaCl, and 112 h of growth on YPD with 1.4 M NaCl. (B) Growth on YPD with 0.4 M LiCl is compared with growth on YPD with 1 M KCl. (C) Growth on YPD with 1.2 M KCl is compared with growth on YPD with 1.2 M NaCl. Serial dilutions of log-phase cells grown in YPD were plated directly onto these media.

mutants all grow on both ions; thus, neither ion is as toxic as sodium to cells bearing *mcm1(Δ2-17)HA*. The mutants exhibit little or no defect in growth on 1 M KCl (Fig. 6B) but do grow more slowly on 0.4 M LiCl (Fig. 6B). This result suggests that Mcm1 may play a more important role in ion stress than in high osmolarity, but the distinction between these pathways has yet to be delineated.

If phosphorylation of the N-terminal domain is important for the function of this domain, we might find that a point mutation that destroys a phosphorylation site also renders the cells sensitive to salt. This is so. In comparison to cells with wild-type *MCM1-HA*, cells bearing *mcm1S2A* show markedly reduced survival when plated directly onto media containing 1.2 M NaCl but not when plated onto media containing 1.2 M KCl (Fig. 6C). The mutations T8A, T10A, and T8A/T10A do not severely affect survival on 1.2 M NaCl (Fig. 6C).

Mcm1 activity is regulated: a new isoform of Mcm1 is induced after treatment with Na^+ or K^+. The simplest explanation of the results that mutation of one amino acid (S2A) or one domain (amino acids 2 to 17) of Mcm1 causes a defect in the response to salt stress while mutation of a different amino acid (T35A) causes enhanced growth on high-salt medium is that Mcm1 participates in transcriptional control of a gene that is important for survival on high-salt medium. Mcm1 could participate either directly or indirectly. Mcm1 could cooperate with a new cofactor whose activity is regulated in response to salt; the mutations in Mcm1 that affect survival on high-salt medium might affect interactions with the cofactors. Alternatively, the activity of Mcm1 itself might be directly regulated in response to the stress induced by high concentrations of NaCl. Our working hypothesis since finding the heterogenous set of Mcm1 isoforms has been that particular isoforms might be regulated in response to external or internal signals. Therefore, we investigated the effect of salt stress on the pattern of Mcm1 isoforms.

As shown in Fig. 7, a new isoform of Mcm1 protein is induced when yeast cells are treated with 1.2 M NaCl or KCl for 90 min. This isoform, labelled "b," migrates as a multiply phosphorylated species (just above the T8-dependent isoform seen in Fig. 5E); the isoform labelled "a," which is even more acidic, is also induced. The concentrations of the isoforms, bands a and b, are induced both in absolute amounts and relative to the concentrations of other similarly phosphorylated Mcm1 species, for example, band c. The induced band is likely Mcm1-HA, since it is not seen in strains bearing Mcm1(1-98)HA or Mcm1(Δ2-17)HA as the sole source of Mcm1 (data not shown). The coinduction of two acidic isoforms can be accounted for by a salt-activated kinase that phosphorylates a specific residue but acts on more than one starting isoform of Mcm1 protein. Treatment of cells with 0.1 M LiCl has two effects on Mcm1: an increase in the amount of Mcm1 protein is observed (note that lane 4 of Fig. 7 contains less total protein than the other lanes), and a slight induction of bands a and b is seen, although it is unclear if these bands represent a greater percentage of the Mcm1 protein, as they do with NaCl and KCl induction.

Therefore, Mcm1 protein itself is regulated in response to high salt concentrations, and Mcm1 function is important for survival on high-salt medium. It seems likely that the Mcm1

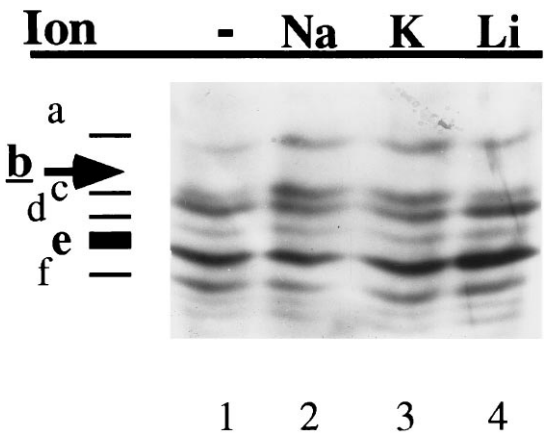


FIG. 7. Analysis of Mcm1-HA isoforms by IEF gel electrophoresis from cells treated with different salts. Cells were grown in YPD, diluted into YPD media containing the indicated slats, grown for 90 min at 30°C, harvested, washed, and frozen. Crude extracts were prepared with VSIEF buffer. Lanes were loaded with 50 μg of protein (except for lane 4, which contains 40 μg of protein) from strains grown in YPD without salt (lane 1), with 1.2 M NaCl (lane 2), with 1.2 M KCl (lane 3), or with 0.1 M LiCl (lane 4).

isoform induced by salt exhibits altered transcriptional regulatory activity on a gene(s) that is important for survival on high-salt medium.

DISCUSSION

In vivo Mcm1 protein is phosphorylated; many distinct isoforms of Mcm1 exist. We have shown here that Mcm1 protein, an essential global transcription regulator, is phosphorylated in vivo, with two major sites in the nonessential N-terminal domain. Mcm1 protein is not a single species: several distinct isoforms of Mcm1-HA exist, many of which differ solely by phosphorylation. Furthermore, we find that two particular isoforms are induced in response to high salt concentrations and that Mcm1 is important for survival on high concentrations of NaCl. These results provide direct evidence that the Mcm1 protein is posttranscriptionally regulated and provide a new framework in which to consider how Mcm1 regulates such a large set of diverse genes.

Most Mcm1 molecules are phosphorylated in vivo with the principal site at S2 and a second major site at T8. Both full-length Mcm1 and a truncated derivative of Mcm1 lacking the C-terminal two-thirds of the protein are labelled in vivo with ^{32}P. We find that major phosphorylation sites lie within the first 17 amino acids, based on three observations. (i) The major ^{32}P-labelled tryptic peptides, resolved in two dimensions, are susceptible to Glu-C protease digestion, as expected for the M1-to-K16 tryptic peptide. (ii) ^{32}P labelling of a derivative of Mcm1 from which amino acids S2 to E17 had been deleted was repeatedly unsuccessful. (iii) The major isoforms of Mcm1-HA are phosphatase sensitive, indicating that most molecules are phosphorylated; the major isoforms of Mcm1(Δ2-17)HA are not phosphatase sensitive and thus not phosphorylated. The assignment of specific phosphorylation sites to S2 and T8 is based on the observations that mutations S2A and T8A result in changes in the set of Mcm1 isoforms. The S2A mutant Mcm1 protein migrates as a more basic set of isoforms than wild-type Mcm1 protein, consistent with the idea that most Mcm1 molecules are phosphorylated at this site. The T8A mutation (alone or in combination with an S2A mutation) causes loss of an acidic isoform relative to its parent form. The S2 site matches a potential casein kinase II site while T8 matches a potential casein kinase I site, but the identities of the actual kinases that phosphorylate these sites are unknown.

Many different types of Mcm1 molecules, most of which are different phosphoforms of the protein, are seen. At least eight isoforms of Mcm1-HA are separated in acidic IEF gels. Most of the Mcm1-HA molecules are phosphorylated, since they exhibit altered mobility when treated with phosphatase. The differences between different isoforms are likely due to the fact that they carry different numbers of phosphates, because the isoforms collapse to two primary species after phosphatase treatment. We infer that Mcm1 is phosphorylated at low efficiency at additional sites outside the N terminus because neither mutation nor deletion of the primary N-terminal phosphorylation sites fully eliminates the expected acidic isoforms or collapses the isoforms to the same two bands observed with full dephosphorylation with CIP. Thus, phosphorylated species of these mutants apparently exist.

Implications of multiple Mcm1 species for Mcm1's diverse functions. One of the most puzzling questions concerning Mcm1 protein has been how a single protein can participate in such a wide variety of important, regulated processes. One proposal has been that Mcm1 itself is not a regulatory protein but acts as a scaffold for binding other true regulators (see reference 84). However, this model could not account for the fact that strong Mcm1 binding sites work as promoter elements (33), an observation which suggests that Mcm1 alone activates transcription. Partial phosphorylation of Mcm1 at several different sites might create functionally distinct molecules. Since different isoforms exist and are regulated, we think it likely that these isoforms differ in their activity.

Specific phosphorylated forms of Mcm1 might work on specific subsets of the genes on which Mcm1 acts and thus be individually regulated. Individual isoforms may be recruited with specific cofactors to the set of genes that the cofactor binds. Alternatively, some phosphorylation sites may be modified only if Mcm1 is bound with a specific cofactor. Althoefer et al. (2) and Maher et al. (45) demonstrated that Mcm1 plays a key role in G_2-cell-cycle-regulated transcription. Mcm1 molecules involved in G_2-regulated transcription might be phosphorylated at a putative $p34^{CDC28}$ consensus site in Mcm1 in a cell cycle-regulated manner; this event could be required for cooperative binding with Sff or could be essential for transcription activation with Sff. This idea could explain how Mcm1 might act as a regulated participant in several mutually exclusive regulatory loops.

Alternatively, specific phosphorylated forms might not manifest gene selectivity but might have different effects on the transcription apparatus. Phosphorylation might mediate the interactions between Mcm1 and the general transcription apparatus, creating a situation in which general Mcm1 activity is regulated. Regulation of Mcm1 activity might have different consequences for different sets of genes depending upon the activities of the cofactors, but it would impose a general regulatory response on Mcm1.

Mcm1 is implicated in the response to salt stress. We suggest that Mcm1 protein is involved in an additional process in the yeast cell, the cellular response to high salt concentrations. This proposal is based on the following three observations. (i) Deletion of the amino-terminal amino acids 2 to 17 of Mcm1 or mutation of the primary phosphorylation site S2A results in reduced survival in response to high salt concentrations. (ii) Mutation of a MADS box conserved amino acid, T35, results in somewhat more rapid growth on high-salt medium, as well as suppression of the high-salt defect caused by deletion of amino acids 2 to 17. (iii) Growth in 1.2 M NaCl or KCl results in

induction of a new isoform(s) of Mcm1 protein. These results are interesting for several reasons. First, amino acids S2 to E17 are dispensable for essential as well as previously known functions of Mcm1 protein: their deletion caused only marginal effects on transcription activation of a reporter gene, on cell-type-specific transcription, and on minichromosome stability (13). Thus, the salt-sensitive phenotype of a Δ2-17 deletion mutant is most easily explained as a specific defect caused by failure to respond to a particular signal or to work with a single cofactor. While this finding extends the global nature of Mcm1 function, it also supports the idea that individual functions may be accomplished by distinct Mcm1 molecules. Second, it is surprising that mutation of an amino acid conserved in all known MADS box proteins (T35) confers no obvious growth defect but instead confers a growth advantage in specific high-salt conditions. Third, the observation that both increased and diminished growth on high-salt medium can be conferred by mutations in Mcm1 is plausibly interpreted as evidence that Mcm1 plays a central role in the response to high salt concentrations. Fourth, the induction of a new isoform elicited by growth in high-salt medium provides direct evidence that Mcm1 activity is regulated.

We do not think it likely that the salt-dependent phosphorylation site itself lies in the N terminus. The mutation at S2A confers salt sensitivity. Since S2 is nearly completely phosphorylated in the absence of salt, it is hard to envision that increased phosphorylation of this amino acid is required for salt resistance. The mutations at T8 and T10 do not confer salt sensitivity; thus, it is unlikely that phosphorylation of these amino acids is required for the response to salt. Furthermore, we observe salt-dependent induction of a new isoform in cells bearing *MCM1(Δ2-17)HA*, *MCM1S2A*, *MCM1T8A*, and *MCM1T10A* alleles (data not shown). Thus, the most plausible model is that the amino terminus (possibly phosphoserine at amino acid 2) is necessary but not sufficient for induction of the salt-response genes.

Two signal transduction systems that result in transcriptional regulation and might plausibly involve Mcm1 are important in the response to salt. First, salt shock results in induction of glycerol synthesis and *GPD1* gene expression via the HOG MAP kinase pathway and Sln1 protein (1, 44, 56, 65). Since mutations in *sln1* enhance Mcm1-dependent transcriptional activation from a strong Mcm1 binding site (93), it is reasonable that *mcm1(Δ2-17)* mutants might be defective in a response to Sln1 activation and that Mcm1 could participate in the HOG pathway-mediated *GPD1* expression. The induction of the Mcm1 isoform by both NaCl and KCl is consistent with effects mediated by the HOG pathway, but the required concentration is higher than that required for other HOG1-mediated effects. Second, upon salt shock, a Na^+ efflux system, encoded by the *ENA1* gene, is activated transcriptionally such that the efflux of Na^+, and of its analog Li^+, is enhanced (20, 26, 87). The observation that *mcm1(Δ2-17)* mutants are sensitive to NaCl and LiCl but not to KCl suggests but does not prove that these mutants are defective in the *ENA1*-dependent pathway. Induction of ENA1 transcription is affected both by calcineurin (51, 60) and by PPZ protein phosphatases (70). Calcineurin is a conserved Ca^{2+}/CaM-dependent protein phosphatase implicated in regulation of intracellular ion homeostasis and maintenance of cell integrity (reference 21 and references therein). Interestingly, we find that a high-affinity Mcm1 binding site present in our library of genomic Mcm1 binding sites (41) lies around 1 kb upstream of the *CNA2* gene, coding for one subunit of calcineurin (our unpublished observation). The PPZ phosphatases appear to play a role opposite to that of calcineurin in expression of the *ENA1* gene (70).

Comparison to SRF. The existence of multiple isoforms of Mcm1 could provide important clues to understanding a human protein, SRF, that is highly homologous to Mcm1 protein. In humans, SRF is essential for activation of immediate-early genes such as c-*fos* and c-*jun* (22, 83) when cells are stimulated by serum growth factors or mitogens. In addition, the ubiquitously expressed human beta-actin gene appears to be under the control of the serum response element (15), the binding site for SRF. The mechanism by which SRF responds to mitogenic signals is not completely understood. Information on the signal transduction systems that mediate growth signals and on the physical modifications of SRF and its cofactors have not yet converged. Transcriptional induction in response to Ca^{2+} and serum is mediated via the serum response element, the binding site for SRF and its cofactor(s) the ternary complex factor (53, 54, 83). The response to mitogenic stimuli involves at least two signal transduction pathways: a MAP kinase cascade, which exerts its effects via the SRF cofactor ternary complex factor (89), and a Rho family of GTPases, which appear to act via SRF (30). The amino-terminal portion of the SRF MADS box appears to be essential for the response to serum (29, 34), but it is unknown if posttranslational modification of this domain or recruitment of an additional factor is important for the Rho-mediated response to serum (30). On the other hand, SRF, like Mcm1, is phosphorylated in residues surrounding the MADS box by several protein kinases, casein kinase II, Ca^{2+}/CaM-dependent protein kinase II, and $pp90^{RSK}$ kinase (reviewed in reference 64). While phosphorylation by these kinases modulates the DNA-binding activity of SRF (47, 72), none of the modified residues seems to be important for serum response (55). Functionally distinct classes of SRF molecules may exist with a small number of molecules modified at a particular essential site. If only a small percentage of the SRF molecules are physically modified in the MADS box domain in response to the Rho GTPases, then this modification may be difficult to observe over the background of nearly fully phosphorylated sites.

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