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Accessibility of α2-Repressed Promoters to the Activator Gal4

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It has been proposed that eukaryotic repressors of transcription can act by organizing chromatin, thereby preventing the accessibility of nearby DNA to activator proteins required for transcription initiation. In this study, we test this idea for the yeast α2 repressor using a simple, artificial promoter that contains a single binding site for the activator protein Gal4 and a single binding site for the repressor α2. When both the repressor and the activator are expressed in the same cell, the artificial promoter is efficiently repressed. In vivo footprinting experiments demonstrate that Gal4 can occupy its binding site even when the promoter is repressed. This result indicates that α2-directed repression must result from interference with some stage in transcription initiation other than activator binding to DNA.

Negative regulation of transcription in eukaryotes occurs by a variety of mechanisms. Some repressors act by preventing the DNA binding of activators, some bind DNA and interact with nearby activators, "quenching" their activation surface, and some communicate directly with the general transcription machinery, blocking its function or assembly (for reviews, see references 14, 16, 18, and 26). Still other repressors appear to organize repressive forms of chromatin that block the accessibility of proteins to DNA (for reviews, see references 31, 33, and 45). For some repressors, more than one of these mechanisms is thought to function simultaneously, resulting in a very low level of gene expression under repressing conditions.

One case in which two mechanisms of repression have been proposed is that of the yeast α2 protein. This protein is responsible for repressing the expression of two sets of cell-type-specific genes, **a**-specific genes and haploid-specific genes (for reviews, see references 7, 15, and 17). To repress **a**-specific genes, α2 binds cooperatively with the Mcm1 protein to a 34-bp DNA sequence called the **a**-specific gene operator. α2/Mcm1 binds a second protein complex composed of the Tup1 and Ssn6 proteins. Tup1 and Ssn6 are required for the repression of at least five sets of yeast genes and have been proposed to function as a general repression machine in *Saccharomyces cerevisiae*, recruited to DNA by a variety of sequence-specific DNA-binding proteins (21, 24, 41, 42).

The **a**-specific gene operator will bring about repression when placed in many positions upstream of a target gene, and models for repression by α2/Mcm1/Ssn6/Tup1 (referred to as the α2 repression complex) must account for this action at a distance (20, 32). One model proposes that the α2 repression complex interacts directly with the general transcription machinery at the promoter, blocking its assembly or maturation (13, 20). A second model proposes that the α2 repression complex positions nucleosomes over promoter elements, blocking the accessibility of nearby DNA to proteins (23, 34, 35, 37). In this work, we wished to determine whether an α2-repressed promoter is accessible to Gal4, a yeast activator protein that binds DNA.

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MATERIALS AND METHODS

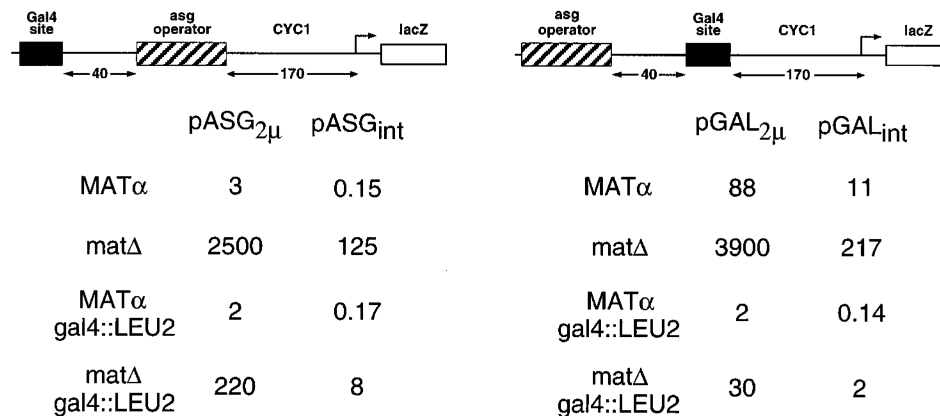
Plasmids. The **a**-specific gene operator used in this study is derived from *STE6* (20). The Gal4-binding site is the consensus site (CGGAGGACTGTCCTCCGT GCA) (44). The Gal4-binding site and the *STE6* operator were ligated into the *Pst*I site and the *Sal*I site, respectively, of the Bluescript polylinker and were subsequently subcloned into the blunted *Sal*I site of pΔSS (19) in either orientation to produce $pASG_{2\mu}$ and $pGAL_{2\mu}$. Promoter regions were then sequenced. Integrating plasmids were constructed by removing the 2μm sequences, resulting in $pASG_{int}$ and $pGAL_{int}$.

Yeast strains and β-galactosidase assays. All four yeast strains used in this study are derivatives of EG123 (*MAT***a** *trp1 leu2 ura3 his4*). *mat*Δ is KT23αx8, created by deletion of *MAT*α from 246-1-1 (*MAT*α *trp1 leu2 ura3 his4*) (36, 39). Plasmid pSJ4*LEU* was used to make a deletion insertion of *LEU2* at the *GAL4* gene (10). Plasmids $pASG_{int}$ and $pGAL_{int}$ were integrated into the *ura3-52* allele. Integrations were confirmed by Southern analysis (38). β-Galactosidase assays were performed as described by Goutte and Johnson (12). Cells were grown initially on synthetic medium minus uracil plus 2% glucose and then transferred to synthetic medium minus uracil plus 2% galactose, 2% ethanol, and 3% glycerol for several cell doublings.

Competitive PCR for quantitation of mRNA. The levels of repression of an **a**-specific gene, STE2, were compared at the RNA level between *MAT*α and *mat*Δ cells. Quantitative PCR (9) was used to detect the very low levels of **a**-specific gene mRNA present in α cells. Briefly, RNA was isolated from cells, reverse transcribed (Superscript II; BRL) by using a *STE2*-specific primer, and added to PCR mixtures containing known amounts of a competitor DNA that was amplified with the same *STE2* primers as the cDNA but that resulted in a smaller PCR product due to an internal deletion in the *STE2* gene. The relative amounts of target cDNA versus competitor can be measured by direct scanning of ethidium-stained gels (1-D Multi-Lane Scan, IS-1000 Digital Imaging System), and these amounts can be compared between *MAT*α and *mat*Δ cells to determine the level of repression of an **a**-specific gene.

Genomic footprinting. In vivo footprinting was performed as previously described, with modifications (1). Yeast strains were grown in 100 ml of synthetic medium minus uracil plus 2% galactose, 3% glycerol, and 2% ethanol to a density of 10^7 cells per ml. The cells were pelleted and resuspended in ice-cold medium to a final volume of 1 ml. A 5-μl volume of dimethyl sulfate was added with vigorous mixing. The cells were incubated at 20°C for 5 min, after which the reaction was quenched with 50 ml of ice-cold 10 mM Tris (pH 7.5)–1 mM EDTA. The cells were pelleted and resuspended in 900 μl of lysis buffer (50 mM morpholinepropanesulfonic acid [pH 7.0], 200 mM NaCl, 5 mM EDTA, 0.5% Triton X-100). The cells were lysed with glass beads (0.5-mm diameter) for 45 s in a bead beater (Biospec Products). The lysate was removed from the glass beads and diluted in 3.5 ml of additional lysis buffer. The lysate was treated with RNase A (250 μg/ml) and proteinase K (100 μg/ml) for 1 h at 37°C. The cellular debris was pelleted (12,000 × *g* for 20 min), and genomic DNA was prepared by loading the supernatant onto a Qiagen column (Qiagen Inc., Studio City, Calif.). DNA was then digested with *Hae*III, phenol chloroform extracted, ethanol precipitated, and resuspended in 100 μl of Tris-EDTA. Finally, the DNA was dialyzed against water (12,000- to 14,000-Da exclusion limit) for 2 h.

Methylated bases were detected by multiple rounds of primer extension with *Taq* polymerase. A 0.5-μg amount of DNA from cells with 2μm plasmids or 10 μg from cells with single-copy reporters, 1 pmol of end-labeled primer, 1 U of *Taq* polymerase, 200 μM each deoxynucleoside triphosphate, and 1× *Taq* buffer (40 mM NaCl, 10 mM Tris [pH 8.9], 5 mM $MgCl_2$, 0.01% gelatin [30]) were combined in a total volume of 50 μl. Mineral oil was layered over the samples,



	$pASG_{2\mu}$	$pASG_{int}$		$pGAL_{2\mu}$	$pGAL_{int}$
MATα	3	0.15	MATα	88	11
matΔ	2500	125	matΔ	3900	217
MATα gal4::LEU2	2	0.17	MATα gal4::LEU2	2	0.14
matΔ gal4::LEU2	220	8	matΔ gal4::LEU2	30	2

FIG. 1. α2 represses test constructs activated by Gal4. The test constructs are diagrammed at the top of the figure. Each construct consists of a single Gal4-binding site and a single **a**-specific gene (asg) operator upstream of a *CYC1 lacZ* promoter fusion. The distances in base pairs between the promoter elements are indicated. At the bottom of the figure are the results of β-galactosidase activity assays performed with four different strains. Values are the averages of assays performed in duplicate on three independent transformants.

which were then subjected to 10 to 20 rounds of thermal cycles (1 min at 94°C, 2 min at 55 to 63°C, and 1 min at 72°C). The mineral oil was extracted with chloroform, and the samples were ethanol precipitated. The pellets were washed with 70% ethanol, dried briefly, and resuspended in 4 μl of formamide loading buffer. The primer extension products were then electrophoresed through a 6% polyacrylamide sequencing gel. The gels were dried and exposed to Kodak XAR-5 film for 12 to 24 h. Note that many methylated guanines appear as doublets by *Taq* polymerase primer extension because of the variable addition of an extra nucleotide. This does not affect the interpretation of these results.

Plasmid DNA was methylated in vitro as described by Maxam and Gilbert (28), and 10 ng was used for primer extension as described above. Neither the methylated plasmid DNA nor the genomic DNA was treated with piperidine, since this step is unnecessary (4).

The primers used in this study were as follows. For plasmid $pASG_{2\mu}$, the bottom-strand primer (5′-ATCCACGCTATATACACGCCTGGC-3′) anneals to top-strand sequences in the *CYC1* promoter from positions −236 to −212 with respect to the first codon. The $pGAL_{2\mu}$ primer (5′-CTAAAGTTGCCTGGCCA TCCACGC-3′) anneals to the top strand of the *CYC1* promoter from positions −220 to −196 with respect to the first codon. The primers used for the coding and noncoding strands of plasmid $pGAL_{2\mu}$ were 5′-AACTGTATTATAAGTAA ATGCATG-3′ and 5′-TGCCATATGATCATGTGTCGTCGC-3′, respectively. For the integrating constructs, primers were designed that hybridized to sequences in both the *CYC1* promoter ($pASG_{int}$) and the *URA3* gene ($pGAL_{int}$), as well as in the *STE6* operator, in order to avoid background from the native yeast genes. For $pASG_{int}$, the primer used was 5′-CGGATCTGCTCGACGA GCGTGTAA-3′. The primer used for $pASG_{2\mu}$ yielded the same results. For $pGAL_{int}$, the primer used was 5′-TCAGTTATTACCCTCGACCTCGTCG-3′.

Isolation and analysis of chromatin. Chromatin was isolated from four strains (*MAT*α, *mat*Δ, *MAT*α *gal4*::*LEU2*, and *mat*Δ *gal4*::*LEU2*) containing promoter constructs $pGAL_{2\mu}$, $pGAL_{int}$, or $pASG_{2\mu}$, according to the Nonidet P-40-permeabilized spheroplast method (22). Briefly, the cells were grown in the medium used for the β-galactosidase assays to an optical density (A_{660}) of 0.8, washed with 1 M sorbitol, and digested with 0.5 mg of Zymolyase T100 (ICN) per ml. Nuclei were washed and resuspended in buffer containing 1 M sorbitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol, and 0.075% Nonidet P-40. The nuclei were digested for 5 min at 37°C with micrococcal nuclease (MNase) (Worthington Biochemical Corp.) concentrations ranging from 0 to 25 U/ml. DNA was purified by phenol extraction after digestion with proteinase K and RNase A. Naked DNA was prepared in this manner before MNase digestion with 7.5, 15, or 30 U/ml for 1 min at 37°C. Indirect end label analysis was used to determine the positions of nuclease-sensitive regions according to the method described by Thoma et al. (40). Chromatin and naked DNA were cut with a variety of restriction enzymes that cut either in the *lacZ* gene or in the *URA3* gene. The enzymes used that cut in *lacZ* (with the distance from the start of the **a**-specific gene operator in pGAL constructs or from the start of the Gal4-binding site in pASG constructs indicated in parentheses) were *Hpa*I (853 bp), *Dde*I (534 bp), and *Fsp*I (451 bp). The enzymes used that cut in *URA3* were *Stu*I (441 bp) and *Dde*I (160 bp). Probes were generated by PCR and varied in length from 50 to 238 bp.

RESULTS

α2 represses Gal4-activated promoters. The chromatin reorganization model for repression predicts that DNA near the operator should be less accessible to proteins than is naked DNA. To determine whether an α2-repressed promoter is accessible to Gal4, hybrid promoters containing a single Gal4-binding site and a single **a**-specific gene operator upstream of a *CYC1* β-galactosidase promoter fusion were constructed (Fig. 1). The Gal4-binding site was placed either upstream (pASG) or downstream (pGAL) of the **a**-specific gene operator with respect to the *CYC1* promoter. The plasmid names reflect the DNA element, either the Gal4-binding site or the **a**-specific gene operator, that lies adjacent to the *CYC1* promoter. Promoter constructs either were placed on multicopy 2μm yeast plasmids ($pASG_{2\mu}$ and $pGAL_{2\mu}$) or were integrated into the chromosome at the *URA3* locus ($pASG_{int}$ and $pGAL_{int}$). To assess whether these test promoters were activated by Gal4 and whether activated transcription could be repressed by α2, the constructs were transformed into the following four different cell types: cells containing both α2 and Gal4 (*MAT*α *GAL4*), cells containing only Gal4 (*mat*Δ *GAL4*) or only α2 (*MAT*α *gal4*::*LEU2*), and cells lacking both proteins (*mat*Δ *gal4*::*LEU2*). In the presence of galactose, the promoters are activated 10- to 130-fold by Gal4 (Fig. 1; compare values from *mat*Δ *GAL4* cells with those from *mat*Δ *gal4*::*LEU2* cells). Furthermore, α2 represses transcription approximately 800-fold relative to the activated level when the operator is positioned between the Gal4 site and the *CYC1* promoter ($pASG_{2\mu}$ and $pASG_{int}$) and about 30-fold when the operator is positioned upstream of the Gal4-binding site ($pGAL_{2\mu}$ and $pGAL_{int}$; compare expression from *MAT*α *GAL4* cells with that from *mat*Δ *GAL4* cells). These results indicate that α2 is capable of efficiently repressing activated transcription from these constructs. The fact that the repression is greater when the operator is between the Gal4-binding site and the promoter than when the operator is upstream of the Gal4-binding site is consistent with the behavior of the operator in other test constructs (19). The expression of the constructs in *mat*Δ *gal4*::*LEU2* strains is presumably due to activation by the MCM1 protein bound to the **a**-specific gene operator (2, 20).

The level of repression of an a-specific gene correlates with the repression of the hybrid reporters. We wished to know whether the strong repression (20- to 800-fold) of the test promoters is comparable to that of a bona fide **a**-specific gene. To determine the magnitude of α2 repression of the **a**-specific gene *STE2*, we employed quantitative RNA PCR analysis (9).

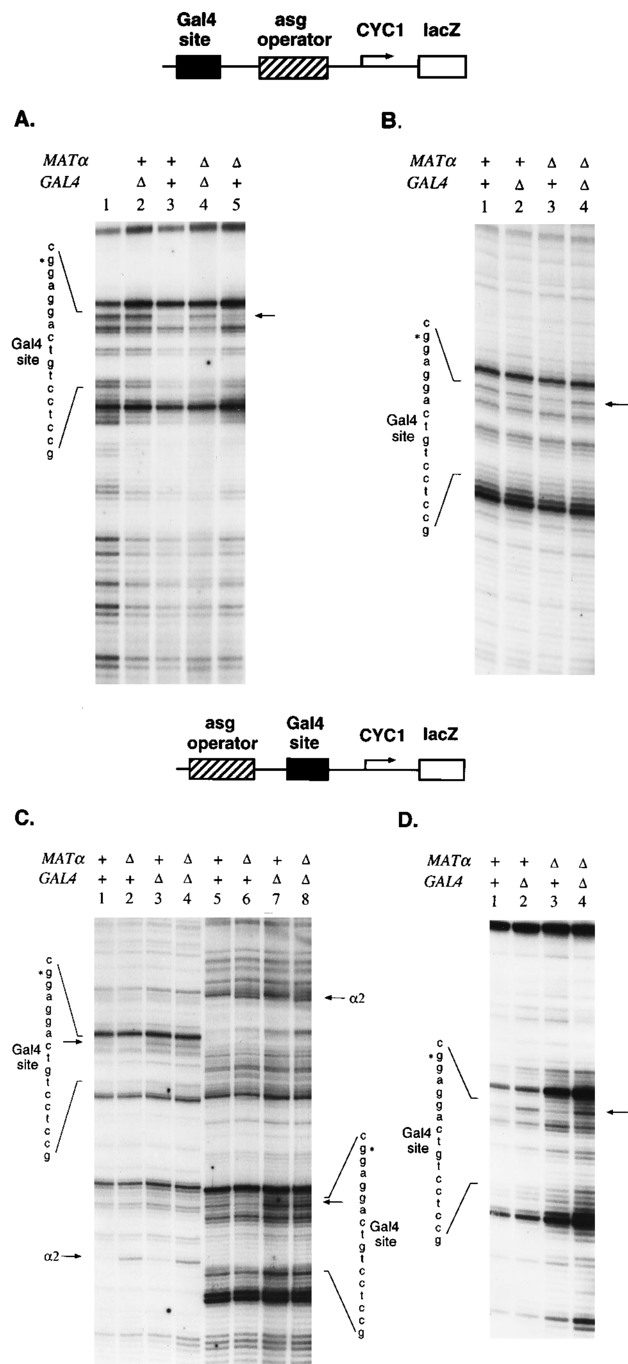


FIG. 2. Gal4 can occupy its site when the test constructs are repressed. Each panel shows the primer extensions from in vivo methylated DNA of the indicated test promoters in four different cell types: *MAT*α, *mat*Δ, *MAT*α *gal4*::*LEU2*, and *mat*Δ *gal4*::*LEU2*. The Gal4-binding site is indicated. The strong bands that bracket the Gal4-binding site are sequence-specific stops for *Taq* polymerase. The Gal4 footprint is clearly detected in $GAL4^{+}$ strains and is indicated by an arrow. The constructs are diagrammed over the appropriate panels. (A) Primer extension of the noncoding strand of promoter construct $pASG_{2\mu}$. Lane 1, extension products from in vitro-methylated plasmid DNA. Coding strand primer extension yields similar results (not shown). (B) Primer extension of the coding strand of promoter construct pASG integrated at *URA3*. (C) Primer extension of the coding strand (lanes 1 to 4) and the noncoding strand (lanes 5 to 8) of promoter construct $pGAL_{2\mu}$; (the α2 footprint is indicated by an arrow). (D) Primer extension of the noncoding strand of promoter construct pGAL integrated at *URA3*. asg, **a**-specific gene.

The results indicate that *STE2* transcription is repressed 200-fold in α cells relative to **a** cells (which lack α2), a result that is comparable to that observed in the test promoters, in which the α2 operator is located between the Gal4-binding site and the promoter (data not shown). This result indicates that the test promoters used in this study provide a legitimate model system in which to analyze α2 repression.

Gal4 can occupy its site when the test constructs are repressed. In principle, α2 repression of the test promoters could result either from interference with Gal4 DNA binding or from interference with a subsequent step in transcription initiation. In order to determine whether α2 interferes with Gal4 DNA binding in vivo, we performed dimethyl sulfate footprinting experiments on growing yeast cells. When bound to DNA, Gal4 protects a single guanine on each strand of its binding site from methylation by dimethyl sulfate (11). This protection can be seen in Fig. 2A by comparing the results from DNA isolated from strains that contain Gal4 (lanes 3 and 5) with those that lack it (lanes 2 and 4). In the case of constructs $pASG_{2\mu}$ and $pGAL_{int}$, a Gal4 footprint can be detected both in the activated state (*mat*Δ *GAL4* cells) and in the repressed state (*MAT*α *GAL4* cells) (compare lanes 3 and 5 in Fig. 2A and lanes 1 and 3 in Fig. 2D). For construct $pGAL_{2\mu}$, a clear Gal4 footprint is visible when the construct is active, and a weaker footprint is visible under repressed conditions (Fig. 2C; compare lanes 5 and 6). In the case of construct $pASG_{int}$, a Gal4 footprint is seen in *mat*Δ cells but cannot be detected in α cells (Fig. 2B; compare lanes 1 and 3). In three of four of the test promoters (including the most strongly repressed), Gal4 occupies its binding site under conditions in which transcription is tightly repressed (*MAT*α *GAL4* cells). These results indicate that α2 must repress transcription by some means other than preventing the DNA binding of activator proteins. We do not know the reason why Gal4 fails to occupy one of the repressed templates; however, the results obtained with the other three templates prove that repression can occur even though Gal4 is bound. We also note that the α2 footprint can be seen in these experiments (Fig. 2C, lanes 1, 3, 5, and 7, as indicated).

Nucleosomes are not positioned over test promoters. It has been observed that α2 bound to DNA positions nucleosomes adjacent to it, and it has been proposed that this positioning can contribute to transcriptional repression. In contrast to the behavior of α2, DNA-bound Gal4 is able to disrupt binding of the core histone particle both in vitro and in vivo (29, 46). To assess the role of nucleosome positioning in transcriptional repression of the test constructs used in this study, we mapped the distribution of nucleosomes over these constructs in both active and repressed states. Chromatin was isolated and digested with MNase, and the relevant regions of the DNA were displayed by indirect end labeling (40). Digestion patterns across the test promoter $pGAL_{2\mu}$ resembled those of the naked DNA controls (Fig. 3), indicating a lack of positioned nucleosomes even when Gal4 is absent (*MAT*α *gal4*::*LEU2*). Moreover, the digestion patterns across test construct $pGAL_{2\mu}$ were not observably different in the presence or absence of α2, even though α2 had a dramatic effect on the expression of this construct. In the same chromatin preparations, positioned nucleosomes were seen across the *URA3* gene (in accordance with reference 3), which is located immediately upstream of the test promoter (Fig. 3; note the patterns of enhanced and protected bands in the chromatin preparations which are indicative of positioned nucleosomes [lanes 1 to 4] compared with naked DNA [lane 5]). This last observation indicates that the experiments shown in Fig. 3 are of sufficient resolution to detect positioned nucleosomes. Moreover, we detected positioned nucleosomes across the promoter of the **a**-specific gene

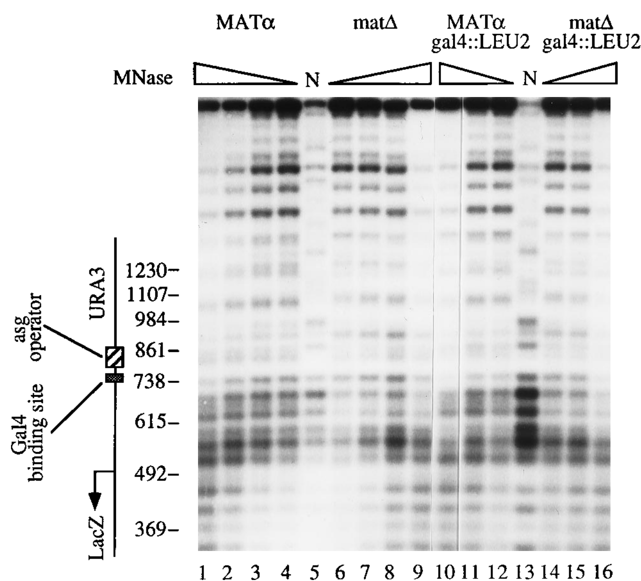


FIG. 3. MNase mapping of the pGAL$_{2\mu}$ promoter region. The indirect-end-labeling method was used to display the results of MNase digestion of chromatin isolated from four strains. Chromatin and naked DNA were cut with *Hpa*I after digestion with MNase. *Hpa*I cuts in the *lacZ* gene, 853 bp downstream of the beginning of the **a**-specific gene (asg) operator. The labeled primer used for indirect end labeling is 238 bp long, extending from the *Hpa*I site in *lacZ* toward the **a**-specific gene operator. Lanes 1 to 4, chromatin isolated from the *MAT*α strain and digested with decreasing amounts of MNase (6, 3, 1.5, and 0.75 U/ml); lanes 6 to 9, chromatin isolated from the *mat*Δ strain and digested with the same but increasing amounts of MNase; lanes 10 to 12, chromatin isolated from the *MAT*α *gal4*::*LEU2* strain and digested with decreasing amounts of MNase (6, 1.5, and 0.75 U/ml); lanes 14 to 16, chromatin isolated from the *mat*Δ *gal4*::*LEU2* strain and digested with increasing amounts of MNase (0.75, 1.5, and 3 U/ml). N, naked DNA digested with 15 U of MNase (lane 5) or 30 U of MNase (lane 13) per ml. Size markers in base pairs are indicated on the left, along with a diagram indicating the positions of the **a**-specific gene operator and the Gal4-binding site, as well as the *lacZ* and *URA3* genes.

STE2 (in accordance with the results described by Ganter et al. [8]), again suggesting that the failure to observe positioned nucleosomes across the artificial promoters is not due to a problem in detecting nucleosomes (data not shown). We repeated nucleosome mapping with the additional promoters (pGAL$_{int}$ and pASG$_{2\mu}$) and, in agreement with the results of Fig. 3, observed no evidence of positioned nucleosomes over any of the hybrid promoters in any of the four strains used in this work (data not shown).

DISCUSSION

This study demonstrates that α2 can efficiently repress transcription of a simple, artificial test promoter while still allowing access of the activator protein GAL4 to its binding site on the DNA. Thus, the α2 repressor must block transcription at a step subsequent to activator binding. On the surface, the presence of GAL4 on the DNA of the repressed promoters seems at odds with the proposal that α2 represses transcription by positioning nucleosomes around its binding site. On the basis of experiments performed in vivo and in vitro (29, 46), DNA-bound GAL4 appears to disrupt nucleosomes. One might have predicted that GAL4 would prevent the nucleosome positioning on the constructs described in this article. This idea was tested experimentally, and the results indicate a lack of specifically positioned nucleosomes regardless of whether GAL4 is present on the DNA.

The failure to detect positioned nucleosomes in the absence of Gal4 was initially surprising in light of the strong nucleosome positioning produced by α2 on native **a**-specific genes. However, the test promoter differs from those of **a**-specific genes in several ways. The TATA boxes and the transcription start site of the hybrid promoters are derived from the *CYC1* promoter. One feature of the *CYC1* promoter that might explain the absence of positioned nucleosomes is the constitutive binding of TBP to the TATA box of this promoter as proposed by Chen et al. (6). These investigators found that a derivative of the *CYC1 lacZ* promoter lacking upstream repressor or activator sites was free of positioned nucleosomes. Furthermore, in vivo footprinting indicated that TBP was bound to the TATA elements of this silent *CYC1 lacZ* promoter (also see reference 5). Our results could be explained by the model that TBP is bound to the TATA elements and prevents the *CYC1* promoter from being packaged in nucleosomes. With respect to TBP binding, the *CYC1* promoter may differ from other yeast promoters, including those of some **a**-specific genes. Despite this fact, the *CYC1* promoters used in this study were very strongly activated by Gal4 and were strongly repressed by α2, suggesting that the differences in initial TBP binding among promoters is relatively unimportant for regulation by these proteins. Finally, if TBP bound to the *CYC1* TATA elements does prevent nucleosomes from forming over this promoter, one might have predicted that a repressor that acts solely by nucleosome positioning would be unable to repress the *CYC1* promoter. As shown here and elsewhere (19, 21), α2 can tightly repress this promoter and the level of repression can be even higher than that of a bona fide **a**-specific gene.

If α2 does not repress transcription by controlling access of activator proteins to DNA, how does it work? Since α2 can repress basal transcription in vitro (13), it has been proposed that the α2 repression complex may act directly on the basal transcription machinery, interfering with a step in transcription initiation. In further support of this model is the discovery that components of the RNA polymerase II holoenzyme are required for efficient α2 repression (25, 27, 43). Direct interference with the basal transcription machinery seems an apt mechanism for a repressor such as α2 that must efficiently repress a large number of genes that utilize a variety of activator proteins.

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