EUKARYOTIC CELL, Nov. 2006, p. 1925–1933 Vol. 5, No. 11
1535-9778/06/$08.00+0 doi:10.1128/EC.00105-06

Effect of Sequence-Directed Nucleosome Disruption on Cell-Type-Specific Repression by α2/Mcm1 in the Yeast Genome▽

Nobuyuki Morohashi,[1] Yuichi Yamamoto,[1] Shunsuke Kuwana,[1] Wataru Morita,[1] Heisaburo Shindo,[2] Aaron P. Mitchell,[3] and Mitsuhiro Shimizu[1]*

Department of Chemistry, Meisei University, Hino, Tokyo 191-8506, Japan[1]; School of Pharmacy, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-0392, Japan[2]; and Department of Microbiology, Columbia University, New York, New York 10032[3]

Received 12 April 2006/Accepted 31 August 2006

In *Saccharomyces cerevisiae*, a-cell-specific genes are repressed in *MAT*α cells by α2/Mcm1, acting in concert with the Ssn6-Tup1 corepressors and the Isw2 chromatin remodeling complex, and nucleosome positioning has been proposed as one mechanism of repression. However, prior studies showed that nucleosome positioning is not essential for repression by α2/Mcm1 in artificial reporter plasmids, and the importance of the nucleosome positioning remains questionable. We have tested the function of positioned nucleosomes through alteration of genomic chromatin at the a-cell-specific gene *BAR1*. We report here that a positioned nucleosome in the *BAR1* promoter is disrupted in *cis* by the insertion of diverse DNA sequences such as poly(dA) · poly(dT) and poly(dC-dG) · poly(dC-dG), leading to inappropriate partial derepression of *BAR1*. Also, we show that *isw2* mutation causes loss of nucleosome positioning in *BAR1* in *MAT*α cells as well as partial disruption of repression. Thus, nucleosome positioning is required for full repression, but loss of nucleosome positioning is not sufficient to relieve repression completely. Even though disruption of nucleosome positioning by the *cis*- and *trans*-acting modulators of chromatin has a modest effect on the level of transcription, it causes significant degradation of the α-mating pheromone in *MAT*α cells, thereby affecting its cell type identity. Our results illustrate a useful paradigm for analysis of chromatin structural effects at genomic loci.

In *Saccharomyces cerevisiae*, **a**-cell-specific genes are repressed in *MAT*α cells by the α2/Mcm1 repressor, in concert with the corepressors Tup1-Ssn6, and several models for repression mechanisms have been proposed (46). Tup1-Ssn6 may interact with the general transcriptional machinery to inhibit transcription directly, or it may interfere with transcriptional activator function (12, 18, 20, 21, 29, 34). Nucleosomes are precisely positioned in the promoters of **a**-cell-specific genes in the repressed state in *MAT*α cells but are not positioned in the activated state in *MAT***a** cells (9, 14, 37, 42), and the presence and absence of nucleosome positioning is not a consequence of transcription (9). Tup1 interacts with histones and histone deacetylases (5, 10, 11, 51, 52), and a *tup1* mutation causes both disruption of nucleosome positioning and repression (9, 57). Also, the Isw2-Itc1 chromatin remodeling complex (17) is involved in regulation of Tup1-Ssn6-repressed genes (56), including **a**-cell-specific genes (16, 38). Thus, it has been proposed that positioned nucleosomes may modulate the accessibility of promoters to transcription factors to repress the genes (44).

However, some studies suggest that nucleosome positioning is not essential for repression by α2/Mcm1. When the **a**-cell-specific *STE6* TATA box is placed at different locations in a positioned nucleosome and in the internucleosomal linker in *STE6-lacZ* reporter plasmids, no expression is detectable, even with the TATA box located in a linker region (31). Nucleosomes are not positioned in a test *CYC1* promoter containing the α2 operator and Gal4 binding site and Gal4 can occupy its site, even though the test promoter is repressed by α2/Mcm1 (35). α2/Mcm1-dependent repression occurs in a naked DNA template in vitro (18). Also, the role of nucleosome positioning in repression of **a**-cell-specific genes has been examined by introducing mutations in histones and in other factors, such as Tup1, Ssn6, and histone deacetylases (37, 51). However, interpretation of these mutations is complicated by the fact that they have highly pleiotropic effects. Thus, although positioned nucleosomes have been observed in a number of promoters in yeast and mammalian cells, the importance of the positioning has remained questionable.

DNA can adopt several types of conformations as dictated by its sequence (45), and genomic analyses show that alternative DNA structure-forming sequences are represented in eukaryotic genomes (7, 39). Among such sequences, poly(dA) · poly(dT) and poly(dG) · poly(dC) as well as Z-DNA-forming sequences do not form nucleosomes reconstituted from purified histone octamer (2, 6, 15, 43), whereas CTG repeats preferentially bind to histone octamers in vitro (50). We have shown that the unusual *B′* conformation, adopted by longer poly(dA) · poly(dT) sequences, disrupts an array of positioned nucleosomes in yeast cells (41). Poly(dA) · poly(dT) sequences in the yeast *HIS3* promoter (19) and the *Candida glabrata AMT1* gene (58) stimulate transcription by improving accessibility to the promoter in vivo. The nucleosome-free sequences were evolutionarily conserved and are enriched in poly(dA) · poly(dT) sequences as revealed by genome-scale analysis of yeast chromosome III (55). Poly(dA) · poly(dT) as well as $(CCGNN)_n$, both of which do not favor nucleosome formation, can act as efficient boundaries of silent chromatin (4, 54). Z-DNA is required for the activation of the human CSF1

* Corresponding author. Mailing address: Department of Chemistry, Meisei University, 2-1-1 Hodokubo, Hino, Tokyo 191-8506, Japan. Phone: 81-42-591-7483. Fax: 81-42-591-8181. E-mail: shimizum@chem.meisei-u.ac.jp.

▽ Published ahead of print on 15 September 2006.

promoter by the SWI/SNF-like BAF complex, and it is thought that Z-DNA formation promoted by the BAF complex stabilizes the open chromatin structure at the promoter (23, 24). Thus, DNA structural properties may be used to modulate the positioning of nucleosomes in vivo to alter gene expression.

In this report, we have developed a systematic strategy to test critically the role of nucleosome positioning in repression of **a**-cell-specific gene *BAR1* using nucleosome-disrupting sequences. We show here that longer poly(dA) · poly(dT) and poly(dC-dG) · poly(dC-dG) inserts block formation of a positioned nucleosome in the promoter to cause partial derepression of *BAR1*, while shorter inserts, CTG and GAGCTC repeats, are incorporated into a positioned nucleosome to maintain the repressed *BAR1* state. These results indicate that nucleosome positioning contributes to full repression by α2/Mcm1, but it is solely responsible for repression.

MATERIALS AND METHODS

Yeast strains and plasmids. Yeast strains used were FY23 (*MAT***a** *ura3-52 trp1Δ63 leu2Δ1*) and FY24 (isogenic to FY23 except for *MAT*α), which were obtained from the Yeast Genetics course at Cold Spring Harbor Laboratory. To construct strains with a modified *BAR1* promoter, we cloned the −500 to +51 region of *BAR1* into pRS306ΔKI, a pRS306 derivative in which the KpnI site in pRS306 was filled in, forming pYY1-2. Then, mutations in the sequence AATGT at a region −158 to −154 to GTACC were introduced to create a KpnI site in the *BAR1* promoter by PCR, forming pAS1-8. A pair of oligonucleotides synthesized chemically was annealed and cloned into the KpnI site of pAS1-8. The portions of the *BAR1* promoter sequence were replaced with $(CTG)_{12}$ or $(CG)_7$ by two-step PCR, and the modified promoter fragments were recombined with pAS1-8 in vivo in yeast. All the modified promoters were verified by DNA sequencing. Plasmids containing the modified *BAR1* promoters were digested with XbaI and were integrated into the genomic *BAR1* locus in FY23 and FY24, and the plasmid portions were looped out by two-step gene replacement. *isw2*Δ strains, MHS303 and MHS314, were constructed from wxy292 and wxy293, respectively, by one-step gene replacement using pFA6aMXHIS3 (53). Strains constructed in this study are listed in Table 1.

Halo assay. To assay for the generation of α-factor halo (27), *MAT***a** *sst1* cells were grown for 24 h. Aliquots were then diluted to an optical density at 600 nm (OD_{600}) of 0.5, and 100 μl of the diluted culture (~10^6 cells) was spread on a YEPD (1% yeast extract, 2% peptone, 2% glucose) plate and allowed to dry. Spots of strains of interest were grown on YEPD plates overnight, the plates were replica plated to the *sst1* spread plates, and the replica plates were incubated for 2 days.

Northern blot analysis. Cells were grown to an OD_{600} of 0.5 to 1.0, harvested, and snap-frozen in a dry ice-ethanol bath. RNA was prepared by a hot phenol method (47). Northern blot analysis was performed as described previously (3). A *BAR1* fragment (+1 to +500) was prepared by PCR using 5′-ATG TCT GCA ATT AAT CAT CTT TGT TTG AAA-3′ and 5′-ACG GGT GTC GTA GCA TAC TTG GCA ACT CCG-3′ as 5′-forward and 3′-reverse primers, respectively. A Northern probe for *BAR1* was prepared by a random priming reaction with the *BAR1* fragment or an *ENO1* fragment as described elsewhere (3).

Analysis of chromatin structure. Yeast cells were cultured in YEPD medium until the OD_{600} reached ~1.0. Nuclei were isolated, and micrococcal nuclease (MNase) digestion proceeded as described previously (41, 42). Cleavage sites for MNase were analyzed by primer extension mapping using a primer with the *BAR1* −391 to −357 sequence as described elsewhere (42).

TABLE 1. Strains used in this study

Strain	Genotype
FY23	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1*
FY24	*MAT*α *ura3-52 trp1Δ63 leu2Δ1*
MHS114	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-KpnI*
MHS180	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CTG)_{12}$
MHS177	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*A_{20}
MHS191	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*A_{25}
MHS196	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*A_{30}
MHS116	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*A_{34}
MHS170	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-2x*A_{34}
MHS507	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*T_{20}
MHS193	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*T_{25}
MHS552	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CG)_4$
MHS547	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CG)_5$
MHS543	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CG)_6$
MHS526	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CG)_7$
MHS530	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CG)_7TATA(CG)_7$
MHS751	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*Sac_5
MHS752	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*Sac_6
MHS772	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CTG)_{12}SB$
MHS747	*MAT*α *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CG)_7SB$
MHS109	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-KpnI*
MHS713	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CTG)_{12}$
MHS714	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*A_{20}
MHS715	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*A_{25}
MHS716	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*A_{30}
MHS112	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*A_{34}
MHS159	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-2x*A_{34}
MHS717	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*T_{20}
MHS718	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*T_{25}
MHS719	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CG)_4$
MHS720	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CG)_5$
MHS721	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CG)_6$
MHS722	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CG)_7$
MHS723	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CG)_7TATA(CG)_7$
MHS749	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*Sac_5
MHS750	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*Sac_6
MHS768	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CTG)_{12}SB$
MHS745	*MAT***a** *ura3-52 trp1Δ63 leu2Δ1 BAR1-*$(CG)_7SB$
MHS303	*MAT***a** *ura3 trp1 leu2 his3 lys2 ho::LYS2 isw2::HIS3*
MHS314	*MAT*α *ura3 trp1 leu2 his3 lys2 ho::LYS2 isw2::HIS3*

RESULTS

Experimental design. Previous studies indicate that the yeast α2/Mcm1 repressor positions nucleosomes adjacent to the α2 operator in **a**-cell-specific genes, such as *BAR1*, *STE2*, and *STE6*, in the genome (9, 14, 37, 42), as well as in yeast minichromosomes containing an α2 operator (36, 42). Here, we have implemented a strategy to test the functional significance of the positioned nucleosomes by introducing short, diverse nucleosome-disrupting sequences into the *BAR1* genomic locus. The *BAR1* promoter was modified by the insertion of poly(dA) · poly(dT), poly(dC-dG) · poly(dC-dG), CTG, or GAGCTC repeat sequences (Fig. 1; Table 1) as follows. We cloned the −500 to +51 region of *BAR1* in an integrative plasmid and introduced modification into the genomic *BAR1* locus by two-step gene replacement. In strains constructed in this study, a KpnI site was created at −158 in the *BAR1* promoter and A_n (n = 20, 25, 30, 34), $A_{34}GGTACCA_{34}$ (denoted as $2xA_{34}$), $(CG)_n$ (n = 4 to 7), $(CG)_7TATA(CG)_7$[denoted as $(CG)_{14}$], $(TGC)_{11}T$ [denoted as $(CTG)_{12}$, since this insert becomes $(CTG)_{12}$, including the neighboring KpnI sequence], $AC(GAGCTC)_5GA$ (denoted as Sac_5), or $AC(GAGCTC)_6GT$ (denoted as Sac_6) were inserted into the KpnI site in the *BAR1* promoter. Also, portions of the promoter sequence upstream of the KpnI site were replaced with $(CTG)_{12}$ or $(CG)_7$ [denoted as $(CTG)_{12}SB$ and $(CG)_7SB$, respectively] to maintain the native distance in the *BAR1* promoter. We predicted that longer poly(dA) · poly(dT) and poly(dC-dG) · poly(dC-dG) sequences would act as nucleosome-disrupting sequences (2, 6,

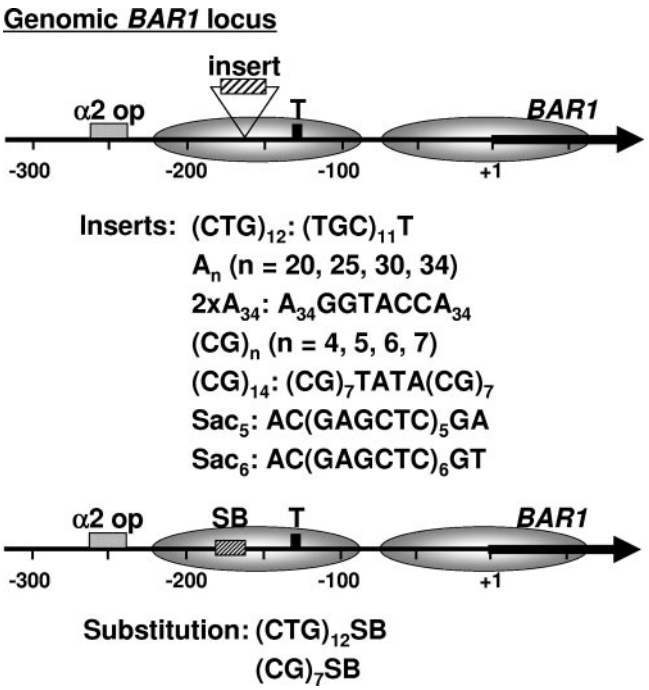


FIG. 1. Experimental design for this study. Nucleosomes (gray ellipses) are positioned at the *BAR1* promoter in *MAT*α cells from the α2 operator (α2 op) to the coding region, and the TATA box (T) is incorporated into the positioned nucleosome (42). The genomic *BAR1* promoter was modified to examine the effect of nucleosome destabilization. The nucleosome-disrupting sequences [poly(dA) · poly(dT) or poly(dC-dG) · poly(dC-dG)], a nucleosome-incorporating sequence, $(TGC)_{11}T$ [denoted as $(CTG)_{12}$; see text], or mixed sequences CA $(GAGCTC)_5GA$ and $CA(GAGCTC)_6GT$ (denoted as Sac_5 and Sac_6, respectively) were inserted into the KpnI site (−158) in the *BAR1* promoter in the genome. Portions of the *BAR1* promoter sequence upstream of the KpnI site were replaced with $(CTG)_{12}$ or $(CG)_7$ [denoted as $(CTG)_{12}$SB and $(CG)_7$SB, respectively, as indicated by a box with SB] to maintain the native distance in the *BAR1* promoter.

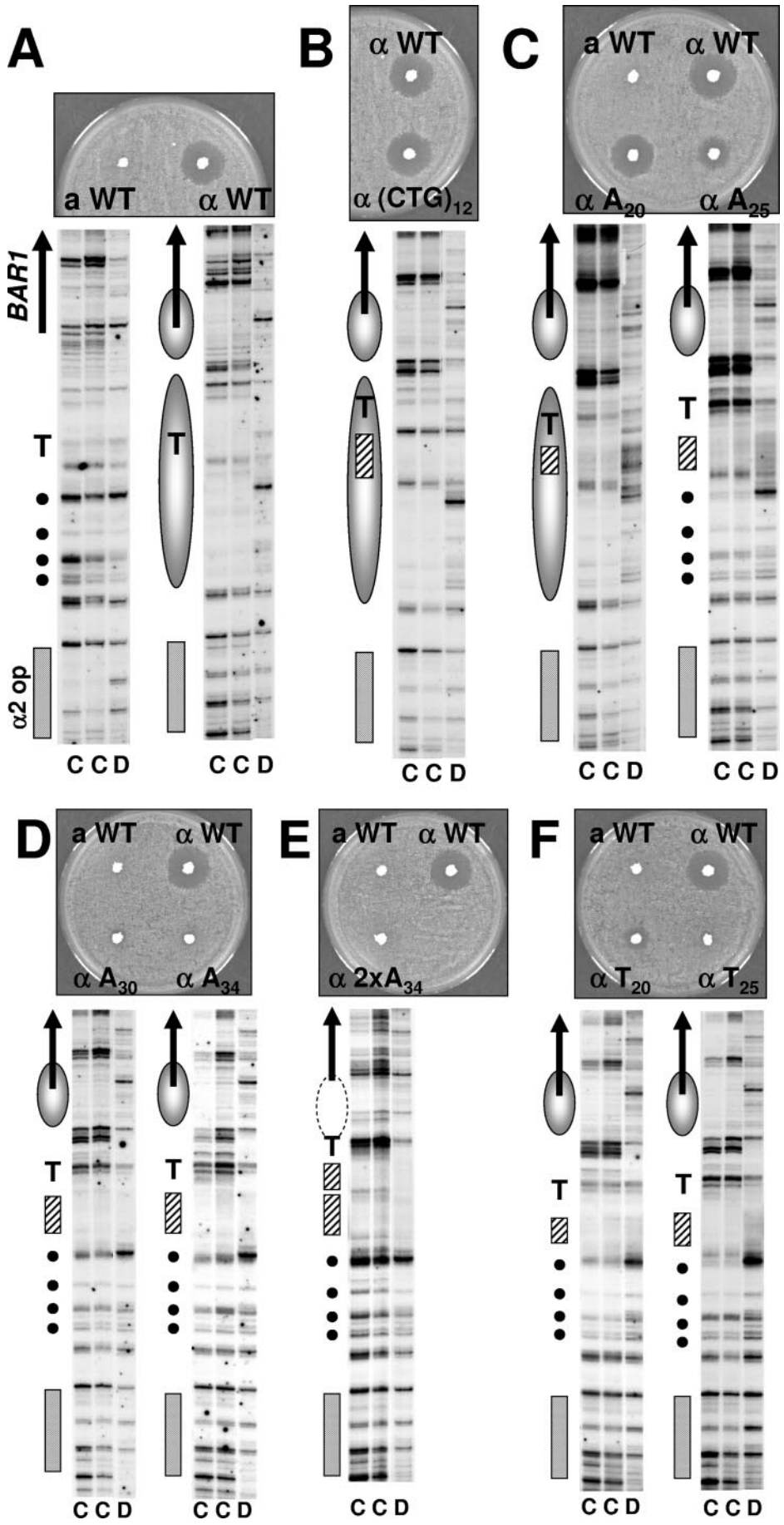
15, 41, 43, 48), whereas $(CTG)_{12}$ and mixed sequences Sac_5 and Sac_6 would serve as control inserts, since CTG and GA GCTC repeats were shown previously to be incorporated into nucleosomes (41, 50).

Effect of introduced sequences on *BAR1* expression. We examined the effect of nucleosome-disrupting sequences, such as poly(dA) · poly(dT) and poly(dC-dG) · poly(dC-dG), on repression of *BAR1* in *MAT*α cells through a halo assay (27), which reflects degradation of the α mating pheromone (α-factor) by the *BAR1* product, a protease. In this assay, strains to be tested for α-factor production were replica plated onto a lawn of the tester strain (*MAT***a** *sst1*), which is supersensitive to α-factor (8); a zone of growth inhibition (halo) in the *sst1* cells surrounding a tested colony indicates that the colony secretes α-factor. If *BAR1* were derepressed in *MAT*α cells, α-factor would degraded and the size of the halo would be diminished.

A halo was observed around control wild-type *MAT*α cells (α WT) but not wild-type *MAT***a** cells (**a** WT), as expected (Fig. 2A, upper portion). Halo size was unaffected by introduction of the KpnI site into the *BAR1* promoter (data not shown) or the insertion of control sequences $(CTG)_{12}$, Sac_5, or Sac_6 (Fig. 2B and 3). Insertion of A_{20} also had no effect on halo formation, but increased length of A_n ($n \geq 25$) decreased the halo size (Fig. 2C to E), indicating that these longer A_n tract insertions caused derepression. In addition to length, the orientation of poly(dA) · poly(dT) also affected derepression. Interestingly, T_n tracts (i.e., the T tract on the top strand of *BAR1*) were more effective than A_n tracts of identical length in causing *BAR1* derepression (Fig. 2C and F, compare α T_{20} and α T_{25} with α A_{20} and α A_{25}). Thus, in both orientations, poly (dA) · poly(dT) increased expression of *BAR1*. $(CG)_n$ also derepressed *BAR1*, as shown in Fig. 2G and H. The mating factor halo was undetectable after insertion of $(CG)_6$ or $(CG)_7$, and halo size was noticeably diminished by insertion of $(CG)_4$ or $(CG)_5$. Thus, $(CG)_n$ acts as a more powerful disruptor than A_n; the insertion of only ~10 to 14 bp leads to derepression of *BAR1*.

We evaluated the effect of spacing between the α2 operator and the TATA box that is altered by the insertions. First, three control inserts, $(CTG)_{12}$ (34 bp), Sac_5 (34 bp), and Sac_6 (40 bp), did not affect repression, as revealed by the halo assay (Fig. 3). This result indicates that spacing alone does not relieve repression. Second, we replaced a portion of the promoter sequence with $(CTG)_{12}$ or $(CG)_7$ to maintain native distance between the α2 operator and the coding region (Fig. 1, bottom). Halo size was unaffected by the $(CTG)_{12}$ substitution, whereas it was severely diminished by the $(CG)_7$ substitution (Fig. 3), which agreed with the results for the insertion of $(CTG)_{12}$ and $(CG)_7$. Thus, the substitutions of these sequences caused the same effect as the insertions (Fig. 2B and H), indicating that changes in promoter distance of the α2 operator from the TATA box are not required to cause changes in repression. Furthermore, it should be noted that the derepression level of *BAR1* increased as the length of $(CG)_n$ (insertions of 8, 10, 12, 14, and 32 bp) increased, as shown by Northern analysis (see Fig. 4, below). Thus, changes in the helical orientation of the α2 operator and TATA box do not affect repression. Therefore, derepression must result from the nature of the inserted sequences, rather than their effects on overall promoter length.

We examined *BAR1* expression by Northern blot analysis to confirm our interpretation of the halo assays (Fig. 4). There was strong expression of *BAR1* mRNA in the control *MAT***a** WT strain, whereas no signal was detected in *MAT*α WT, *MAT*α Kpn, *MAT*α $(CTG)_{12}$, *MAT*α A_{20}, *MAT*α A_{25}, *MAT*α T_{20}, and *MAT*α $(CG)_4$ strains. *BAR1* mRNA was detectable to some extent (1 to 15% of full expression in *MAT***a** WT) in *MAT*α A_{30}, *MAT*α A_{34}, *MAT*α $2xA_{34}$, *MAT*α T_{25}, and *MAT*α $(CG)_n$ ($n \geq 5$) strains. Even though mRNA levels are low in these strains, *BAR1* expression is sufficient to cause substantial α-factor degradation. Thus, the halo assay seems more sensitive than Northern analysis for monitoring changes in *BAR1* expression. Importantly, in keeping with the halo assays in Fig. 2, the derepressed mRNA levels showed length dependence for poly(dA) · poly(dT) and poly(dC-dG) · poly(dC-dG), and $(CG)_n$ affected *BAR1* expression more than A_n or T_n. We also examined expression from the modified *BAR1* promoters in a set of *MAT***a** strains in order to determine whether the inserted sequences cause adventitious promoter activation (Fig. 4). The level of *BAR1* mRNA in this series of *MAT***a** strains varied from 1.0- to 1.6-fold above the level in the *MAT***a** WT strain. Given that *BAR1* mRNA is undetectable in the *MAT*α WT


A
a WT
α WT
BAR1
T
α2 op
C C D
C C D
B
α WT
α (CTG)12
T
C C D
C
a WT
α WT
α A20
α A25
T
C C D
C C D
D
a WT
α WT
α A30
α A34
T
C C D
C C D
E
a WT
α WT
α 2xA34
T
C C D
F
a WT
α WT
α T20
α T25
T
C C D
C C D

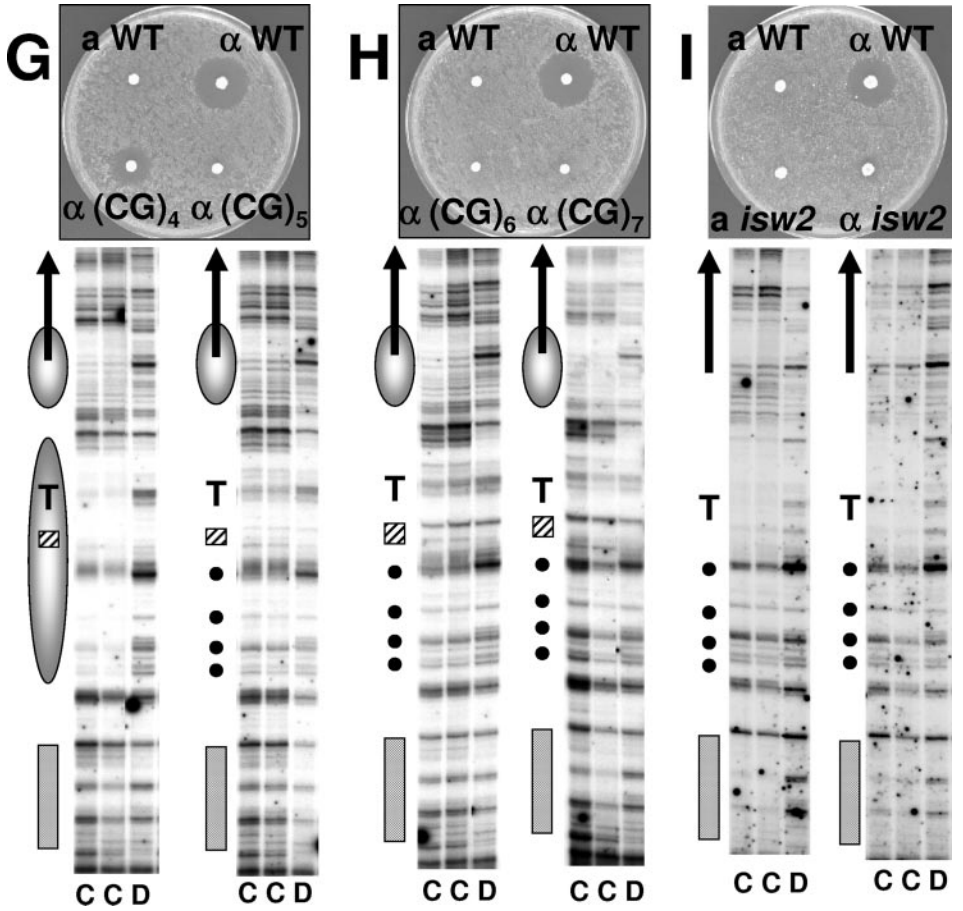


FIG. 2. Chromatin alteration by alternative DNA structure-forming sequences disrupts repression of the **a**-specific gene *BAR1* in the yeast genome. Expression of *BAR1*, as analyzed by halo assay, is shown in photographs of plates. Increased expression of *BAR1* in *MAT*α cells reduces the halo size, as explained in the text. **a** WT and α WT are wild-type strains. The designations $(CTG)_{12}$, A_n, T_n, and $(CG)_n$ refer to the top-strand sequences inserted into the *BAR1* promoter. *MAT***a** *isw2* and *MAT*α *isw2* are isogenic *isw2*Δ mutant strains with a wild-type *BAR1* promoter sequence. Autoradiograms of primer extension mapping of MNase cleavage sites in the genomic *BAR1* gene are shown under the halo assay plates. MNase cleavage sites downstream of the α2 operator were mapped. In each set of data, lanes labeled C indicate MNase digestion of isolated nuclei (chromatin) at two nuclease levels, and lanes labeled D indicate MNase digestion of the naked DNA as a control. Locations of the α2 operator (α2 op; gray shaded box), TATA box (marked with T), inserts (hatched box), the coding region of *BAR1* (arrows), and positioned nucleosomes (gray ellipses) are shown on the left side of each gel. An ellipse with a dotted line indicates a nucleosome whose positioning is uncertain in the *MAT*α $2xA_{34}$ strain (E). Black circles on the left side of the gels indicate characteristic cleavage sites between the α2 operator and TATA box in wild-type *MAT***a** cells.

strain, we infer that the derepression caused by active insertions is much greater than 1.6-fold. These results indicate that the insertion of nucleosome-disrupting sequences partially derepresses *BAR1* in its native genomic context in *MAT*α cells.

Chromatin alteration in the *BAR1* promoter by introduced sequences. We analyzed the chromatin structure of the genomic *BAR1* promoter region by limited digestion of nuclei with MNase and subsequent high-resolution primer extension mapping (Fig. 2, lower portions). The *BAR1* promoter region was cut with MNase in WT *MAT***a** cells, in which *BAR1* is expressed, whereas a region of about 140 bp adjacent to the α2 operator was protected from MNase digestion in WT *MAT*α cells, as indicated by a comparison of digested purified DNA (lanes marked "D") and digested chromatin (lanes marked "C") (Fig. 2A). These results indicate that nucleosomes are positioned adjacent to the α2 operator in *MAT*α cells but are not positioned in *MAT***a** cells, in good agreement with previous studies (9, 14, 37, 42, 44). The effects of promoter insertions on chromatin structure were monitored by the patterns of MNase cleavage between the α2 operator and TATA box (Fig. 2), the region in which the sequences were inserted. Insertion of control $(CTG)_{12}$ or shorter inserts, A_{20} and $(CG)_4$, did not significantly affect formation of positioned nucleosomes (Fig. 2B, C, and G). However, insertion of longer $A_n \cdot T_n$ ($n \geq 25$) sequences in *MAT*α cells led to increasing the MNase cleavage sites characteristic of *MAT***a** cell chromatin (Fig. 2C to F). Similarly, the MNase cleavage sites became stronger as the length of $(CG)_n$ ($n \geq 5$) tracts increased (Fig. 2G and H).

The nucleosome positioning adjacent to the α2 operator was destabilized to roughly the same extent by these longer A_n, T_n, and $(CG)_n$ sequences, although $(CG)_n$ inserts caused greater derepression of *BAR1* than A_n or T_n. At present, it is uncertain why the magnitude of derepression caused by poly(dA) · poly(dT) and poly(dC-dG) · poly(dC-dG) is dif-

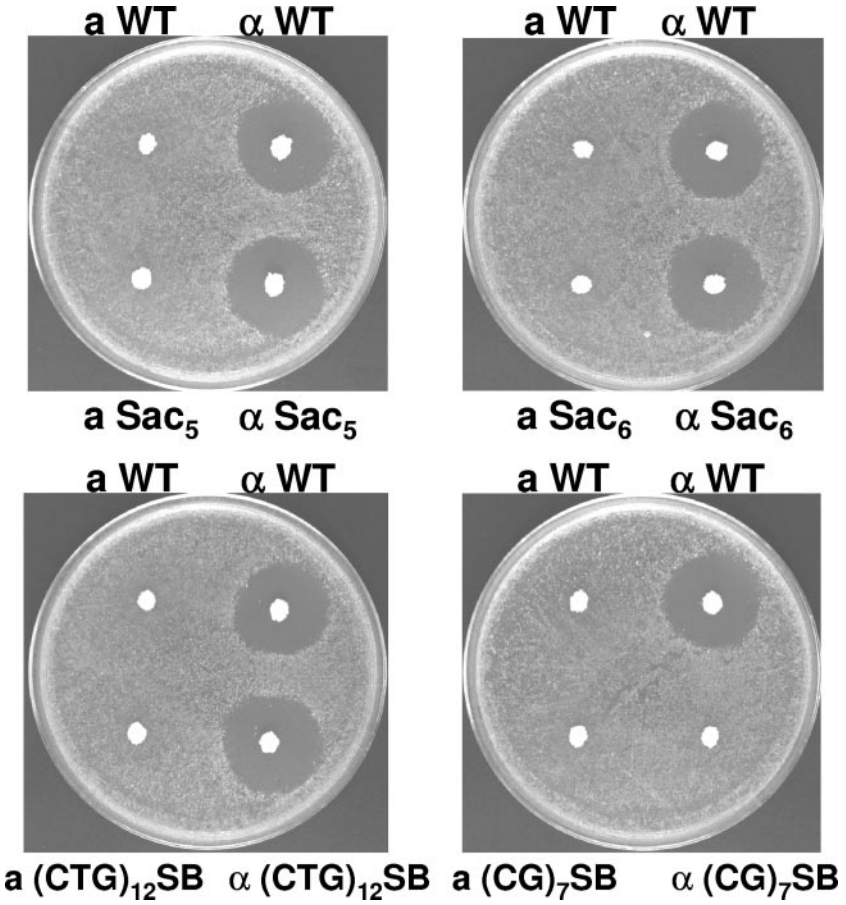


FIG. 3. Lack of effect of the distance of the α2 operator from the TATA box and of the helical orientation of the α2 operator with respect to the TATA box, as revealed by the halo assay. The designations Sac_5 and Sac_6 refer to $CA(GAGCTC)_5GA$ and $CA(GAGCTC)_6GT$ insertion, respectively, into the KpnI site (−158) in the *BAR1* promoter. The designations $(CTG)_{12}$SB and $(CG)_7$SB refer to the replacement of existing promoter sequence upstream of the KpnI site with $(CTG)_{12}$ and $(CG)_7$, respectively, to maintain the native distance between the α2 operator and the TATA box. **a** and α indicate the mating types of the strains.

ferent. It is possible that the types of alternative structures (*B′* conformation or Z-DNA) or intrinsic structural properties (local distortion and stiffness) may cause this effect. Our studies indicate that loss of nucleosome positioning is usually accompanied by partial relief of cell-type-specific repression of *BAR1*, as shown by halo assay and Northern analysis, although there is no simple relationship between the magnitudes of the two effects.

Chromatin alteration and derepression of *BAR1* in an *isw2* mutant. The Isw2 chromatin remodeling complex is required for nucleosome positioning by Crt1 and Tup1 at the DNA damage-inducible gene *RNR3* (12, 16, 57) and for normal chromatin structure of the **a**-cell-specific gene *STE6* in *MAT*α cells (12, 16, 57). In addition, repression of **a**-cell-specific genes requires Itc1 (38), a subunit of the Isw2 complex (17). These prior studies suggest that an *isw2*Δ mutation might have an effect similar to nucleosome-disrupting sequences at *BAR1*.

Figure 2I shows the halo assay and mapping of MNase cleavage sites at *BAR1* in *isw2*Δ isogenic strains. The halo assay indicates that *BAR1* is derepressed in a *MAT*α *isw2*Δ strain, in keeping with the report by Ruiz et al. (38) that an *itc1* mutation causes derepression of **a**-cell-specific genes *ASG7*, *BAR1*, and *STE2*. Decrease in the halo size in the *MAT*α *isw2* strain (Fig. 2I) was similar to that in *MAT*α A_{30}, *MAT*α T_{25}, and *MAT*α $(CG)_5$ strains (Fig. 2D, F, and G). In addition, *BAR1* mRNA in the *MAT*α *isw2*Δ strain was detectable to the same extent (2.3% of full expression in *MAT***a** WT) as in these strains (Fig. 4). As seen in Fig. 2I, the MNase cleavage pattern is nearly identical in *MAT***a** and *MAT*α *isw2*Δ strains. These results reveal that the Isw2 chromatin remodeling complex is required for nucleosome positioning at the genomic *BAR1* locus in *MAT*α cells and that an *isw2* mutation does not have a significant effect on the *BAR1* transcription level. This result is consistent with a report by Zhang and Reese (57) that nucleosome positioning in the DNA damage-inducible gene *RNR3* is disrupted by *isw2* mutation, but the level of *RNR3* mRNA was only slightly increased.

DISCUSSION

We have shown here that both the integrity of the positioned nucleosomes and *MAT*α cell-type-dependent repression of *BAR1* respond to the same *cis*- and *trans*-acting modulators of

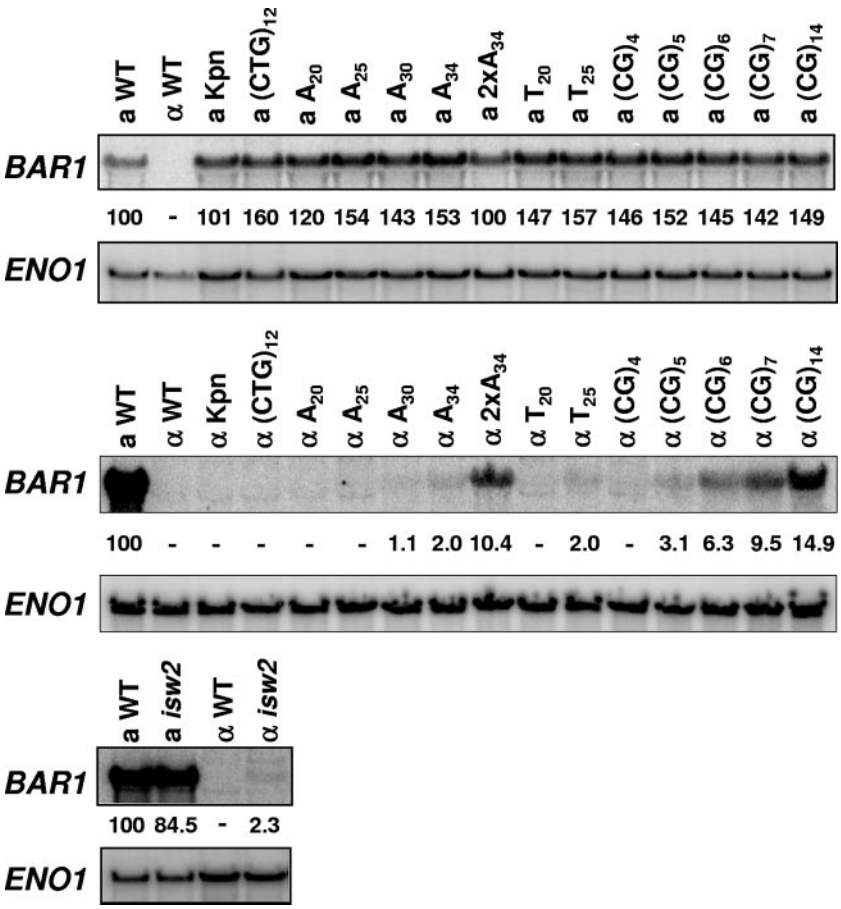


FIG. 4. Northern blot analysis of *BAR1*. Strains analyzed are indicated above each lane. The designations of strains are described in the legend of Fig. 2, except for $(CG)_{14}$, which refers to $(CG)_7TATA(CG)_7$ inserted into the *BAR1* promoter. Northern blots were probed with the *BAR1* probe and then stripped and reprobed with the *ENO1* probe as a loading control, which is not regulated by mating type. The *BAR1* mRNA levels, which are shown under the *BAR1* blot, were determined as the *BAR1*/*ENO1* ratio by using a phosphorimager and were normalized to the intensity of the *MAT***a** WT strain, set as 100. These normalized ratios are shown at the bottom of the *BAR1* blot.

chromatin structure. The sequences we have used are diverse, yet both poly(dA) · poly(dT) and poly(dC-dG) · poly(dC-dG) share the ability to disrupt a positioned nucleosome and to cause inappropriate activation of *BAR1*. Even though chromatin alteration shows a modest effect on the level of *BAR1* mRNA, it has a significant biological consequence, that is, it causes substantial degradation of the α-mating pheromone in *MAT*α cells, thereby affecting its cell type identity. Thus, the most economical model to explain our data is that nucleosome positioning directly contributes to complete repression of the genomic *BAR1* locus by α2/Mcm1.

However, loss of nucleosome positioning is not sufficient to relieve repression of *BAR1* completely. One reason for this could be explained by the absence of activator function of Mcm1 in *MAT*α cells; Mcm1 acts as an activator for **a**-cell-specific genes in *MAT***a** cells, whereas it acts as a repressor with α2 in *MAT*α cells. Since Tup1 has high affinity for underacetylated histones and histone deacetylases (5, 10, 11, 52), complete relief of chromatin-mediated repression may require not only loss of nucleosome positioning but also other activities, such as action of a histone acetyltransferase.

The residual repression that persists despite the disruption of nucleosome positioning is also likely to be achieved by chromatin-independent mechanisms of Tup1-Ssn6 action (29, 56). Two additional mechanisms have been proposed for repression by α2/Mcm1: activator interference (20), in which Ssn6-Tup1 exerts repression while the activator still occupies its target DNA site (35), and general transcription machinery interference, in which Ssn6-Tup1 inhibits the transcription machinery directly and independently of chromatin or activators (18, 29). Our findings here do not rule out any repression mechanism. Rather, our results provide support for the contribution of nucleosome positioning to **a**-specific gene repression.

We note that insertions of longer A_n, T_n, or $(CG)_n$ sequences primarily disrupt one nucleosome in the promoter, while nucleosome positioning is preserved in the coding region. Interestingly, the coding region is separated from the α2 operator by the disrupted nucleosome. This may be explained by the fact that the Isw2 complex is associated with the entire region of the *RNR3* gene (57) and that the Isw2 complex slides nucleosomes to remodel chromatin structure (12, 13). Thus, it is likely that the insertions disrupt only one nucleosome proximal to the site, and the preserved nucleosome positioning in

the *BAR1* coding region may be mediated by the Isw2 complex. This explanation is consistent with our results showing that the chromatin structure of *BAR1* is nearly identical between *MAT***a** WT, *MAT***a** *isw2*, and *MAT*α *isw2* (no positioned nucleosomes).

It may seem possible that proteins that bind to poly(dA) · poly(dT) or poly(dC-dG) · poly(dC-dG) compete with binding of histone octamer; hence, the effects of these sequences might not be a consequence of intrinsic DNA structural properties. Alternatively, the absence of nucleosome positioning might be a consequence of affecting the ability of the α2/Mcm1 complex to recruit Ssn6/Tup1. However, the idea that DNA structural properties alter nucleosome positioning is founded on several lines of evidence. We and others previously demonstrated that longer A_n tracts exist as an unusual *B′* conformation to create a nucleosome-free region in yeast cells (41, 48). Consistent with these reports, we found here that disruption of nucleosome positioning and derepression of *BAR1* showed a length dependent of the A_n tract, indicating that the *B′* conformation excludes histone octamers from the promoter. Interestingly, T_n disrupts *BAR1* repression more effectively than A_n, as monitored in the halo assay. This difference in orientation can be explained by the fact that the unusual conformation of poly(dA) · poly(dT) is asymmetric; that is, the minor groove narrows asymmetrically from the 3′ end towards the 5′ end of a T_n stretch (1, 25, 28). Also, *BAR1* expression was not affected by the *dat1*Δ mutation (data not shown), which lacks the only known poly(dA) · poly(dT) binding protein in *S. cerevisiae* (33). As for poly(dC-dG) · poly(dC-dG), its effects may be explained by the fact that $(CG)_n$ in the Z-form is not incorporated into nucleosomes in vitro (2, 6, 15). The length of $(CG)_n$ is critical for Z-DNA formation and stability in vivo (32), and the B-Z transition occurs from $(CG)_4$ to $(CG)_5$ at natural superhelical densities (22). Also, CG repeats longer than $(CG)_6$ can form Z-DNA stably in vivo in yeast cells (30). These studies argue that it is the Z-DNA conformation of $(CG)_n$ ($n \geq 5$) that disrupts nucleosome positioning at the genomic *BAR1* promoter, though the existence of Z-DNA formation in the *BAR1* promoter was uncertain in the present study. We cannot rule out a contribution of sequence-dependent general properties of the inserted DNA that may alter nucleosome organization (26, 40, 49). Whatever the structure of $(CG)_n$ in the *BAR1* locus is, the key feature is that poly(dC-dG) · poly(dC-dG) as well as poly(dA) · poly(dT) sequences disrupt nucleosome positioning in a genomic context to alter gene expression. The intrinsic properties of these sequences make them useful tools for inquiring into local chromatin function in diverse cells and organisms as well as for artificial alteration of gene expression in vivo.

ACKNOWLEDGMENTS

We thank Toshiki Kobayashi, Atsushi Sato, Yukino Mukai, and Mayu Yamaguchi for technical assistance in constructions of plasmids and strains in their undergraduate studies, Daichi Kurihara for construction of *isw2*Δ strains, and Karen Barwell and Clarissa Nobile for their help in Northern blot analysis. We thank Robert D. Wells of Texas A&M University for stimulating discussion and useful comments.

This work was supported, in part, by a JSPS research grant to M.S. and by NIH grants RO1 GM39531 and AI070272 to A.P.M.

REFERENCES

1. **Alexeev, D. G., A. A. Lipanov, and I. Skuratovskii.** 1987. Poly(dA) · poly(dT) is a B-type double helix with a distinctively narrow minor groove. Nature **325:**821–823.
2. **Ausio, J., G. Zhou, and K. van Holde.** 1987. A reexamination of the reported B-Z DNA transition in nucleosomes reconstituted with poly(dG-m^5dC) · poly(dG-m^5dC). Biochemistry **26:**5595–5599.
3. **Barwell, K. J., J. H. Boysen, W. Xu, and A. P. Mitchell.** 2005. Relationship of *DFG16* to the Rim101p pH response pathway in *Saccharomyces cerevisiae* and *Candida albicans*. Eukaryot. Cell **4:**890–899.
4. **Bi, X., Q. Yu, J. J. Sandmeier, and Y. Zou.** 2004. Formation of boundaries of transcriptionally silent chromatin by nucleosome-excluding structures. Mol. Cell. Biol. **24:**2118–2131.
5. **Bone, J. R., and S. Y. Roth.** 2001. Recruitment of the yeast Tup1p-Ssn6p repressor is associated with localized decreases in histone acetylation. J. Biol. Chem. **276:**1808–1813.
6. **Casasnovas, J. M., and F. Azorin.** 1987. Supercoiled induced transition to the Z-DNA conformation affects the ability of a $d(CG/GC)_{12}$ sequence to be organized into nucleosome-cores. Nucleic Acids Res. **15:**8899–8918.
7. **Champ, P. C., S. Maurice, J. M. Vargason, T. Camp, and P. S. Ho.** 2004. Distributions of Z-DNA and nuclear factor I in human chromosome 22: a model for coupled transcriptional regulation. Nucleic Acids Res. **32:**6501–6510.
8. **Chan, R. K., and C. A. Otte.** 1982. Isolation and genetic analysis of *Saccharomyces cerevisiae* mutants supersensitive to G_1 arrest by a factor and α factor pheromones. Mol. Cell. Biol. **2:**11–20.
9. **Cooper, J. P., S. Y. Roth, and R. T. Simpson.** 1994. The global transcriptional regulators, SSN6 and TUP1, play distinct roles in the establishment of a repressive chromatin structure. Genes Dev. **8:**1400–1410.
10. **Davie, J. K., D. G. Edmondson, C. B. Coco, and S. Y. Dent.** 2003. Tup1-Ssn6 interacts with multiple class I histone deacetylases *in vivo*. J. Biol. Chem. **278:**50158–50162.
11. **Edmondson, D. G., M. M. Smith, and S. Y. Roth.** 1996. Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. Genes Dev. **10:**1247–1259.
12. **Fazzio, T. G., M. E. Gelbart, and T. Tsukiyama.** 2005. Two distinct mechanisms of chromatin interaction by the Isw2 chromatin remodeling complex in vivo. Mol. Cell. Biol. **25:**9165–9174.
13. **Fazzio, T. G., and T. Tsukiyama.** 2003. Chromatin remodeling *in vivo*: evidence for a nucleosome sliding mechanism. Mol. Cell **12:**1333–1340.
14. **Ganter, B., S. Tan, and T. J. Richmond.** 1993. Genomic footprinting of the promoter regions of *STE2* and *STE3* genes in the yeast *Saccharomyces cerevisiae*. J. Mol. Biol. **234:**975–987.
15. **Garner, M. M., and G. Felsenfeld.** 1987. Effect of Z-DNA on nucleosome placement. J. Mol. Biol. **196:**581–590.
16. **Gelbart, M. E., N. Bachman, J. Delrow, J. D. Boeke, and T. Tsukiyama.** 2005. Genome-wide identification of Isw2 chromatin-remodeling targets by localization of a catalytically inactive mutant. Genes Dev. **19:**942–954.
17. **Gelbart, M. E., T. Rechsteiner, T. J. Richmond, and T. Tsukiyama.** 2001. Interactions of Isw2 chromatin remodeling complex with nucleosomal arrays: analyses using recombinant yeast histones and immobilized templates. Mol. Cell. Biol. **21:**2098–2106.
18. **Herschbach, B. M., M. B. Arnaud, and A. D. Johnson.** 1994. Transcriptional repression directed by the yeast α2 protein *in vitro*. Nature **370:**309–311.
19. **Iyer, V., and K. Struhl.** 1995. Poly(dA:dT), a ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure. EMBO J. **14:**2570–2579.
20. **Keleher, C. A., C. Goutte, and A. D. Johnson.** 1988. The yeast cell-type-specific repressor α2 acts cooperatively with a non-cell-type-specific protein. Cell **53:**927–936.
21. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. Cell **68:**709–719.
22. **Kim, J., C. Yang, and S. DasSarma.** 1996. Analysis of left-handed Z-DNA formation in short $d(CG)_n$ sequences in *Escherichia coli* and *Halobacterium halobium* plasmids. Stabilization by increasing repeat length and DNA supercoiling but not salinity. J. Biol. Chem. **271:**9340–9346.
23. **Liu, H., N. Mulholland, H. Fu, and K. Zhao.** 2006. Cooperative activity of BRG1 and Z-DNA formation in chromatin remodeling. Mol. Cell. Biol. **26:**2550–2559.
24. **Liu, R., H. Liu, X. Chen, M. Kirby, P. O. Brown, and K. Zhao.** 2001. Regulation of CSF1 promoter by the SWI/SNF-like BAF complex. Cell **106:**309–318.
25. **Lyamichev, V.** 1991. Unusual conformation of $(dA)_n \cdot (dT)_n$-tracts as revealed by cyclobutane thymine-thymine dimer formation. Nucleic Acids Res. **19:**4491–4496.
26. **Mai, X., S. Chou, and K. Struhl.** 2000. Preferential accessibility of the yeast *his3* promoter is determined by a general property of the DNA sequence, not by specific elements. Mol. Cell. Biol. **20:**6668–6676.
27. **Manney, T. R.** 1983. Expression of the *BAR1* gene in *Saccharomyces cerevisiae*: induction by the α mating pheromone of an activity associated with a secreted protein. J. Bacteriol. **155:**291–301.

28. **McConnell, K. J., and D. L. Beveridge.** 2001. Molecular dynamics simulations of B′-DNA: sequence effects on A-tract-induced bending and flexibility. J. Mol. Biol. **314:**23–40.
29. **Mennella, T. A., L. G. Klinkenberg, and R. S. Zitomer.** 2003. Recruitment of Tup1-Ssn6 by yeast hypoxic genes and chromatin-independent exclusion of TATA binding protein. Eukaryot. Cell **2:**1288–1303.
30. **Oh, D. B., Y. G. Kim, and A. Rich.** 2002. Z-DNA-binding proteins can act as potent effectors of gene expression *in vivo*. Proc. Natl. Acad. Sci. USA **99:**16666–16671.
31. **Patterton, H. G., and R. T. Simpson.** 1994. Nucleosomal location of the *STE6* TATA box and Matα2p-mediated repression. Mol. Cell. Biol. **14:**4002–4010.
32. **Rahmouni, A. R., and R. D. Wells.** 1989. Stabilization of Z DNA *in vivo* by localized supercoiling. Science **246:**358–363.
33. **Reardon, B. J., D. Gordon, M. J. Ballard, and E. Winter.** 1995. DNA binding properties of the *Saccharomyces cerevisiae DAT1* gene product. Nucleic Acids Res. **23:**4900–4906.
34. **Redd, M. J., M. B. Arnaud, and A. D. Johnson.** 1997. A complex composed of tup1 and ssn6 represses transcription *in vitro*. J. Biol. Chem. **272:**11193–11197.
35. **Redd, M. J., M. R. Stark, and A. D. Johnson.** 1996. Accessibility of α2-repressed promoters to the activator Gal4. Mol. Cell. Biol. **16:**2865–2869.
36. **Roth, S. Y., A. Dean, and R. T. Simpson.** 1990. Yeast α2 repressor positions nucleosomes in TRP1/ARS1 chromatin. Mol. Cell. Biol. **10:**2247–2260.
37. **Roth, S. Y., M. Shimizu, L. Johnson, M. Grunstein, and R. T. Simpson.** 1992. Stable nucleosome positioning and complete repression by the yeast α2 repressor are disrupted by amino-terminal mutations in histone H4. Genes Dev. **6:**411–425.
38. **Ruiz, C., V. Escribano, E. Morgado, M. Molina, and M. J. Mazon.** 2003. Cell-type-dependent repression of yeast a-specific genes requires Itc1p, a subunit of the Isw2p-Itc1p chromatin remodelling complex. Microbiology **149:**341–351.
39. **Schroth, G. P., and P. S. Ho.** 1995. Occurrence of potential cruciform and H-DNA forming sequences in genomic DNA. Nucleic Acids Res. **23:**1977–1983.
40. **Sekinger, E. A., Z. Moqtaderi, and K. Struhl.** 2005. Intrinsic histone-DNA interactions and low nucleosome density are important for preferential accessibility of promoter regions in yeast. Mol. Cell **18:**735–748.
41. **Shimizu, M., T. Mori, T. Sakurai, and H. Shindo.** 2000. Destabilization of nucleosomes by an unusual DNA conformation adopted by poly(dA) · poly(dT) tracts *in vivo*. EMBO J. **19:**3358–3365.
42. **Shimizu, M., S. Y. Roth, C. Szent-Gyorgyi, and R. T. Simpson.** 1991. Nucleosomes are positioned with base pair precision adjacent to the α2 operator in *Saccharomyces cerevisiae*. EMBO J. **10:**3033–3041.
43. **Simpson, R. T., and P. Kunzler.** 1979. Chromatin and core particles formed from the inner histones and synthetic polydeoxyribonucleotides of defined sequence. Nucleic Acids Res. **6:**1387–1415.
44. **Simpson, R. T., S. Y. Roth, R. H. Morse, H. G. Patterton, J. P. Cooper, M. Murphy, M. P. Kladde, and M. Shimizu.** 1993. Nucleosome positioning and transcription. Cold Spring Harbor Symp. Quant. Biol. **58:**237–245.
45. **Sinden, R. R.** 1994. DNA structure and function. Academic Press, San Diego, Calif.
46. **Smith, R. L., and A. D. Johnson.** 2000. Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. Trends Biochem. Sci. **25:**325–330.
47. **Spellman, P. T., G. Sherlock, M. Q. Zhang, V. R. Iyer, K. Anders, M. B. Eisen, P. O. Brown, D. Botstein, and B. Futcher.** 1998. Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization. Mol. Biol. Cell **9:**3273–3297.
48. **Suter, B., G. Schnappauf, and F. Thoma.** 2000. Poly(dA · dT) sequences exist as rigid DNA structures in nucleosome-free yeast promoters *in vivo*. Nucleic Acids Res. **28:**4083–4089.
49. **Thastrom, A., L. M. Bingham, and J. Widom.** 2004. Nucleosomal locations of dominant DNA sequence motifs for histone-DNA interactions and nucleosome positioning. J. Mol. Biol. **338:**695–709.
50. **Wang, Y. H., S. Amirhaeri, S. Kang, R. D. Wells, and J. D. Griffith.** 1994. Preferential nucleosome assembly at DNA triplet repeats from the myotonic dystrophy gene. Science **265:**669–671.
51. **Watson, A. D., D. G. Edmondson, J. R. Bone, Y. Mukai, Y. Yu, W. Du, D. J. Stillman, and S. Y. Roth.** 2000. Ssn6-Tup1 interacts with class I histone deacetylases required for repression. Genes Dev. **14:**2737–2744.
52. **Wu, J., N. Suka, M. Carlson, and M. Grunstein.** 2001. TUP1 utilizes histone H3/H2B-specific HDA1 deacetylase to repress gene activity in yeast. Mol. Cell **7:**117–126.
53. **Xu, W., and A. P. Mitchell.** 2001. Yeast PalA/AIP1/Alix homolog Rim20p associates with a PEST-like region and is required for its proteolytic cleavage. J. Bacteriol. **183:**6917–6923.
54. **Yu, Q., J. Sandmeier, H. Xu, Y. Zou, and X. Bi.** 2006. Mechanism of the long range anti-silencing function of targeted histone acetyltransferases in yeast. J. Biol. Chem. **281:**3980–3988.
55. **Yuan, G. C., Y. J. Liu, M. F. Dion, M. D. Slack, L. F. Wu, S. J. Altschuler, and O. J. Rando.** 2005. Genome-scale identification of nucleosome positions in *S. cerevisiae*. Science **309:**626–630.
56. **Zhang, Z., and J. C. Reese.** 2004. Redundant mechanisms are used by Ssn6-Tup1 in repressing chromosomal gene transcription in *Saccharomyces cerevisiae*. J. Biol. Chem. **279:**39240–392450.
57. **Zhang, Z., and J. C. Reese.** 2004. Ssn6-Tup1 requires the ISW2 complex to position nucleosomes in *Saccharomyces cerevisiae*. EMBO J. **23:**2246–2257.
58. **Zhu, Z., and D. J. Thiele.** 1996. A specialized nucleosome modulates transcription factor access to a *C. glabrata* metal responsive promoter. Cell **87:**459–470.