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Transcription of α-Specific Genes in *Saccharomyces cerevisiae*: DNA Sequence Requirements for Activity of the Coregulator α1

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Transcription activation of α-specific genes in *Saccharomyces cerevisiae* is regulated by two proteins, MCM1 and α1, which bind to DNA sequences, called P'Q elements, found upstream of α-specific genes. Neither MCM1 nor α1 alone binds efficiently to P'Q elements. Together, however, they bind cooperatively in a manner that requires both the P' sequence, which is a weak binding site for MCM1, and the Q sequence, which has been postulated to be the binding site for α1. We analyzed a collection of point mutations in the P'Q element of the *STE3* gene to determine the importance of individual base pairs for α-specific gene transcription. Within the 10-bp conserved Q sequence, mutations at only three positions strongly affected transcription activation in vivo. These same mutations did not affect the weak binding to P'Q displayed by MCM1 alone. In vitro DNA binding assays showed a direct correlation between the ability of the mutant sequences to form ternary P'Q-MCM1-α1 complexes and the degree to which transcription was activated in vivo. Thus, the ability of α1 and MCM1 to bind cooperatively to P'Q elements is critical for activation of α-specific genes. In all natural α-specific genes the Q sequence is adjacent to the degenerate side of P'. To test the significance of this geometry, we created several novel juxtapositions of P, P', and Q sequences. When the Q sequence was opposite the degenerate side, the composite QP' element was inactive as a promoter element in vivo and unable to form stable ternary QP'-MCM1-α1 complexes in vitro. We also found that addition of a Q sequence to a strong MCM1 binding site allows the addition of α1 to the complex. This finding, together with the observation that Q-element point mutations affected ternary complex formation but not the weak binding of MCM1 alone, supports the idea that the Q sequence serves as a binding site for α1.

The **a** and α cell types of the yeast *Saccharomyces cerevisiae* express distinct sets of genes: **a** cells transcribe **a**-specific genes, and α cells transcribe α-specific genes. This differential transcription is achieved by a combinatorial strategy involving three proteins, MCM1, which is expressed in all cell types, and α1 and α2, which are expressed exclusively in α cells. At each gene set, unique regulatory complexes are formed by the binding of subsets of the three proteins to specific sites within the transcriptional control regions. Two complexes serve to activate transcription, and a third serves to repress transcription. The sum of their activities limits transcription of **a**- and α-specific genes to the appropriate cell type (for a review, see references 6, 16, and 45).

Cell-type-specific transcription of **a**-specific genes is achieved by the action of MCM1 and α2 (Fig. 1). The upstream control regions of **a**-specific genes contain nearly perfect versions of the palindromic MCM1-binding site, the P box, flanked by α2-binding sites. MCM1 binds to these P boxes and is essential for transcription activation of the gene set in **a** cells (Fig. 1) (2, 8, 17, 20, 21, 25, 32). In α cells, α2 and MCM1 bind cooperatively to the combined α2-P box-α2 site and repress transcription of **a**-specific genes (17, 22, 23, 35).

MCM1 acting with α1 brings about cell-type-specific transcription of α-specific genes (Fig. 1). The upstream region of α-specific genes contains versions of the P box, called P' [e.g., P(*STE3*) of reference 20], in which one-half of the palindrome is degenerate. A conserved 10-bp sequence, the Q box, is present immediately adjacent to the degenerate side of the P' box. In vivo, the 26-bp P'Q sequence confers α-specific expression to reporter genes, but neither P' nor Q alone confers any expression to reporter genes (1, 11, 18–20, 37). The requirements for promoter activity in vivo mirror the requirements for formation of MCM1-containing protein-DNA complexes in vitro (1, 2, 14, 19, 31, 32). MCM1 binds poorly to both P' and P'Q sequences (~30-fold less well than to perfectly palindromic P sequences [3]). Likewise, α1 alone does not bind detectably to these sequences. However, α1 and MCM1 together bind cooperatively to P'Q. These conclusions are based primarily on studies with a P'Q sequence from a particular α-specific gene, *STE3*, but similar conclusions have been reached for P'Q elements from *MFα1* [QP(*MFα1*B)] and from *MFα2* (1, 2, 14, 31, 32). Most strikingly, in vivo footprinting studies showed that MCM1 occupies QP(*MFα1*B) only in α cells when α1 is present (32).

The results described above imply that one role of α1 is to recruit MCM1 to bind to the control regions of α-specific genes and thereby bring about transcription activation. However, other findings suggest that α1 may have additional roles that contribute to activation of α-specific genes. First, α1 may induce a conformational change that makes MCM1 more proficient at transcription activation. This possibility was suggested by the observation that appreciable α1-independent binding of MCM1 to QP(*MFα1*A) is seen both in vitro and in vivo even though transcription from this element

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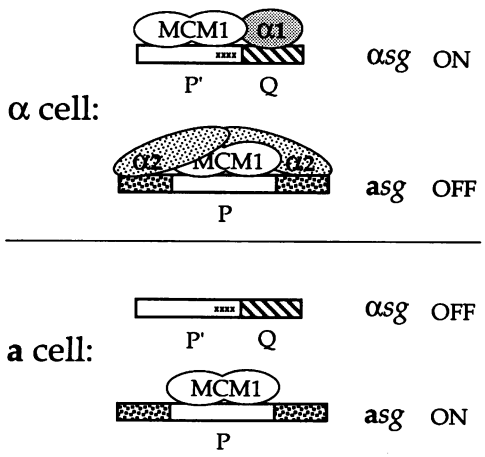

FIG. 1. Model for α- and a-specific gene regulation. Cell-type-specific transcription of α- and a-specific genes (*αsg* and *asg*) is regulated by three proteins—MCM1, which is expressed in all cell types, and α1 and α2, which are expressed exclusively in α cells from the *MAT*α locus. This regulation hinges upon the nature and sequence context of MCM1-binding sites, called P boxes, found in the upstream control regions of a- and α-specific genes. The control regions of α-specific genes contain composite sequences consisting of a degenerate P box (P') and an adjacent 10-bp sequence, the Q box. MCM1 binds to these degenerate P boxes very weakly, resulting in lack of activation of α-specific genes in a cells. In α cells, α1 and MCM1 together bind cooperatively to P'Q elements, leading to transcription activation. The more palindromic P boxes at a-specific genes bind MCM1 strongly and mediate transcription activation in a cells. These P boxes are flanked by α2-binding sites. In α cells MCM1 and α2 bind cooperatively to the composite element and repress transcription.

is strictly dependent on α1 (1, 2, 32). More directly, protease susceptibility assays indicate that MCM1 when bound alone at P'Q elements has a conformation different from that when bound with α1 (48). Second, α1 itself has the ability to activate transcription if it is delivered to DNA as a LexA-α1 hybrid protein binding to LexA operator sites (38). Third, α1 may recruit STE12, a protein required for pheromone-mediated transcription induction of α-specific genes (9, 10, 17). This possibility is suggested by recent studies which show that α1 can interact in solution with STE12 protein (49), although STE12 has not yet been detected as part of protein-DNA complexes at α-specific genes.

The studies summarized above point to the Q sequence as a crucial ingredient in bringing about α-specific transcription. However, the role of the Q sequence has been deduced solely by comparing the properties of two elements, P'Q and P'. To gain a more complete understanding of the molecular mechanisms by which Q elements mediate α-specific transcription, we have pursued two lines of investigation. First, random point mutagenesis of the Q element from a particular α-specific gene, *STE3*, was used to identify critical positions within the element. Second, the promoter and binding properties of novel combinations of P, P', and Q were evaluated. These studies indicate that P' and Q combine to make an active element if and only if Q is adjacent to the degenerate side of P'. Although a 10-bp consensus Q element was deduced by comparison of the control regions from α-specific genes (20), point mutations at only four of the positions lead to a reduction or loss of promoter activity. The promoter activity of the mutations correlates directly with their ability to form a ternary complex with α1 and MCM1 in vitro, supporting the idea that interdependent recruitment of α1 and MCM1 to bind to P'Q sequences is a critical event in transcription activation of α-specific genes.

MATERIALS AND METHODS

Strains and media. The yeast strains 246.1.1 (*MAT*α *leu2 ura3 his4-519 trp1 can1-101*) and EG123 (isogenic with 246.1.1, except *MAT***a**) were both provided by K. Tatchell via A. Mitchell. SY1378 (isogenic with 246.1.1, except *HIS4 his3Δ200::ura3 pep4::ura3*) was provided by B. Stevenson. In addition, DC5 (*MAT***a** *leu2-3 leu2-112 his3 gal2 can1*), which was provided by J. Strathern, served as a source of **a**-factor. *Escherichia coli* SB69 (15), SB115 (17), DH5αF' (Bethesda Research Laboratories), and CJ236 (26) have been described previously. Standard media and methods were used for propagating yeast and bacteria (33, 41).

Plasmids. Plasmids were constructed by standard methods (33). All oligomers were synthesized by the University of Oregon Biotechnology Laboratory with an Applied Biosystems DNA synthesizer model 380B (phosphoramidite chemistry). Plasmid pSL1418, which was used as a template for the site-directed mutagenesis of the *STE3* Q-box region, has the coding region of *HIS3* embedded within *STE3* sequences such that *HIS3* expression is under control of the *STE3* promoter. The *STE3-HIS3-STE3* construct, provided by B. Stevenson, was cloned as a 2,450-bp *Eco*RI fragment into the *Eco*RI site of vector pRS316, a *CEN/ARS URA3* yeast shuttle vector (42) oriented such that the *STE3* upstream region is on the *Kpn*I side of the polylinker. The detailed composition of the *STE3-HIS3-STE3* fragment is 5'-GAAT TCGAGCTCGCCC plus *STE3* coordinates 2 to 817 (GenBank accession number M12239) plus 5'-GGGGATCTC GACC plus *HIS3* coordinates 501 to 1383 (GenBank accession number X03245) plus *STE3* coordinates 2449 to ca. 3170 (terminating at a natural *Eco*RI site of *STE3*).

The *STE3-HIS3-lacZ* reporter plasmids (e.g., pSL2132 with wild-type Q) were constructed by subcloning 1,204-bp *Hpa*I (*STE3* coordinate 49) to *Bgl*II (*HIS3* coordinate 922) fragments from pSL1418 and Q-mutant variants of pSL1418 into the *Sma*I and *Bam*HI sites of pSL57 (20).

The *CYC1-lacZ* reporter plasmids with various combinations of synthetic P, P', and Q elements were constructed in several steps as follows. P and P'Q elements from previously constructed plasmids pSL1195 and pSL904 (17) were first subcloned as *Hin*dIII-*Bam*HI fragments, 162 and 171 bp, respectively, into the *Hin*dIII and *Bam*HI sites of Bluescript KS+ (Stratagene). Site-directed mutagenesis was used to convert P'Q into P' with oligomer D1820, into PQ with oligomer D192, and into QP'Q with oligomer D1605. Likewise, PQ was converted into QPQ with oligomer D1605, and QP'Q was converted into QP' with oligomer D1820. The sequences of the three oligomers used for the site-directed mutagenesis were as follows: D192, 5'-CCTAATTAGGAAA ACAATGAC; D1605, 5'-CCTCGACTGTCATTGTTTTCCT AATTAG; and D1820, 5'-CTAATTAGTGTCGTCGAGGG. Newly constructed P, P', and Q combinations were confirmed by DNA sequencing by the dideoxy method (34). To make the corresponding reporter plasmids, *Hin*dIII-*Bam*HI fragments containing the synthetic promoter elements were subcloned into the *Hin*dIII and *Bam*HI sites of plasmid pSL745 (20). The net result is equivalent to placing the synthetic promoter elements into the upstream *Xho*I site of pSL745 (see Table 2 for sequence details).

The plasmid used to express the maltose-binding protein (MBP)–α1 fusion, pSL2187, was constructed by ligating a

polymerase chain reaction (PCR)-generated, blunt-end DNA fragment containing the entire α1 coding sequence plus 88 nucleotides downstream of the stop codon into *Xmn*I-cut pMALc2 (New England Biolabs). The PCR-generated region in pSL2187 was sequenced to ensure that no mutations had been created during the PCR amplification. Cleavage of the resulting MBP-α1 fusion protein by factor Xa produces full-length α1 with no extra residues at the N terminus.

Isolation and His$^-$ phenotype of Q mutations. Mutations in the Q region of *STE3*'s P'Q element were obtained by site-directed mutagenesis of plasmid pSL1418 with oligomer D1147: 5'-CCTAATTAGtgtcacaatgacagagAAAATTGTACC, which corresponds to *STE3* coordinates 603 to 568. Letters in lowercase indicate 16 positions doped with errors during synthesis at a frequency of 6%, e.g., t indicates 94% T, 2% A, 2% G, and 2% C. Approximately 10,000 *E. coli* colonies were obtained by transformation with the mutagenized DNA, and Q mutations were isolated by two separate protocols. In the first protocol, random *E. coli* clones from the mutagenesis were sequenced and then characterized phenotypically. In the second protocol, a heterogeneous preparation of double-stranded DNA was prepared from the ~10,000 *E. coli* colonies and transformed into yeast strain SY1378. The transformants, isolated on minimal medium lacking uracil (SD-Ura), were tested for growth on minimal medium lacking uracil and histidine (SD-Ura-His). As described above, plasmid pSL1418 carries a *STE3-HIS3* fusion which allows growth of a *MAT*α yeast strain deleted for *his3* (e.g., SY1378) on medium lacking histidine. Plasmid DNA was isolated from strains displaying a His$^-$ phenotype and passaged through *E. coli* DH5αF' for cloning and sequencing.

A semiquantitative assessment of the promoter strengths of Q mutants was made by testing transformants of SY1378 for resistance to 3-amino-1,2,4-triazole (ATZ), which is a competitive inhibitor of the *HIS3* gene product (24, 46). Transformants growing on SD-Ura were diluted in SD, and droplets, each containing ~1,000 cells, were spotted on a series of plates: SD-Ura (control), SD-Ura-His, and SD-Ura-His plus 2, 5, 10, and 20 mM ATZ. The test spots were incubated at 30°C for 48 h and scored qualitatively for growth. Routinely, determinations were made on three separate transformants of each mutant.

β-Galactosidase assays. Yeast strains with *lacZ* reporter constructs were grown, prepared, and assayed for β-galactosidase activity as described previously (20). In experiments to measure response to **a**-factor, a culture filtrate (0.2-μm-pore-size Nalgene filter unit) of strain DC5 grown to saturation in rich medium at 30°C was used as the source of **a**-factor. For pheromone treatment, exponentially growing cultures were mixed 1:1 with this filtrate, while uninduced cells were mixed 1:1 with fresh medium; both were incubated for an additional 2.5 h at 30°C.

DNA binding assays. DNA fragments containing the Q mutations were prepared by the PCR from pSL1418 and Q-mutant derivatives of pSL1418 with primers D2061 (5'-CCGTCGACTCTGGGTATGGGGTGC [the last 18 bases correspond to *STE3* coordinates 530 to 547]) and D1176B (5'-CCAGATCTCCGCCATTATTCCTTC [the last 17 bases correspond to *STE3* coordinates 647 to 631]). The 125-bp fragments were purified by agarose gel electrophoresis, cleaved with *Sal*I and *Bgl*II, and labelled by filling in with the Klenow fragment of DNA polymerase and [α-^{32}P]dATP (6,000 Ci/mmol; New England Nuclear). DNA fragments containing wild-type P'Q and the different P, P', and Q combinations were isolated from the Bluescript KS+ plasmids containing these sequences (see above). *Hin*dIII-*Sst*I fragments (ranging in length from 148 to 168 bp) were purified by agarose gel electrophoresis and ^{32}P labelled as described above.

The MBP-α1 protein used in the band shift experiments was expressed in TB1 cells with pSL2187, described above, and partially purified with an affinity column for MBP as follows. Freshly transformed cells were grown in 1 liter of LB [LB is 20 mM Tris (pH 7.4), 150 mM NaCl, 10 mM EDTA, 10 mM ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid [EGTA], 10 mM β-mercaptoethanol] plus Amp plus 0.2% glucose at 30°C to an optical density at 600 nm of ~0.5, 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and growth was continued for 2 h. Cells were harvested, suspended in 50 ml of LB150 (LB with 150 mM NaCl) plus protease inhibitors and frozen at −70°C (protease inhibitors used were phenylmethylsulfonyl fluoride, [70 μg/ml], pepstatin [1.5 μg/ml], and leupeptin [7 μg/ml]). After thawing, lysozyme was added to 0.7 mg/ml, and the cell suspension was incubated on ice for 20 min and then frozen at −70°C. The lysed cells were then thawed, NaCl was added to 500 mM, and cellular debris was removed by centrifugation. The extract was then diluted approximately fivefold with LB, the NaCl concentration was adjusted to 200 mM, and glycerol was added to 20% before loading on an Amylose (New England Biolabs) column equilibrated in CB (CB is 20 mM Tris [pH 7.4], 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, and 20% glycerol). MBP-α1 was eluted from the affinity column with CB plus 10 mM maltose, and peak fractions, as monitored by determining the A_{280}, were pooled and concentrated with a Centricon 30 device (Amicon) to ~0.5 mg/ml, estimated with the Bio-Rad Protein Assay Kit. This preparation was ~50% MBP-α1 as judged by Coomassie blue staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was used in band shift reactions at a final concentration of ~10 μg/ml. For cleavage of α1 from the fusion protein, 1 μg of factor Xa (New England Biolabs) was added per 50 μg of partially purified MBP-α1 and incubated at room temperature for 90 min. Phenylmethylsulfonyl fluoride (10 ng/μl) was then added to stop the cleavage reaction.

Crude protein extracts containing MCM1(1–98), a truncated version of MCM1, were prepared as described elsewhere (4) from strain SY2531, which is deleted for the chromosomal copy of *MCM1* and bears a *CEN/ARS TRP1* plasmid expressing MCM1(1–98) from the *MCM1* promoter. Band shift reaction mixes were incubated for 20 min at room temperature in a final volume of 15 to 20 μl containing 20 mM $NaPO_4$ (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 120 mM NaCl, 5 μg of crude yeast extract, 60 ng of double-stranded poly(dI-dC) (Pharmacia) per μg of extract, and 100 pM labeled DNA fragment. Protein-DNA complexes were resolved on 4% polyacrylamide–bisacrylamide (38:1.25) containing 10% glycerol in 1× TGE as described previously (4). For quantitation, regions of the gels containing free DNA and protein-DNA complexes were excised from the gel and the radioactivity present at each region was measured by scintillation counting.

RESULTS

Mutagenesis of the *STE3* Q-box region. Four genes with α-specific transcription have been identified in *S. cerevisiae*: *STE3*, encoding the receptor for the pheromone **a**-factor; *MFα1* and *MFα2*, both encoding the pheromone α-factor;

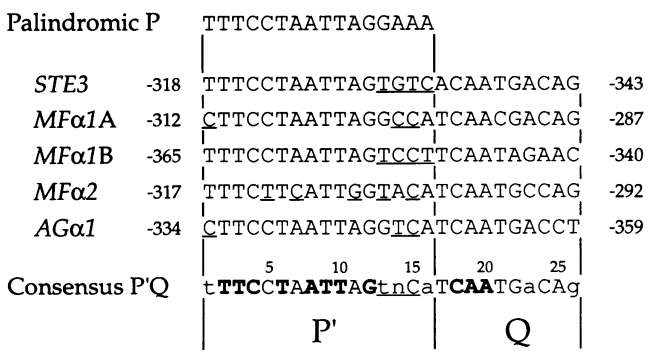

FIG. 2. The P'Q regulatory elements of α-specific genes. The P'Q elements of four α-specific genes—*STE3* (18, 20), *MFα1* (11, 18), *MFα2* (43, 44), and *AGα1* (27)—are aligned for sequence comparison. *MFα1* has two such elements, designated A and B. Coordinates are given relative to the initiation codons. The perfectly palindromic P sequence is shown above, and bases in the P' sequences differing from P are underlined. A consensus P'Q sequence is shown below with bases found in all five examples of P'Q in boldface uppercase type, bases found in four of the five in normal uppercase type, and bases found in three of the five in lowercase type. The base represented by "n" has no consensus. The positions in the consensus are numbered according to the convention used in this paper. The G at position 23 in the P'Q of *MFα1*B is C in reference 18.

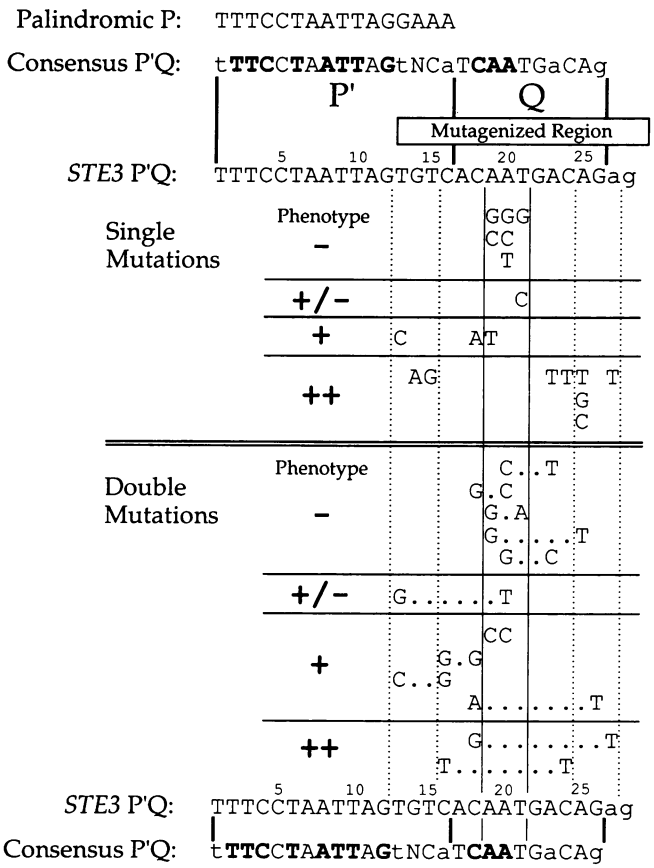

FIG. 3. Summary of Q mutations and His⁻ phenotypes. The region of the P'Q sequence of *STE3* mutagenized by site-directed mutagenesis is indicated with a box. The palindromic P and consensus P'Q sequences are included for reference. Single point mutations and their respective phenotypes are presented in the upper portion. Several separate, single mutations with comparable phenotypes are presented on a single line. Double point mutations are listed in the lower portion. Each double mutation is presented on a separate line, and dots are used to emphasize that there are two changes. Phenotypes of the Q mutants in the context of the *STE3-HIS3* reporter construct were determined by a growth assay on minimal agar medium lacking histidine (SD-Ura-His) and containing the inhibitor ATZ at a range of concentrations from 0 to 20 mM. ++, substantial growth at 20 mM; +, some growth at 5 mM and little or no growth at 10 mM; +/−, some growth at 2 mM and little or no growth at 5 mM; −, slow growth without inhibitor and no growth at 2 mM.

and *AGα1*, encoding the α-specific agglutinin. Deletion analysis and DNA sequence comparisons revealed an element, termed the P'Q box, common to these genes that is necessary and sufficient for the observed α-specific expression (20). *STE3*, *MFα2*, and *AGα1* each have a single P'Q element, while *MFα1* has two. The five P'Q examples and the derived consensus sequence are illustrated in Fig. 2. As discussed in the introduction, the 16-bp P'-box part of the control element is thought to be an imperfect binding site for the activator MCM1. The degenerate MCM1-binding site is designated here P' to distinguish it from a functional MCM1-binding site, P. In order to define further the role of the Q region of the P'Q element and to determine the essential as well as the unessential base pairs, we created variants of the *STE3* Q box by site-directed mutagenesis.

The mutagenesis was performed on a fusion construct in which the *HIS3* gene was placed under control of the *STE3* promoter on a single-copy *CEN/ARS* plasmid. This starting plasmid (pSL1418) confers an α-specific His⁺ phenotype to *his3Δ* cells and therefore allowed the effects of the Q mutations to be monitored easily. For the in vitro mutagenesis, an oligomer-primer that was lightly doped with nucleotide changes for 16 bases in the vicinity of the Q box was synthesized (see Fig. 3 and Materials and Methods). Mutant plasmids were isolated from the pool of mutagenized DNA by two protocols. In the first procedure, the DNA was transformed into *E. coli* and plasmids from isolated colonies were sequenced. Plasmids from clones with sequence changes in the Q region were then transformed into yeast cells for phenotypic characterization. In the second procedure, the mutagenized pool of DNA was first amplified by passage through *E. coli* and transformed directly into the yeast. Plasmid DNA from yeast colonies that showed a His⁻ phenotype was then isolated and sequenced. The two methods were complementary. More than half of the plasmids sequenced by the first method had no mutations, but the remainder had Q mutations with a full spectrum of phenotypes, including some with no discernible phenotype. The second method efficiently yielded Q mutations, primarily ones with strong phenotypes. The combined collection of Q mutations is presented in Fig. 3.

Promoter activity of the Q mutants. The promoter activity of the Q mutants was characterized by two assays. First, the original *STE3-HIS3* fusions were evaluated for their ability to restore growth to a *MATα his3Δ* strain on minimal medium lacking histidine. This assay was made semiquantitative by the use of the inhibitor ATZ. Mutants that could grow on plates lacking histidine were challenged with increasing concentrations of ATZ and compared with a wild-type control. The results of these assays are included in Fig. 3. The single-base-pair changes in Q that cause a strong His⁻ phenotype are all localized to coordinates 19 to 21, AAT, of the P'Q element. These changes were isolated multiple times, mostly by the second isolation method discussed

above. The 19G, 19C, 20G, 20C, 20T, and 21G mutations were recovered in 3, 2, 5, 2, 4, and 10 instances, respectively. Other mutations—21C (one isolate), 19T (two isolates), 13C (one isolate), and 18A (two isolates)—caused less-severe phenotypes but can easily be distinguished from the wild type by ATZ sensitivity. The phenotypes of the remainder of the single-base-pair changes (each with a single isolate, except for 23T with two) were similar to that of the wild type. Although we have not isolated all possible single-base-pair changes in the mutagenized region, it is probable, in view of the repeated isolation of some mutations, that most if not all of the single mutations that cause a strong His^- phenotype have been isolated.

As shown in Fig. 3, several double mutations were isolated from the mutagenesis and analyzed for their ability to promote expression of the *HIS3* reporter. Except for 19C-20C (see below), the phenotypes of the double mutations were consistent with those of the single mutations in that each double mutation that resulted in a strong His^- phenotype had a mutation within bases 19 and 21.

One mutation that conferred a strong His^- phenotype fell outside the bounds of coordinates 19 and 21 (data not shown; see Table 1, below). This mutation, 14GG, has two G's in place of one at position 14 and therefore separates P′ and Q by 1 bp. Thus, the implication is that the spacing between the P′ and Q sequence elements is crucial for promoter activity.

A more quantitative assessment of promoter strength was made by converting many of the *STE3-HIS3* fusions into *STE3-HIS3-lacZ* fusions and performing β-galactosidase assays on strains bearing the new constructs. As shown in Table 1, this approach yielded results that were fully consistent with the His phenotypes and allowed a finer discrimination of differences. Two minor conclusions also emerged from the data in Table 1. First, with the exception of the double mutation 19C-20C, none of the mutations conferred activity in an **a** cell background. The double mutation 19C-20C exhibited a moderate level of β-galactosidase activity in both α and **a** cells, implying that the mutation created a new, non-cell-type-specific promoter element. This implication is supported by the observation that a novel protein-DNA complex is formed on 19C-20C DNA (see below). Second, two mutations in the degenerate side of the P′ element (Fig. 3 and Table 1) had modest effects on expression. Mutation 14A, which brings the MCM1-binding site closer to the palindromic P sequence, boosted expression of the reporter and bound MCM1 better than the wild-type P′Q sequence in band shift assays (data not shown). Mutation 13C, on the other hand, reduced expression of the reporter and bound MCM1 approximately twofold less well than the wild type in band shift assays (data not shown; note that none of the naturally occurring P′Q elements have C at position 13 [Fig. 2]).

The 26-bp P′Q sequence confers a second mode of regulation to α-specific genes: increased transcription in response to pheromone (17, 20). To determine whether any of the Q mutations allowed a separation of these two modes of expression, β-galactosidase activities were measured before and after pheromone treatment. No mutation caused a loss of inducibility and still conferred the wild-type basal signal, nor were there any that caused a loss of the basal level while retaining the full inducibility (Table 1). Several of the mutants that displayed a reduced basal level of expression had higher levels of induction than the wild type. For example, 21C increased expression more than 30-fold when growth was in the presence of pheromone, whereas the wild type increased expression less than 6-fold. These differences may be somewhat artifactual, however, as the induced level of expression in the wild type may have reached an upper limit for expression.

TABLE 1. Promoter and DNA binding activity of Q mutants

Mutation[a]	His phenotype[b]	β-Galactosidase activity[c] of:			Binding activity[d] (%)
		α cells	α cells + aF	**a** cells	
None (wild type)	++	730	4,500	1	100
13C	+	66	1,500	1	62
14A	++	1,400	4,800	2	108
15G	++	720	4,100	1	85
18G-27T	++	330	2,900	1	54
18A	+	110	1,900	1	15
19T	+	53	1,500	1	19
19C	−	2	59	1	≤2
19G	−	1	3	1	≤2
20G	−	1	2	1	≤2
20C	−	1	2	1	≤2
20T	−	1	5	1	≤2
21G	−	2	53	1	≤2
21C	+/−	31	1,000	1	12
23T	++	240	2,700	1	46
24T	++	470	3,200	1	73
25T	++	730	4,100	1	ND[e]
25G	++	1,100	4,800	1	ND
25C	++	1,500	5,800	ND	ND
27T	++	730	4,800	ND	ND
19C-20C	+	26	70	25	≤2
14GG	−	6	340	ND	4
No P′Q	−	≤0.1	≤0.1	≤0.1	≤2

[a] The Q mutations are identified according to the convention established in Fig. 3. The double mutation 18G-27T is included among the single mutations because the single change 27T has little or no effect.

[b] His phenotype values are repeated from Fig. 3. Plasmid pRS316 (42), lacking *STE3-HIS3*, served as a negative control (no P′Q).

[c] The Q mutations in the *STE3-HIS3-lacZ* context of plasmid pSL2132 were transformed into isogenic *MAT***a** and *MAT*α strains (246.1.1 and EG123, respectively), and transformants were assayed for β-galactosidase as described in Materials and Methods. The α cells were grown in either the absence or presence of pheromone **a**-factor (**a**F). Activities are reported in modified Miller units (20, 29), and each is the mean determination of three independent transformants. The assay of wild-type Q in α cells was performed six times (three transformants each assayed twice), and the calculated standard deviation of 16% of the mean value typifies the variability experienced in these assays. Cells transformed with pSL57, which lacks all *STE3* and *HIS3* sequences, served as a negative control (no P′Q).

[d] The relative ability of P′Q fragments bearing Q mutations to form complexes with MCM1 and α1 was determined by band shift assays as described in the legend to Fig. 4 and Materials and Methods. The amount of P′Q-MCM1-α1 complex formed is expressed as a percentage of that formed by the wild-type P′Q fragment, designated 100%, and is the mean of at least two independent determinations. The fragment used as a negative control (no P′Q) has a 14-bp deletion that eliminates most of the P′ sequence.

[e] ND, values not determined.

DNA binding of MCM1 and α1: stoichiometry. MCM1 and α1 together bind tightly to P′Q-containing DNA fragments in vitro (1, 2, 14, 19, 31, 47). Although MCM1 exists in extracts as a stable dimer and binds to the palindromic P sequence as a dimer (1, 4, 19, 31), the possibility that MCM1 binds as a heterodimer with α1 to P′Q sequences existed. Thus, the DNA-binding specificity of MCM1 could change in a manner analogous to the conversion of the yeast α2 repressor from a repressor of **a**-specific genes in which it binds DNA as a homodimer to a repressor of haploid-specific genes in which it binds as a heterodimer with **a**1 (7, 12). To determine how many MCM1 molecules are present in the P′Q-MCM1-α1 complexes observed in band shift assays, we used extracts made from cells that express both full-length MCM1 and a truncated version, MCM1(1–98) (4). These extracts formed

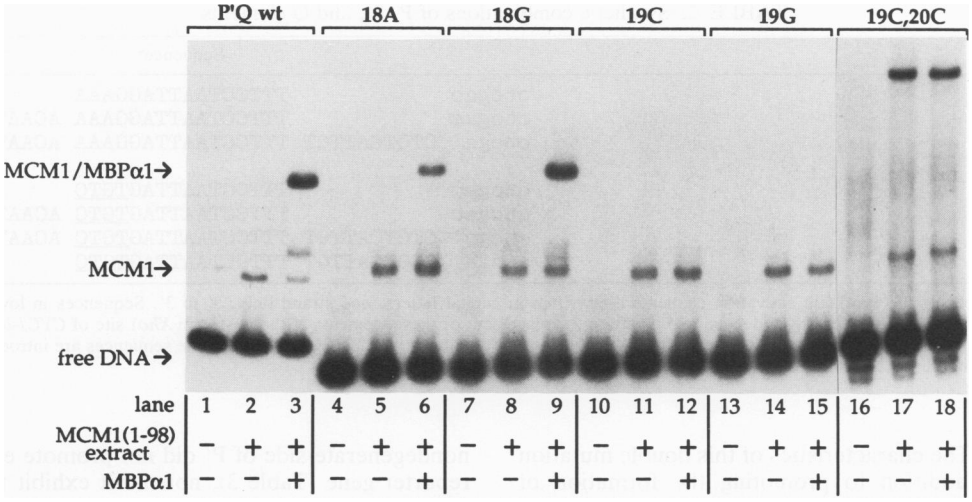

FIG. 4. DNA binding of MCM1 and α1 to Q-box mutants. Band shift assays were performed as described in Materials and Methods using crude yeast extracts containing MCM1(1–98) and partially purified MBP-α1. Radiolabelled DNA fragments containing the wild-type *STE3* P'Q sequence and representative Q mutations were incubated with no extract, MCM1 extract alone, and MCM1 extract plus MBP-α1 as indicated. P'Q-MCM1 and P'Q–MCM1–MBP-α1 complexes, indicated by arrows, were separated on a polyacrylamide gel in 1× TGE. Note that the DNA fragment containing wild-type P'Q is 158 bp long with the P'Q site near the end of the fragment while DNA fragments containing the mutant P'Q elements are 125 bp long with the P'Q sites in the middle of the fragment. The protein-DNA complexes formed on the mutant fragments migrate more slowly than expected on the basis of their size, suggesting that the DNA in the complexes may be bent in some manner. Other experiments showed that complexes formed on a similar fragment containing wild-type P'Q also migrated slowly (data not shown), demonstrating that the slow mobility is not caused by the Q mutations.

three complexes on P'Q DNA, corresponding to α1 bound with both types of homodimers and with the heterodimer (data not shown). This result is consistent with previous observations (32) and suggests that MCM1 remains a dimer when it binds with α1 to P'Q sequences.

To examine the stoichiometry of α1 in the DNA-protein complexes, we used two different forms of α1—an MBP-α1 fusion protein purified from *E. coli* and bona fide α1 prepared by cleaving MBP-α1 at a factor Xa site that separates the two moieties of the fusion protein. We first compared the properties of α1 and MBP-α1. These comparisons, which included measuring the half times for association and dissociation of the P'Q–MCM1–MBP-α1 and P'Q-MCM1-α1 complexes (data not shown), revealed no important differences. Having two versions of α1 protein provided us with the opportunity to test whether the P'Q-MCM1-α1 complexes observed in band shift assays contain one or more molecules of α1. When both versions of α1 were included in the same band shift reaction, only two α1-containing complexes were seen (data not shown). This result suggests either that a single molecule of α1 is present in the complexes or that α1 forms stable multimers that do not readily dissociate and reassociate in solution.

DNA binding of MCM1 and α1: Q mutations. We tested the collection of Q mutants for their ability to bind MCM1 and α1 using MCM1(1–98) prepared as a crude extract from yeast cells that are deleted for the chromosomal copy of *MCM1* and bear a single-copy plasmid with the *MCM1*(1–98) construct (4). One advantage of using MCM1(1–98) is that this truncated version allows detection of a small amount of binding of MCM1 alone to P'Q sequences that is not seen in band shift assays with full-length MCM1. Thus, we were able to quantitate the ability of the P'Q-box mutants to bind MCM1 alone as well as their ability to bind both α1 and MCM1. We used the MBP-α1 fusion protein in these assays because it greatly accentuated the mobility differences between P'Q-MCM1 and P'Q-MCM1-α1 complexes and therefore facilitated the analysis. As noted above, the fusion protein is similar to α1 in its DNA binding properties.

The results from band shift experiments using DNA fragments with representative Q mutations are presented in Fig. 4. In all cases, a faint band representing the P'Q-MCM1 complex was seen with the MCM1(1–98) extract (Fig. 4 and data not shown), demonstrating that none of the Q mutations has an observable defect in ability to bind MCM1 alone. With both MCM1 and α1 present, a new band representing the P'Q-MCM1-α1 complex was seen, but only for those Q mutations that did not result in a loss of transcriptional activity (Table 1). Quantitation of band shift experiments with these mutations (and others not shown in Fig. 4), revealed that the degree to which the ternary complex was observed correlated strongly with the in vivo transcriptional activity of the Q mutation (Table 1).

Some of the double mutants were also tested for their ability to bind MCM1 and α1 in band shift assays. The DNA fragment with the 14GG change, which inserted 1 bp between the P' and Q elements, showed a marked decrease in ability to form P'Q-MCM1-α1 complexes, in concert with its decreased ability to promote expression of the reporter genes (Table 1). The characteristics of this mutant and the precise P' and Q spacing observed in the natural P'Q elements (Fig. 2) suggest that the relative positioning of MCM1 and α1 is critically important for the ability of MCM1 and α1 to bind cooperatively to these sequences. When the DNA fragment with the 19C-20C changes, which conferred nonspecific gene expression, was used in band shift assays, a new DNA-protein complex that was not observed with any of the other P'Q fragments formed (Fig. 4, lanes 16 to 18). Formation of this complex was not dependent upon inclusion of α1 in the band shift reaction, nor did it seem to include MCM1, as its mobility did not change when different-size versions of MCM1 were used (data not shown). The observation of this novel complex supports the idea that the 19C-20C double mutation creates a new promoter element as

TABLE 2. Synthetic combinations of P, P', and Q elements

Name	Sequence[a]
P	ctcgac TTTCCTAATTAGGAAA gtcgag
PQ	ctcgac TTTCCTAATTAGGAAA ACAATGACAG tcgag
QPQ	ctcga CTGTCATTGT TTTCCTAATTAGGAAA ACAATGACAG tcgag
P'	ctcgac TTTCCTAATTAGTGTC gtcgag
P'Q	ctcgac TTTCCTAATTAGTGTC ACAATGACAG tcgag
QP'Q	ctcga CTGTCATTGT TTTCCTAATTAGTGTC ACAATGACAG tcgag
QP'	ctcga CTGTCATTGT TTTCCTAATTAGTGTC gtcgag

[a] The DNA sequences of the synthetic activation elements are written in capital letters, one strand only, 5' to 3'. Sequences in lowercase are adjacent sequences, either *Xho*I-*Sal*I or *Sal*I-*Xho*I hybrid sites, and illustrate the placement of these elements at the upstream *Xho*I site of *CYC1-lacZ* reporter plasmid pSL745. The left side of each sequence is proximal to the TATA region and ATG initiator of *CYC1-lacZ*. Gaps in the sequences are introduced in the table for alignment purposes. Underlined bases are those in the P' elements that differ from the P element.

discussed above. The characteristics of this double mutation suggest that, in addition to promoting the formation of P'Q-MCM1-α1 complexes, the conserved sequence of the Q box may be important for excluding binding of other regulatory proteins which could perturb proper α-specific gene expression.

Promoter and DNA binding activity of rearranged P and Q boxes. All α-specific control elements contain a single Q sequence adjacent to the degenerate side of the P' sequence. As noted in the introduction, this geometry is thought to allow interactions between α1 and MCM1 to compensate for their deficiencies in binding to particular parts of the total control element, α1 to Q and MCM1 to P'. To investigate the significance of this geometry, we synthesized several new combinations of P, P', and Q sequences (Table 2) and tested their ability to bind MCM1 and α1 in vitro and to drive reporter gene expression in vivo. First, we examined the properties of QP and QP'. Previous studies established that perfectly symmetric versions of the P sequence [referred to as P(PAL)] conferred non-cell-type-specific expression to reporter genes (19, 20) (Table 3). MCM1 bound with high affinity to the symmetric sequence, but a ternary complex including α1 was not observed (2, 20). As shown in Table 3 and Fig. 5, addition of a Q sequence to the symmetric P box caused a modest increase in expression of the reporter gene and allowed formation of PQ-MCM1-α1 ternary complex. In contrast, a QP' construct in which Q is adjacent to the nondegenerate side of P' did not promote expression of the reporter gene (Table 3), nor did it exhibit ternary complex formation (Fig. 5).

We also examined the properties of P and P' elements flanked on both sides by Q. QPQ and QP'Q both activated reporter gene expression (Table 3), although the level of expression was lower than that seen for P'Q or PQ. This reduction may be another manifestation of the observation that a Q box can sometimes interfere with P-box-mediated expression (5, 19, 20). Another possibility is that the added Q boxes affect the spacing between the P box and downstream promoter elements (e.g., the TATA box) in a manner that reduces activity. Stable DNA-MCM1-α1 complexes were readily detected with both QPQ and QP'Q (Fig. 5). The predominant complexes had the same mobility as those formed with PQ or P'Q, implying that only one α1 molecule was present in the complexes. However, with both fragments we also observed a small amount of α1-dependent complex with mobility consistent with that expected for complexes containing two molecules of α1 with MCM1 (Fig. 5). Additional band shift experiments showed that the relatively small amount of this complex did not increase when higher concentrations of MBP-α1 (or bona fide α1) were used or when full-length MCM1 was used in place of MCM1(1–98) (data not shown).

TABLE 3. Promoter activities of synthetic combinations of P, P', and Q elements

Promoter[a]	β-Galactosidase activity[b] of:		
	α cells	α cells + aF	a cells
None	8	8	10
P	860	1,500	790
PQ	1,200	1,800	600
QPQ	980	1,600	380
P'	10	13	10
P'Q	170	830	10
QP'Q	35	250	8
QP'	7	9	9

[a] The promoter elements are the sequences described in Table 2 cloned into reporter plasmid pSL745.

[b] The plasmids were transformed into isogenic *MAT*α and *MAT*a strains (246.1.1 and EG123, respectively), and transformants were assayed for β-galactosidase activity as described in Table 1, footnote *c*, and Materials and Methods. aF, a-factor.

DISCUSSION

In this study we have investigated in detail the sequence requirements for α-specific transcription by manipulating the Q sequence, a conserved promoter element from α-specific genes. We sought to determine the importance of individual base pairs within the Q-box sequence by point mutagenesis and to further define the role of the Q box in α-specific regulation by analyzing several novel juxtapositions of P-, P'-, and Q-box sequences.

Two strategies were used to identify point mutations within the Q sequence. In one strategy we used a screen to identify mutants that failed to express an α-specific reporter gene. In the second, we identified random point mutations in the Q sequence and then assessed the phenotypic consequences of the mutations. Together, these strategies resulted in the identification of mutations in most positions of the 10-bp Q sequence. Given the length of the conserved Q sequence, it was surprising that mutations that caused strong phenotypes were confined to three positions: 19A, 20A, and 21T (Fig. 3). Most mutations at these three positions caused a severe loss in activity, although the changes 19T and 21C had less effect than the others, and the change 21A was not

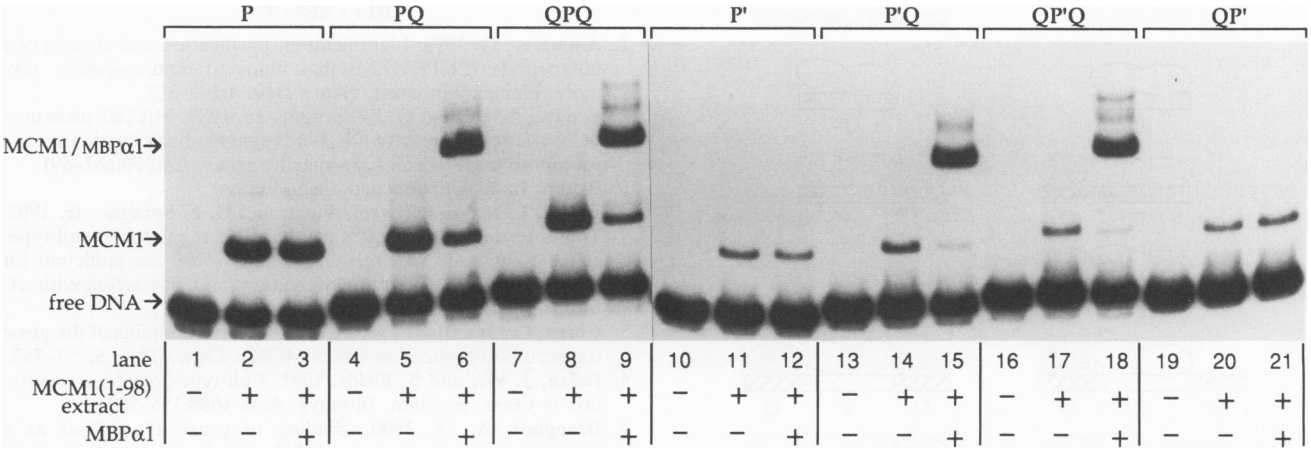

FIG. 5. DNA binding of MCM1 and α1 to synthetic P, P', and Q combinations. Band shift assays were performed as described in the legend to Fig. 4 with DNA fragments containing the synthetic P, P', and Q combinations as indicated. Free DNA, complexes containing MCM1, and complexes containing both MCM1 and one molecule of MBP-α1 are indicated by arrows.

isolated. Although the base at coordinate 18 is C in all five of the naturally occurring P'Q sequences (Fig. 2), the mutations isolated at this position (18A and 18G, as part of double mutations) had only modest effects. The double point mutations had phenotypes consistent with those of the single mutations and again emphasized the importance of residues 19, 20, and 21 (Fig. 3). Ammerer has shown that bp 22 to 26 can be deleted without loss in α-specific expression (1), and consistent with that finding, mutations that we isolated in this zone had little or no effect on activity of the P'Q element.

Previous studies have shown that deletion of the Q element from P'Q sequences abolished both transcription activation in vivo and ability to form DNA-MCM1-α1 complexes in vitro. With the Q-box point mutations we found a direct correlation between the degree to which transcription was activated in vivo and the ability to form P'Q-MCM1-α1 complexes in band shift assays (Table 1). For example, Q mutations that confer intermediate levels of reporter gene expression in vivo have intermediate ability to form DNA-MCM1-α1 complexes in vitro. Coupled with the in vivo footprinting results of Primig et al. (32), these results indicate that at some P'Q sequences, e.g., QP(*STE3*) and QP (*MFα1*B), an essential function of α1 is to promote the formation of stable protein-DNA complexes and thereby recruit a known transcription activator, MCM1. Within these P'Q-MCM1-α1 complexes, α1 may contribute three other functions important for transcription. α1 may induce a conformational change in MCM1 that converts it to a more effective transcription activator (48). Independent of MCM1, α1 is capable of promoting transcription activation (38). Finally, α1 may recruit STE12 (49). At other P'Q sequences, e.g., QP(*MFα1*A), appreciable binding of MCM1 is observed in the absence of α1 both in vitro (1, 2) and in vivo (32). Nonetheless, little or no transcription is observed unless α1 is present. Thus, at these P'Q sequences, the essential function of α1 is likely to be to convert MCM1 to an active form and/or to act as an independent transcription activator.

Band shift analysis of the Q mutants showed that the small amount of P'Q-MCM1 complex formed in the absence of α1 is not affected by the base pair changes in the Q region (Fig. 4; quantitative data not presented). Thus, the Q mutations that cause a severe phenotype in vivo and are defective in forming the ternary complex in vitro still display the same low level of MCM1 binding displayed by wild-type P'Q. This result suggests that the deficiency of the Q-box mutants in forming P'Q-MCM1-α1 complexes is in α1 binding, not in MCM1 binding. Although other models are possible, the simplest interpretation of these findings is that the role of the Q box is to recruit α1 to these sites, perhaps by providing direct contacts with α1 that contribute to the formation of stable P'Q-MCM1-α1 complexes. Sequence-specific binding of α1 to Q sequences has yet to be observed; however, α1 does seem to have some nonspecific DNA binding affinity (3, 14), consistent with the idea that it may contact DNA. The finding that the Q mutations which severely affected formation of DNA-MCM1-α1 complexes were all located at positions 19 to 21 (AAT) of the P'Q sequence suggests that these 3 bases may be important for making sequence-specific contacts with α1. Another possibility is that this sequence is important for allowing a particular structure of the DNA and/or proteins in the P'Q-MCM1-α1 complex necessary for stable complex formation. Given that TA dinucleotides have been implicated as a preferred kink site in DNA (28), an intriguing possibility is that residues 20A and 21T are important for bending of the DNA in this region.

Further evidence for the idea that the Q element serves as a binding site for α1 comes from experiments in which the position and number of Q sequences were manipulated. DNA binding studies with the synthetic combinations of P and Q sequences showed that addition of a single Q box to a P sequence enabled α1 to bind strongly with MCM1. Moreover, addition of a second Q box in the QP'Q and QPQ elements enabled the formation of a modest amount of a complex that seems to contain a second molecule of α1 (Fig. 5). The QPQ and QP'Q complexes with two molecules of α1, however, did not form as readily as those with one, suggesting that they may be unstable. In addition, the QP' construct, in which the Q box is located on the side of P' opposite the degeneracies, did not appreciably form stable ternary complexes.

Our interpretation of these observations, summarized in Fig. 6, is that when α1 contacts the Q sequence, the adjacent MCM1 monomer of the MCM1 dimer is displaced somewhat from its half of the P element. For efficient complex formation, one monomer of MCM1 can be displaced, provided that

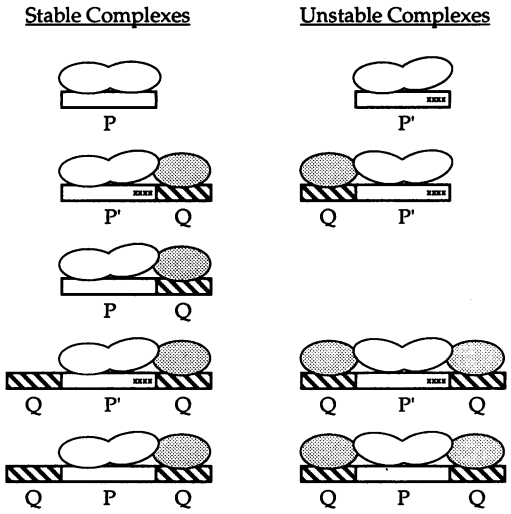

FIG. 6. Stable and unstable MCM1-DNA complexes. Complexes of MCM1 and/or α1 bound to different PQ elements are illustrated. MCM1 and α1 are depicted as in Fig. 1. Stable complexes are efficiently observed in band shift experiments, whereas unstable ones are not. Instability may reflect a kinetic barrier to the formation of the complexes, or it may reflect rapid dissociation of complexes once formed (see text for discussion).

it is anchored by α1 bound to an adjacent Q box and by the other monomer bound to a strong P half-site. Displacement of both monomeric units of MCM1, for example, by α1 binding to Q boxes on both sides of P or by unfavorable half-site sequences within P, results in a decreased ability to form stable complexes.

In conclusion, our findings highlight a number of similarities in expression of α-specific genes in yeast cells and expression of serum-responsive genes in mammalian cells. Transcription of serum-responsive genes requires serum response factor (SRF), which is homologous to MCM1 (30), and $p62^{TCF}$. $p62^{TCF}$ does not bind autonomously to DNA but binds synergistically with SRF to form a ternary complex (40). This complex contains a dimer of SRF bound to a symmetric P-box-like sequence, called the CArG box, and $p62^{TCF}$ bound to a sequence upstream of this site (36). The sequence thought to contact $p62^{TCF}$, CAGGA (13, 39, 40), is found immediately adjacent to the CArG element in the serum response element. Thus, there are two similarities in the requirements for binding to DNA by $p62^{TCF}$ and α1, the coregulatory proteins that play a key role in transcription from either the serum response element or the P'Q sequence. DNA binding for both proteins is dependent upon interaction with a second protein, either SRF or MCM1. In addition, the geometry of the sequence elements that direct ternary complex formation is similar.

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