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Nucleosomal Location of the *STE6* TATA Box and Matα2p-Mediated Repression

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It has been proposed that yeast *MAT*a cell-specific genes are repressed in *MAT*α cells by the Matα2p repressor-directed placement of a nucleosome in a position that incorporates the TATA box of the *MAT*a-specific gene close to the nucleosomal pseudodyad. In this study, we address this proposal directly with a series of plasmids designed to place the *MAT*a-specific *STE6* TATA box at different locations in a nucleosome and in the internucleosomal linker. These plasmids contain different lengths of synthetic random DNA between the Matα2p operator and the TATA box of the *STE6* promoter, which is located upstream of a *lacZ* reporter gene in a multicopy plasmid. We show that in *MAT*α cells, a nucleosome is retained in an identical translational frame relative to the Matα2p operator in all the constructs investigated, irrespective of the sequence of the DNA wrapped onto the histone octamer. This result shows that the nucleosomal organization of the *STE6* promoter in MATα cells is not conferred by the sequence of the promoter itself. No expression of the *lacZ* reporter gene was detectable in *MAT*α cells in any of the constructs, even with the TATA box located in a short internucleosomal linker. These data indicate that repression of *MAT*a-specific genes in *MAT*α cells does not require the precise translational placement of the TATA box close to the nucleosomal pseudodyad; the gene remains repressed when the TATA box is located within the investigated 250-bp region in the organized chromatin domain abutting the Matα2p operator in *MAT*α cells and may remain repressed with the TATA box located anywhere within this organized repression domain.

The successful mating of haploid *Saccharomyces cerevisiae* cells requires the unambiguous expression of either a *MAT*a or a *MAT*α cell mating phenotype. This exclusive expression of only one mating type-specific gene set, despite the presence of both in each haploid cell, is accomplished by a combinatorial mechanism involving a cell type-independent protein, Mcm1p, and the gene products of the *MAT*α locus, the Matα1p and Matα2p proteins (1, 21, 57, 58). In the presence of Matα1p and Matα2p, *MAT*a-specific genes are repressed and *MAT*α-specific genes are expressed, generating a *MAT*α cell phenotype. If *MAT*a is present at the *MAT* locus, Matα1p and Matα2p are absent, leading to an inverted expression pattern of the type-specific genes and resulting in a *MAT*a cell phenotype (for reviews, see references 3 and 16).

Repression of the *MAT*a-specific genes by the *MAT*α2 gene product is conferred by a 32-bp Matα2p operator (21, 38, 58), located about 200 bp upstream of the translation initiation codon of the five known *MAT*a-specific genes (21, 38). In a *MAT*α cell, the Matα2p operator, which contains a weak twofold sequence symmetry, is cooperatively bound by an Mcm1p homodimer at the operator center and by a Matα2p homodimer contacting both ends of the operator, a binding configuration requiring the Matα2p homodimer to straddle the centrally located Mcm1p homodimer (23, 46, 52, 55). In addition to Matα2p and Mcm1p, repression requires the non-DNA-binding proteins Ssn6p (47) and Tup1p (24, 32, 40), which are associated in a complex (56) and may act as a general repressor of transcription in yeast cells (24). It has been shown that transcription from a heterologous *CYC1* promoter containing a LexA binding site is repressed by a LexA-Ssn6p fusion protein in a Tup1p-dependent fashion, and it was proposed that the role of Matα2p is to recruit the Ssn6p-Tup1p complex which in turn mediates repression of the *MAT*a-specific gene (24). Although several mechanisms for Matα2p-mediated *MAT*a-specific gene repression have been proposed (15, 21, 23, 44, 45, 48), the molecular basis of this repression is not understood.

The most striking structural feature of a repressed *MAT*a-specific gene in a *MAT*α cell is the presence of a regular array of nucleosomes covering the promoter area and extending into the structural gene (48). The generation of this Matα2p-dependent nucleosome array adjacent to the Matα2p operator has been characterized in the TRP1ARS1-derived multicopy TASTE and TALS plasmids (44), as well as in the single-copy genomic *MAT*a-specific *STE6* and *BAR1* genes (48). In the TALS plasmid, two nucleosomes were shown to resolve in precise translational frames (44) and to occupy a dominant rotational frame (48) on either side of the Matα2p operator. In contrast, the chromatin structure around the Matα2p operator of genomic *MAT*a-specific genes in *MAT*α cells differs from this arrangement, since a nucleosome is detectable only downstream of the *MAT*α2p operator (48), a structural asymmetry that may be related to the direction of replication fork passage or upstream transcriptional activity in the genome (48). The translational placement of the nucleosome in the genome, however, also appears precise and occurs 15 or 16 bp from the edge of the Matα2p operator in the two *MAT*a-specific genes studied (48).

Roth et al. (44) have shown that the steady-state *TRP1* mRNA level is significantly reduced in a *MAT*α cell when the Matα2p operator is present upstream of the *TRP1* structural gene in the TALS plasmid, an arrangement under which the *TRP1* gene is organized in a more stable nucleosomal structure than that present in a *MAT*a cell. Since the Matα2p operator appears within a narrow range from 90 to 100 bp upstream of the TATA box in three of the *MAT*a-specific genes for which

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the sequences are known (50), it was suggested that a contribution to the repression mechanism of Matα2p is the precise placement of a nucleosome over essential promoter elements, impeding the formation of a preinitiation complex (44, 45, 48, 50). The accommodation of the TATA box sequence in the nucleosome at a position close to the pseudodyad was suggested to contribute to this cell-type-specific repression (50). It is well established that DNA wrapped in a nucleosome is generally less accessible to proteins, particularly in the central 80 bp of the nucleosome (for a review, see reference 50). This implied augmented repressive ability of a nucleosome was clearly demonstrated in the study by Simpson (49), in which the shifting of the A domain of the ARS1 replication element to within the central 80 bp of nucleosomal DNA resulted in an abrupt and significant decrease in plasmid copy number.

Repression of transcription initiation in vitro by the incorporation of promoter elements in nucleosome cores has been shown by various groups (28, 33–35, 61). It was also reported that derepression could be effected by the preincubation of template DNA with HeLa cell extract prior to nucleosome assembly (35), a manipulation that specifically required the presence of TFIID (62). The addition of TFIID to a nucleosomal template did not result in recovery of transcriptional competence (62), suggesting that TFIID cannot bind to a TATA box sequence on the surface of a nucleosome. The substoichiometric assembly of a DNA template with nucleosomes, in which the TATA box is more likely to be accessible in a subpopulation of molecules, can be transcribed (28). It was also shown that histone depletion results in derepression of the *PHO5* gene in vivo (14). This proposed chromatin-mediated repression (for reviews, see references 13 and 50) was further investigated for the *MAT***a**-specific genes, and it was shown that the expression of N-terminally deleted histone H4 mutants in *MAT*α cells resulted in increased nuclease accessibility of the nucleosome located immediately downstream of the *STE6* *MAT*α2p operator and in partial derepression of this *MAT***a**-specific gene (45). Moreover, in the absence of either Ssn6p or Tup1p, the chromatin structure of the partially derepressed genomic *STE6* promoter in a *MAT*α cell resembles that of the promoter in a *MAT***a** cell (6).

Here we directly address the possible involvement of the precise location of the TATA box in a nucleosome in repression of the *MAT***a**-specific genes (35, 50, 57, 58). We have investigated the expression of a β-galactosidase reporter gene, driven by the *STE6* promoter, in various constructs containing the TATA box at different locations in a nucleosome and in a short internucleosomal linker.

MATERIALS AND METHODS

Yeast strains and parental plasmid construction. The yeast strains FY23 (*MAT***a** *ura3-52 trp1Δ63 leu2Δ1*) and FY24 (*MAT*α *ura3-52 trp1Δ63 leu2Δ1*), which are isogenic except at the *MAT* locus (60), were transformed with yeast shuttle vector DNA by a dimethyl sulfoxide-enhanced lithium chloride procedure (18). The *STE6* promoter, comprising the sequence from positions −546 to −2 (37, 57) (GenBank accession number X15428), was fused to the coding sequence of the β-galactosidase gene (positions 1283 to 4404 [22]) (GenBank accession number J01636) at a *Bsp*HI site. This manipulation required the substitution of the sequence CTATGA, present at position 1282 in the β-galactosidase gene, with the restriction recognition sequence of *Bsp*HI and places the translation initiation codon of the β-galactosidase coding sequence in a position identical to that previously occupied by the *STE6* translation start site. The 6-bp sequences present at positions −546 and 4399 of the *STE6* and β-galactosidase sequences, respectively, were also substituted with *Bam*HI recognition sequences. The *STE6* promoter sequence was further altered by substitution of the sequence CATATA, present at position −142, with a *Sty*I restriction enzyme recognition site, CCT TGG. All the base substitutions were performed by a two-step symmetrical PCR procedure by using a pair of complementary template-mismatched central primers containing the desired sequence (17).

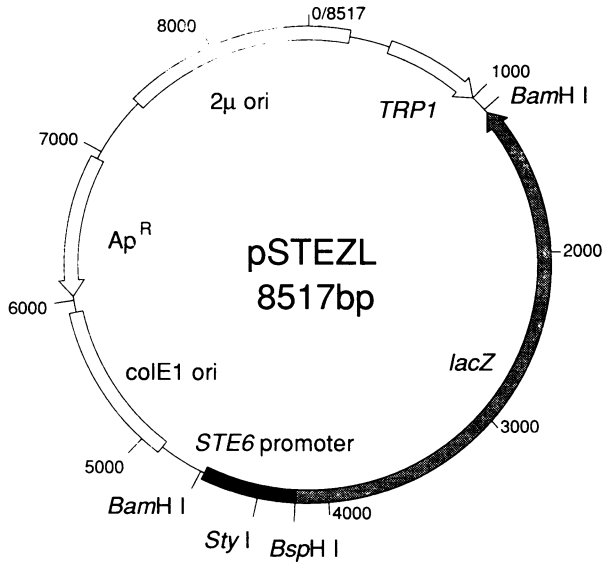

FIG. 1. Structural organization of the yeast shuttle vector pSTEZL. The location of the *STE6* promoter fused to a *lacZ* reporter gene is shown, with the direction of *lacZ* transcription opposite to that of *TRP1*, the selectable marker as indicated. The numbering scheme shown is relative to the numbering origin for the parental plasmid, pRS424 (5). Sequence positions given in the text are relative to the major transcription start site of *STE6* in the genome (58), which corresponds to position 4349 in this figure.

The 3,661-bp *STE6-lacZ* fragment was ligated into the *Bam*HI site of plasmid pRS424ΔZ in an orientation such that the transcription direction of *lacZ* is opposite to that of *TRP1*, the selectable marker, and the construct was denoted pSTEZL (Fig. 1) (the nucleotide sequence of this plasmid is available from us upon request). The plasmid pRS424ΔZ is a derivative of the 2μm origin of replication yeast shuttle vector, pRS424 (5), where the *Hin*dIII-*Pst*I fragment of pRS424 was substituted with a 214-bp *Hin*dIII-*Pst*I fragment from TRP1ARS1 (54) containing the corresponding 3′ region of the *TRP1* gene but lacking the f1 filamentous phage replication origin and N-terminal *lacZ* sequences. All cloning procedures were performed according to standard protocols (2).

Oligonucleotide ligations and isolations. The two complementary 25-nucleotide oligonucleotides, CAAGAACGTCG TAGTACAAGCTCGC and CTTGGCGAGCTTGTACTAC GACGTT, were synthesized with a 380B DNA synthesizer (Applied Biosystems) and purified by high-performance liquid chromatography on a Gen-Pac Fax anion-exchange column (Waters) (2). The sequence of these complementary oligonucleotides was generated randomly, except the four 5′ bases, which are compatible with a *Sty*I restriction enzyme-generated overhang, allowing directional ligation. A 1-μg amount of each of the purified primers was dispensed into 40 μl of 50 mM Tris-HCl (pH 7.6)–10 mM $MgCl_2$–10 mM dithiothreitol–1 mM

ATP and phosphorylated with 20 U of T4 polynucleotide kinase at 37°C for 1 h. The primers were annealed by directly equilibrating the sample at 55°C for 3 min, followed by cooling to 4°C at a rate of 1°C/min. The sample volume was then increased to 80 μl with the above-described buffer, 10 μl was removed for the monomer, and the remainder of the sample was adjusted to 50 μg of bovine serum albumin per ml and ligated with 8 U of T4 DNA ligase at 16°C for exactly 10 min. The ligation was quenched by the addition of EDTA to a final concentration of 50 mM, and the ligation ladder was resolved on a 12% polyacrylamide gel in 90 mM Tris-borate–2 mM EDTA (1× TBE). Bands corresponding to required multimers were cut out under brief UV illumination after the gel had been stained with 0.5 μg of ethidium bromide per ml, and the DNA was isolated by passive elution into 500 mM ammonium acetate–1 mM EDTA (pH 8.0) (2). Fragments containing one to eight repeats of the monomer were ligated into the *Sty*I site of pSTEZL with T4 DNA ligase (2), and the inserts in the plasmid, isolated from *Escherichia coli* SURE (Stratagene), were confirmed by chain-terminating dideoxy nucleotide sequencing. Plasmids pSTEZLΔ25 and pSTEZLΔ50, which contain 25- and 50-bp deletions in the *STE6* promoter sequence between positions −132 and −106 and between positions −132 and −81, respectively, were constructed by a two-step PCR procedure with appropriate 36-nucleotide central primers (17). An identical protocol was used to substitute the two pheromone-responsive elements (PREs) at positions −132 and −114 with the sequence TGATCGC, to give the plasmid pSTEZLΔPRE.

Nucleus preparations, nuclease digestions, and primer extensions. Transformed yeast cells were grown at 30°C in SC medium (43) without tryptophan, and nuclei were isolated from 1-liter cultures (optical density at 600 nm of 0.7 to 1.0) as described by Gross et al. (12). Digestion of nuclei with micrococcal nuclease (MNase; EC 3.1.31.1) and the primer extension of the digested DNA were performed as described by Shimizu et al. (48) with the modification that 1.2 μl of deoxyribonucleoside triphosphate (5 mM for each of the four deoxyribonucleotides) and 1.25 U of *Taq* DNA polymerase (EC 2.7.7.7) were added prior to the initial denaturation step at 94°C. The nuclease concentrations used are given in the legends to the relevant figures. A 25-nucleotide [γ-^{32}P]ATP-end-labeled oligonucleotide primer, which anneals at position −361 in the *STE6* upstream promoter area, was used for all the extensions reported in this study. The primer extension samples, resuspended in formamide, were electrophoresed on 6% polyacrylamide (acrylamide-bisacrylamide, 19:1) gels containing 8 M urea in 1× TBE at 55 W. The gels were dried and exposed at −70°C to X-Omat AR film (Kodak) backed with an intensifying screen (DuPont) for 1 to 3 days as appropriate.

β-Galactosidase assays. The activities of the *lacZ* gene in the various constructs and cell types were measured by determining the amount of *o*-nitrophenol hydrolyzed from *o*-nitrophenol-β-D-galactopyranoside (ONPG) per time unit as described by Rose et al. (43). Briefly, cells were harvested from 50-ml cultures of yeast cells grown in SC medium without tryptophan (optical density at 600 nm of 0.7 to 1.0), resuspended in 1 ml of 10 mM KCl–65 mM sodium phosphate (pH 7)–1 mM $MgSO_4$–40 mM 2-mercaptoethanol, and permeabilized by vigorous agitation following the addition of 50 μl of chloroform and 50 μl of 0.1% sodium dodecyl sulfate. After the sample had been briefly incubated at 28°C, ONPG was added to a final concentration of 2 mM and the reaction was allowed to proceed for a specified time (approximately 5 min) at the same temperature. The reaction was quenched by addition of sodium acetate to a final concentration of 300 mM, cellular debris was removed by centrifugation, and the A_{420} of the supernatant was measured. The β-galactosidase activity was expressed in Miller units (43).

RESULTS

The chromatin structures of the *STE6* promoter in the genome and in a multicopy plasmid are identical. The nucleosomes immediately downstream of the Matα2p operator in the single-copy genomic *STE6* and *BAR1* genes occupy similar translational positions in MATα cells (48) and also resolve in a dominant rotational frame on the *STE6* promoter (45). Since the chromatin structure of the genomic *STE6* promoter was characterized at nucleotide resolution in both *MAT***a** and *MAT*α cells (45, 48), this promoter was chosen to investigate the effect of the nucleosomal location of a *MAT***a**-specific gene TATA box on the expression of a *lacZ* reporter gene in the multicopy plasmid pSTEZL. The chromatin structure of the *STE6* promoter region in the plasmid pSTEZL was investigated first, to establish that this was comparable to that in the genome and therefore relevant to an investigation of the role of the precise position of the nucleosome located over the TATA box in Matα2p-mediated repression. The use of a multicopy plasmid was not expected to titrate out factors required for the establishment of the chromatin structure, since we have previously shown that the presence of a multicopy plasmid in a *MAT*α cell did not change the chromatin structure of the *STE6* genomic gene (48).

In Fig. 2, primer extension of pSTEZL following MNase digestion of the purified plasmid (lanes 5 and 6) and of isolated nuclei prepared from yeast cells transformed with the plasmid and grown under selection (lanes 7 to 10) is shown. It is seen that MNase digestion of free pSTEZL (lanes 5 and 6) generates a nonuniform cleavage pattern, due to the sequence specificity of MNase (10). Although a very similar digestion profile is obtained for pSTEZL in *MAT***a** cell nuclei (Fig. 2, lanes 7 and 8), several positions can be identified that show either enhanced cleavage or protection relative to that of the free DNA. This reflects the MNase accessibility of the promoter, which can no longer support transcriptional initiation (20) and is probably partially stripped of protein factors, as well as the conformational distortions of the promoter DNA (10). In the case of *MAT*α cell nuclei (Fig. 2, lanes 9 and 10), a markedly different digestion pattern is seen, including a broad region spanning positions +1 to −140 that is cut significantly less than the corresponding region in both *MAT***a** cell nuclei (lanes 7 and 8) and free DNA (lanes 5 and 6) at comparable total extents of digestion. The length of this protected area is consistent with the presence of a translationally positioned nucleosome. It is well established that the tight winding of DNA onto the histone octamer surface in a nucleosome results in the protection of the central 146 bp of DNA from MNase cleavage, while the internucleosomal linker DNA is digested more readily (see reference 50 for a review). One may therefore assign the regions at positions +1 and −140, which show extents of cleavage comparable to that of free DNA, to the internucleosomal linker. The partial accessibility of the DNA for 20 to 30 bp on either side of position +1 is likely due to the weak association of the terminal nucleosomal DNA with the octamer surface (reviewed in reference 36) or to the weak exonucleolytic activity of MNase (51).

A second region of decreased cleavage in the pSTEZL chromatin of the *MAT*α cell is visible, starting at the +1 position and extending into the *lacZ* reporter gene for a distance of approximately 140 bp. A continuation of this

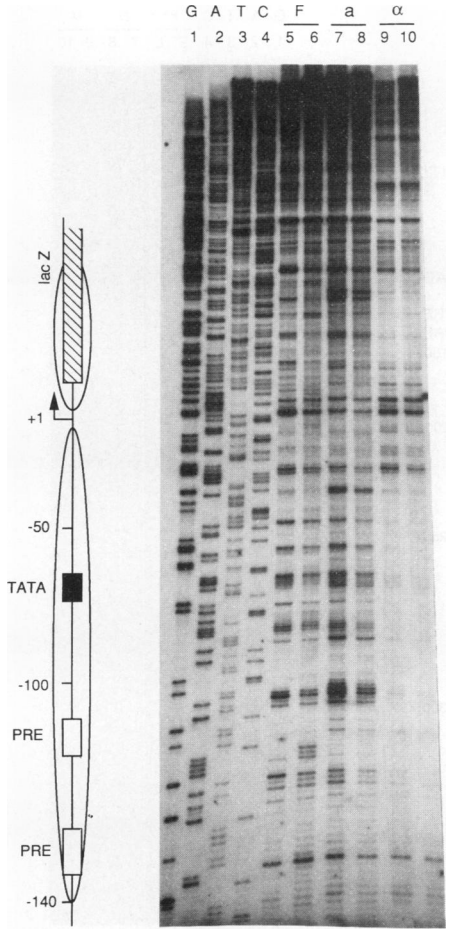

FIG. 2. The nucleosomal organization of the *STE6* promoter region of plasmid pSTEZL in *MAT***a** and *MAT*α yeast cells. The primer extension products of MNase-digested free pSTEZL DNA (lanes 5 and 6) and pSTEZL chromatin in the isolated nuclei of *MAT***a** (**a**) (lanes 7 and 8) and *MAT*α (α) (lanes 9 and 10) yeast cells are shown. The extended ^{32}P-end-labeled primer products were electrophoresed on a 6% polyacrylamide—8 M urea gel, of which an autoradiograph is shown. Nuclei were digested with 5 U (lanes 7 and 9) or 2.5 U (lanes 8 and 10) of MNase per ml. The free DNA samples in lanes 5 and 6 were digested with 0.5 and 0.25 U of MNase per ml, respectively. Dideoxy chain-terminating sequencing standards of the sample used for the free DNA digestions are shown in lanes 1 to 4 and correspond to the nucleotide on the template strand as indicated above each lane. The locations of various promoter elements and the *lacZ* gene in the sequence are shown to the left and include the TATA box (solid rectangle), the PREs (open rectangles), the major transcription start site (bent arrow), and the structural *lacZ* gene (hatched rectangle). The 32-bp Matα2p operator is located at positions −152 to −183, below the bottom margin of the gel. The ellipses demarcate regions in the chromatin of *MAT*α cells protected from MNase cleavage because of the presence of positioned nucleosomes. The sequence numbering is relative to the major start site of transcription (58). The appearance of cleavage sites within the nucleosome DNA termini is likely due to the weak exonucleolytic activity of MNase (51) and the weak association of the terminal DNA regions with the octamer surface (reviewed in reference 36).

pattern of alternating protection and cleavage appears to extend even further into the *lacZ* gene, consistent with the presence of an organized array of nucleosomes downstream of the Matα2p operator in pSTEZL.

The region protected from MNase cleavage in *MAT*α cells does not give well-defined cleavage maxima with a period of approximately 10 bp following DNase I digestion (data not shown), suggesting that the rotational placement of the nucleosome immediately downstream of the Matα2p operator is qualitatively less precise than for some in vitro positioning sequences (reviewed in reference 50). It remains possible, however, that during the nucleus isolation procedure, when the Matα2p repressor is lost from the Matα2p operator (41), some randomization in the rotational placement of this nucleosome takes place.

Insertion of random DNA adjacent to the Matα2p operator does not affect the location of operator-flanking nucleosomes. The similarities in the chromatin structures of the *STE6* promoter in the genome and in pSTEZL, particularly in terms of the translational placement of the nucleosome adjacent to the Matα2p operator and the nucleosomal array extending further downstream, suggested that an investigation of the role of the nucleosomal location of the *STE6* TATA box in Matα2p-mediated repression is relevant in the pSTEZL multicopy plasmid. The ability of the Matα2p operator to position a nucleosome translationally on different sequences in *MAT*α cells (27, 44, 45, 48, 49) was exploited in this study, during which directly repeated multimers of a synthetic random 25-bp sequence were inserted into the *Sty*I restriction site between the Matα2p operator and the TATA box in pSTEZL. It is expected that these plasmids, assembled into chromatin in yeast cells, will contain the *STE6* TATA box at different locations in the nucleosome abutting the Matα2p operator and in the internucleosomal linker DNA. We also note that this length of insert will change the rotational orientation of the TATA sequence by approximately half a helical turn with each additional insert. Here, however, we confine our attention to the effect of the translational setting of the TATA box, particularly at a position close to the nucleosomal pseudodyad and in the internucleosomal linker. A 25-bp as opposed to a multiple-of-10-bp random sequence was chosen to ensure that a structural feature was not repeated with a period close to that of the DNA duplex, thereby introducing an anisotropic signal that may compete for a positioned nucleosome (8, 53). The 25-bp random sequence, furthermore, contained none of the protein recognition sequences listed in the TFD data base (release 6.3) (11) and is not expected to bind any known endogenous yeast transcription factor.

In Fig. 3, the MNase digestion pattern of pSTEZL-3, containing three 25-bp inserts, is shown for the plasmid DNA and for the plasmid in nuclei isolated from *MAT***a** and *MAT*α cells. The accessibility of the DNA to MNase in *MAT***a** cells (Fig. 3, lanes 7 and 8) is very similar to that of the free DNA (lanes 5 and 6), as was found for pSTEZL as described above. In the case of the *MAT*α cell (Fig. 3, lanes 9 and 10), broad areas of protection are once again visible. Note, however, that the accessible region present at position +1 in pSTEZL is absent in pSTEZL-3, and a new area which is cleaved to an extent similar to that for the free DNA is present at approximately position −70 in pSTEZL-3. The length of the protected area, which is demarcated on one side by this accessible region at position −70, is approximately 140 bp (indicated by the lower ellipse on the left of Fig. 3). This is followed by a second protected area extending into the structural *lacZ* gene (indicated by the upper ellipse on the left of Fig. 3), also approximately 140 bp.

An identical result was obtained for pSTEZL-8, which contains a 200-bp (8× 25-bp tandem repeat) random sequence insert between the Matα2p operator and the TATA box (Fig. 4). Two regions of approximately 140 bp which are protected

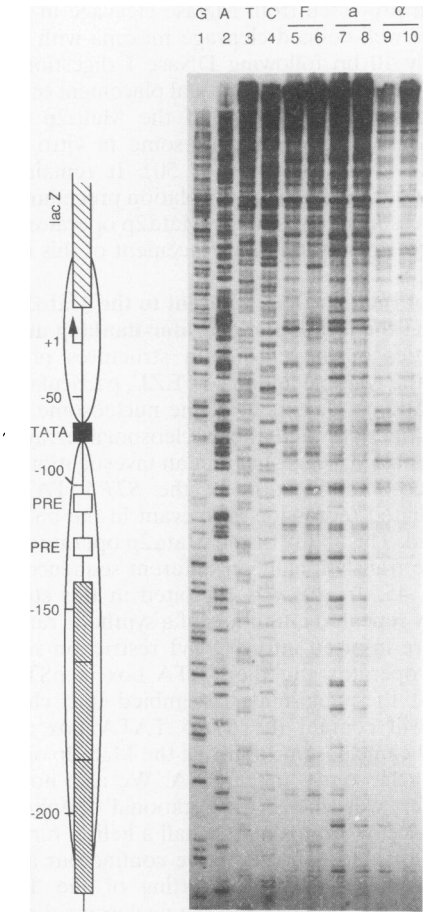

FIG. 3. The nucleosomal organization in *MAT***a** and *MAT*α yeast cells of the *STE6* promoter region in pSTEZL-3. Purified DNA was used as a template for the extension of a ^{32}P-end-labeled primer following MNase digestion of free pSTEZL-3 DNA (F) (lanes 5 and 6) and pSTEZL-3 chromatin in the isolated nuclei of *MAT***a** (**a**) (lanes 7 and 8) and *MAT*α (α) (lanes 9 and 10) yeast cells. The primer extension products were electrophoresed on a 6% acrylamide—8 M urea gel, of which an autoradiograph is shown. The digestion conditions for the free DNA and chromatin were identical to those described in the legend for the corresponding lanes in Fig. 2. Sequencing standards of the sample used for the free DNA digestions are shown in lanes 1 to 4. The position of the 75-bp random DNA insert and the locations of various promoter elements and the *lacZ* gene in the sequence are shown on the left. The 75-bp insert is shown as three densely hatched rectangles towards the bottom of the figure, corresponding to the three 25-bp directly repeated monomers. The other identified features are as described in the legend to Fig. 2.

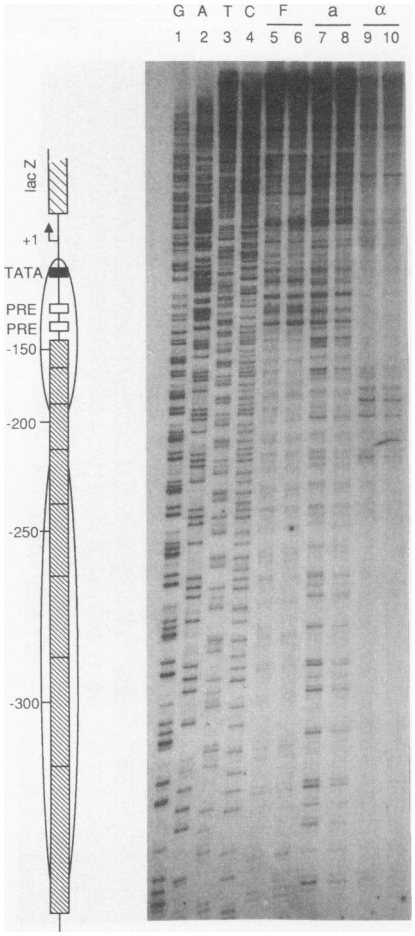

FIG. 4. The nucleosomal organization of the *STE6* promoter region in pSTEZL-8. The primer extension products of DNA purified following MNase digestion of free pSTEZL-8 DNA (F) (lanes 5 and 6) and pSTEZL-8 chromatin in the isolated nuclei of *MAT***a** (**a**) (lanes 7 and 8) and *MAT*α (α) (lanes 9 and 10) yeast cells were electrophoretically separated as described in the legend to Fig. 2. An autoradiograph of the gel is shown. The digestion conditions for individual samples were identical to those described in the legend for the corresponding lanes in Fig. 2. Sequencing standards are shown in lanes 1 to 4 and correspond to the nucleotides indicated above the lanes. The positions of the different promoter elements, the *lacZ* structural gene, and the 200-bp insert in the sequence are indicated on the left and are labeled as described in the legends to Fig. 2 and 3.

from MNase cleavage in the *MAT*α cell (Fig. 4, lanes 9 and 10) but not in the *MAT***a** cell (lanes 7 and 8) and protein-free DNA (lanes 5 and 6) are present (indicated by the two ellipses on the left of the figure). In pSTEZL-8, the nucleosome immediately downstream of the Matα2p operator resides on DNA composed entirely of the inserted random sequence. The location of both nucleosomes downstream of the Matα2p operator in similar translational frames in pSTEZL, pSTEZL-3, and pSTEZL-8 indicates that these nucleosomes retain their translational positions relative to the operator as opposed to following a sequence-directed positioning signal inherent in the promoter sequence itself.

This ability of the Matα2p-Mcm1p nucleated complex to position a nucleosome translationally irrespective of the bordering DNA sequence shifts the downstream sections of the *STE6* promoter, including the TATA box, to consecutive nucleosomal locations with each additional 25-bp fragment inserted into the *Sty*I site. In pSTEZL-3 (Fig. 3), the TATA box is seen to be located in the internucleosomal linker. If the precise placement of the TATA box within the nucleosome was the only feature of chromatin structure responsible for repression of *MAT***a**-specific gene transcription, it would be expected that this location of the TATA box should allow expression of the *lacZ* gene in *MAT*α cells containing the pSTEZL-3 plasmid.

The presence of the *STE6* TATA box in a short internucleosomal linker does not allow transcription in *MAT*α cells. The expression of the *STE6*-driven *lacZ* reporter gene was determined for both *MAT***a** and *MAT*α cells (Fig. 5). In the case of

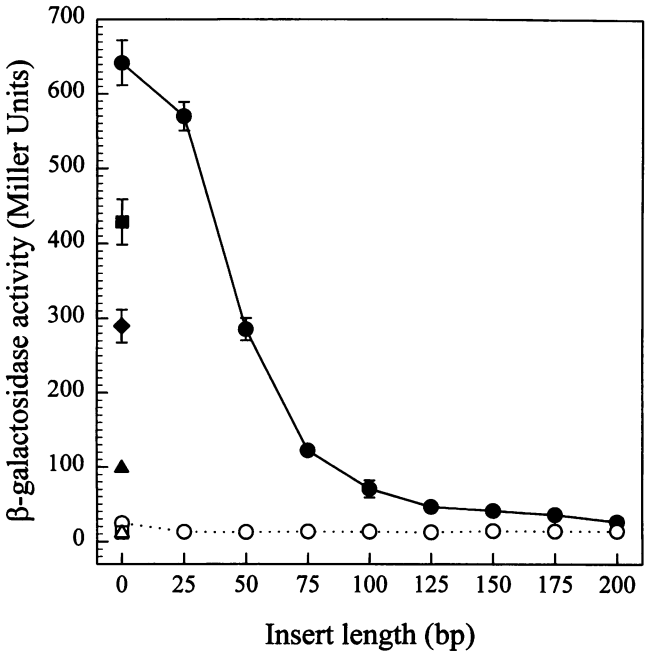

FIG. 5. The *STE6*-driven expression of the *lacZ* reporter gene from different constructs in yeast *MAT***a** and *MAT*α cells. The activity of β-galactosidase in permeabilized yeast cells was measured, and the averages of several determinations (stated below) are given in Miller units. The activity for pSTEZL containing between zero and eight inserts of the 25-bp random DNA in the *Sty*I site is shown for *MAT***a** cells (solid circles; $n = 8$) and *MAT*α cells (open circles; $n = 8$). The activity was also measured for pSTEZLΔ25 in *MAT***a** and *MAT*α cells (solid and open squares, respectively; $n = 4$ for both samples) and for pSTEZLΔ50 in *MAT***a** and *MAT*α cells (solid and open triangles, respectively; $n = 4$ for both samples). The β-galactosidase activity of a *MAT***a** cell transformed with pSTEZLΔPRE is shown (solid diamond; $n = 3$). Note that the datum points for pSTEZLΔ25 and pSTEZLΔ50 determined with *MAT*α cells are superimposed in the lower left corner of the figure. The standard deviation at 3σ is shown by error bars when this exceeds the width of the datum point.

the *MAT***a** cell, indicated by the solid circles in Fig. 5, it is seen that the systematic increase in the distance between the TATA box and the *STE6* Matα2p operator results in a monotonic decrease in the measured β-galactosidase activity. Since the sequences of the TATA box and all associated downstream promoter elements, as well as that of the β-galactosidase structural gene, are identical in all the constructs, this reduction in β-galactosidase activity is most likely due to a decrease in the *STE6*-driven transcription of *lacZ* as opposed to an effect on mRNA translation or protein stability.

No significant β-galactosidase activity was detectable in any of the constructs tested in *MAT*α cells (indicated by open circles in Fig. 5). Some expression in the parental construct containing no inserts may be due to slight leakage in the repression, as found previously (21, 38, 58). This residual expression is reduced with increasing numbers of inserts, probably by the same mechanism whereby expression in *MAT***a** cells decreases (see Discussion also). It was shown above that the TATA box is located in the internucleosomal linker in the pSTEZL-3 construct. The ratio of *lacZ* expression for this construct in *MAT***a** versus *MAT*α cells is 10, which is comparable to the ratio of 30 for the parental pSTEZL construct (Fig. 5). This result clearly shows that a particular nucleosomal location of the TATA box sequence is not necessary for Matα2p-mediated repression of the mating type-specific genes.

We also investigated the expression of *lacZ* in the deletion constructs pSTEZLΔ25 and pSTEZLΔ50, in which 25 and 50 bp, respectively, were deleted between the Matα2p operator and the TATA box. Since we have demonstrated above that the nucleosome abutting the Matα2p operator is retained in an identical translational frame irrespective of the sequence which is wrapped onto the histone octamer, we expect the TATA box in the deletion constructs to be shifted towards the opposite nucleosomal terminus, in the direction of the operator. As shown in Fig. 5, no expression of *lacZ* was detectable in *MAT*α cells in either of the deletion constructs, in agreement with the above result.

Interestingly, the expression level of *lacZ* in pSTEZL-8 is approximately 60-fold less than that of pSTEZL (Fig. 5), yet the structural organization of the *STE6* promoter in both constructs appears qualitatively similar in *MAT***a** cells (Fig. 2 and 4). Although the correspondence between transcriptional activity and promoter chromatin structure is not known, this result suggests that the structure of the *STE6* promoter in a *MAT***a** cell is not due solely to transcriptional activity but may rather reflect the transcriptional competence of the promoter.

PREs are not required for expression from the *STE6* promoter. An enhancement in *MAT***a**-specific gene expression in response to the exposure of a yeast *MAT***a** cell to α mating factor is mediated by the STE12 protein (7), which binds at multiple PREs in the upstream promoter region of *MAT***a**-specific genes (30; reviewed in reference 16). In the absence of α mating factor, STE12 protein still confers stimulation, but to a lesser extent (9, 19). In the pSTEZL-3 construct, where the TATA box is located in the linker DNA, two PRE sequences are incorporated into the nucleosome adjacent to the Matα2p operator (Fig. 3). We investigated the possibility that the failure of the STE12 protein to recognize and bind to the PREs incorporated into the nucleosome may preclude the formation of a stable preinitiation complex on the *STE6* promoter, even though the TATA box is exposed in the linker.

The STE12 recognition sequences, present at positions −132 and −114, were both substituted with a sequence differing from the consensus sequence at four of seven base pairs, which is expected to abolish STE12 binding. It was found (Fig. 5) that removal of the STE12 binding sites reduced the expression of β-galactosidase by approximately twofold in the *MAT***a** cell. This modest reduction suggests that the absence of β-galactosidase expression in the construct containing the TATA box in the linker is not due to the inability of STE12 to bind to its recognition sequence on the surface of a nucleosome.

DISCUSSION

We have shown that placement of the *STE6* TATA box in the internucleosomal linker, a location which is accessible to MNase (44, 45, 48) and *dam* methyltransferase (27), is not sufficient to allow transcription in a *MAT*α cell. This result can be interpreted in terms of the occlusion of TBP from the TATA box, even though this element is exposed in an internucleosomal linker, or the inability of the DNA-bound TBP to nucleate the assembly of a preinitiation complex that leads to productive transcription initiation.

Considering first the binding of TBP to the TATA box, it is noted that the center-to-center distance between the two regions adjacent to the Matα2p operator protected from MNase cleavage by nucleosomes in pSTEZL-3 in *MAT*α cells is approximately 160 bp (Fig. 3), which is comparable to the

length of DNA required to make two full turns around the histone octamer (29). The length of DNA in the linker, which is not associated with the octamer surfaces, is therefore very short and appears to result in a unique spatial arrangement of adjacent nucleosomes relative to the shared linker DNA (51). The binding of yeast TBP to the TATA box sequence in vitro protects approximately 10 bp from hydroxyl radical cleavage (31). In the yeast TBP-DNA cocrystal structure (26), the protein was shown to follow the minor groove, binding to an 8-bp stretch incorporating the TATA sequence. Although the length of DNA required for TBP binding does not appear incompatible with that available in the yeast linker, this binding severely distorts the DNA in a fashion that may not be accommodated by the internucleosomal linker and particularly the flanking nucleosomes. This is quite clear in the cocrystal structure (26), where the insertion of two phenylalanines between the terminal base steps of the bound 8-bp sequence increases the roll angle of these base steps to approximately 40° and the hydrophobic association of the concave surface of TBP (42) with the base edges exposed in the minor groove decreases the average twist angle to approximately 19° over the 8-bp stretch bound, resulting in an unwinding of one-third of a helical turn in the DNA between the binding site edges (26). This unwinding is topologically compensated for by the DNA assuming positive writhe (25, 26), contributing to the severe bending of the DNA by approximately 80° over only 8 bp (25, 26).

The accommodation of the TBP-induced unwinding of the DNA appears possible if the nucleosomes on either side of the TATA box are free to rotate. However, if the bordering nucleosomes are engaged in a rigid spatial arrangement, the unwinding of 45 bp of nucleosomal DNA on either side of the TATA box, corresponding to the fraction of yeast nucleosomal DNA with a torsional flexibility equal to that of free DNA (39), will result in the unwinding of the terminal four helical turns to an average local helical twist of 10.7. It is unclear whether this degree of TBP-induced unwinding can be accommodated in a canonical yeast nucleosome. The severe bending of the TBP-bound DNA may prove structurally more challenging. There appears to be no spatial arrangement between adjacent nucleosomes that can accommodate an 80° bend in the short linker DNA without encountering a severe steric clash that will require the disruption of one or both nucleosomes on either side of the bend.

A second consideration, in the absence of *lacZ* expression in the construct containing the TATA box in the linker, is the recruitment by TBP of the basal polymerase II transcription factors. Others have shown that the sequential assembly of transcription factors into a complex supporting transcription initiation in vitro resulted in the protection of approximately 80 bp from DNase I cleavage (4). This suggests that an extended length of free DNA may be required to facilitate transcription of *MAT***a**-specific genes in *MAT*α cells. The recruitment by TBP of basal factors to form a preinitiation complex may therefore require the disruption of the nucleosomes abutting the TATA box, or the shifting of these nucleosomes to expose a length of DNA sufficient for the formation of the preinitiation complex. It is likely that the recruitment of the basal transcription factors to a promoter area cannot facilitate the simultaneous disruption of the surrounding chromatin structure, and the process may require the involvement of specialized chromatin-remodeling proteins, such as the SNF/SWI activators (see reference 59 for a review).

The spacing between the wild-type *STE6* promoter elements gives optimal promoter activity. Data discussed above show that the repression of a *MAT***a** cell-specific gene is manifest irrespective of the exact location of the *STE6* TATA box within an organized domain of chromatin. Since the precise nucleosomal placement of the *STE6* TATA box is not the sole determinant of Matα2p-mediated repression of the *MAT***a**-specific genes, it is reasonable to ask why the Matα2p operator is located within such a narrow range upstream of the TATA box in the three *MAT***a**-specific genes for which the sequences are known. It was shown above that the systematic increase in the distance between the operator and the TATA box resulted in a monotonic decrease in the expression of β-galactosidase in *MAT***a** cells (Fig. 5). This suggests that the spacing of elements in the wild-type *STE6* promoter is optimized for promoter activity. Interestingly, the observed decrease does not exhibit an oscillation that might be expected if full activity required direct contact between rigid structures on either side of the *Sty*I site. Such an oscillation would be brought about by the introduction of the 25-bp shifts, alternately placing the structures on opposite sides of the DNA helix. The obtained monotonic decrease suggests that if the reduction in the promoter strength is due to the weakening of required protein-protein contacts, these contacts are made between regions that are flexible. A reduction was also observed in constructs lacking the PRE consensus sequences (data not shown), suggesting that STE12 is not involved in this possible protein-protein interaction. Although, contrary to expectation, the expression of *lacZ* in the 25- and 50-bp deletion constructs was also reduced in comparison with that in the wild-type *STE6* promoter, this may be due to the deletion of sequence content.

Relation to other work. Several models for the molecular mechanism of Matα2p-mediated repression have been proposed. These include direct and indirect steric hindrance of preinitiation complex formation (21, 23, 44, 45, 48), obstruction of the sliding path of factors and polymerase molecules from upstream regions to the promoter by Matα2p-Mcm1p (21), the masking of the activation domain of Mcm1p by Matα2p repressor (23), and the stable retention of the transcription complex on the promoter by interaction with the Matα2p-Mcm1p complex (15, 23), possibly via Ssn6p and Tup1p (24).

The Matα2p operator repressed transcription of a *CYC1-lacZ* fusion gene in *MAT*α cells when the operator was present at a location either upstream or downstream of activating sequence elements (21), suggesting that repression does not occur by the occlusion of *trans*-acting factor binding sites by the Matα2p-Mcm1p complex. It seems clear that repression of transcription is not effected by the masking of the activation domain of Mcm1p by Matα2p, since a heterologous *CYC1* promoter is repressible by Matα2p (21), although this promoter does not require Mcm1p for activity. It was also proposed that the Matα2p-Mcm1p complex contacts the basal transcription complex, physically restraining polymerase II from leaving the promoter area and passing into and transcribing the structural gene (15, 23). However, in yeast *MAT*α cells expressing an N-terminally deleted histone H4 mutant, distinct G residues in the Matα2p operator were protected from methylation by dimethyl sulfate, which suggested the presence of the Matα2p-Mcm1p complex at the operator even though the *MAT***a**-specific *STE6* gene was partially derepressed (45). However, without careful quantitation of operator occupancy, it remains possible that a fraction of operators were not bound by Matα2p.

We have shown above that the precise placement of a *MAT***a**-specific promoter TATA box within a nucleosome is not required for Matα2p-mediated repression. This result does not address the general occlusive nature of chromatin, particularly where the assembly of a preinitiation complex may require an

extended length of free DNA. In this regard, the unique dinucleosomal chromatin organization of the *STE6* gene (51), suggestive of a precise spatial structure, may contribute to Matα2p-mediated repression. This broader effect of the chromatin structure of the *STE6* promoter is currently under investigation.

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