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A Transcriptionally Active tRNA Gene Interferes with Nucleosome Positioning In Vivo

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Incorporation into a positioned nucleosome of a *cis*-acting element essential for replication in *Saccharomyces cerevisiae* disrupts the function of the element in vivo [R. T. Simpson, Nature (London) 343:387–389, 1990]. Furthermore, nucleosome positioning has been implicated in repression of transcription by RNA polymerase II in yeast cells. We have now asked whether the function of *cis*-acting elements essential for transcription of a gene transcribed by RNA polymerase III can be similarly affected. A tRNA gene was fused to either of two nucleosome positioning signals such that the predicted nucleosome would incorporate near its center the tRNA start site and essential A-box element. These constructs were then introduced into yeast cells on stably maintained, multicopy plasmids. Competent tRNA genes were transcribed in vivo and were not incorporated into positioned nucleosomes. Mutated, inactive tRNA genes were incorporated into nucleosomes whose positions were as predicted. This finding demonstrates that the transcriptional competence of the tRNA gene determined its ability to override a nucleosome positioning signal in vivo and establishes that a hierarchy exists between *cis*-acting elements and nucleosome positioning signals.

Nucleosomes appear capable of contributing to transcriptional repression in vivo (23). The partial loss of nucleosomes in *Saccharomyces cerevisiae* causes activation of reporter genes linked to various TATA elements (24), and altered histone stoichiometry affects promoter utilization at the *HIS3* locus when a Ty transposable element is present just upstream of this promoter (14). Such effects could be due to direct interference or occlusion at essential promoter elements or to changes in higher-order chromatin structure (72). Other examples suggest that nucleosomes can affect transcription at a local level. In *S. cerevisiae*, activation of the *PHO5* promoter is accompanied by disruption of several positioned nucleosomes just upstream of the gene (3, 10). Substitution of sequences expected to lead to stronger or weaker nucleosome positioning leads to repression or constitutive activation of this normally inducible gene (61). In another example from *S. cerevisiae*, the α2-MCM1 complex has been shown to actively position nucleosomes in yeast α cells with great precision, leading to incorporation of promoter elements of genes repressed in α cells (47, 55). Moreover, mutations in the histone H4 amino terminus which lead to destabilization of the nucleosomes adjacent to the α2-MCM1 operator also lead to derepression of reporter genes in α cells, indicating that these nucleosomes serve as repressors of transcription in vivo (48).

In higher eukaryotes, activation of the mouse mammary tumor virus promoter by the glucocorticoid receptor is accompanied by structural alteration of a specific nucleosome (12, 45). When the appropriate sequences are reconstituted into a nucleosome in vitro, the same nucleosome or a similarly positioned nucleosome is formed (6, 43, 44). This reconstituted nucleosome does not prevent binding of the hormone-bound glucocorticoid receptor but does prevent binding of the essential transcription factor NF-1, suggesting that it plays a role in maintenance of the repressed state of this promoter (7).

Nucleosomes could inhibit transcription at a local level either by sterically preventing access of essential transcription factors or by rendering *cis*-acting elements unrecognizable to appropriate *trans*-acting factors by bending, twisting, or kinking of the DNA (40). Support for the idea of nucleosomes repressing transcription at this level, in addition to the above-mentioned studies, comes from in vitro experiments correlating transcriptional repression with nucleosome assembly (18, 30, 37, 75). When nucleosome assembly was preceded by incubation of the transcriptional template with essential *trans*-acting factors, this inhibition was removed, suggesting that competition between these factors and histones determined the ultimate transcriptional competence of the promoter (9, 37, 75). Other studies have shown that incorporation of specific promoter sequences within a nucleosome is sufficient to inhibit transcription in vitro by SP6 (33) or T7 RNA polymerase (74) or RNA polymerase II (33) or III (38, 54, 70).

In spite of these various data suggesting that nucleosomes can inhibit transcription, gene promoters in vivo are able to function in an environment in which most DNA is packaged into chromatin. One possible explanation is that histones in vivo are essentially transparent to *trans*-acting factors, perhaps because of modifications of the histones or the presence of nucleosome assembly factors leading to a less stable nucleosome structure in vivo than that observed in vitro (39). In this case, the examples of the *PHO5* and mouse mammary tumor virus promoters would have to be regarded as somehow anomalous. Moreover, a *cis*-acting element active in replication in yeast cells has been shown to be inactivated by incorporation into a positioned nucleosome in vivo (58), and so at least in this case this explanation is insufficient. Other possibilities are that most promoters have sequences which have evolved to be unfavorable for the formation of strongly positioned or very stable nucleosomes or that access to *cis*-acting sequences could be regulated by various events and factors during replication, including relative abundance of *trans*-acting factors and histones.

To gain more insight into the role which nucleosomes play

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in regulating transcription in vivo, we have engineered competition between a nucleosome positioning signal and a transcription unit in yeast cells. Two kinds of nucleosome positioning signals were employed. In both cases, transcription was unaffected, and the predicted positioned nucleosome was present only in mutated, transcriptionally inactive templates.

MATERIALS AND METHODS

Plasmids and transfection into yeast cells. The *sup4-o* $tRNA^{Tyr}$ gene, carried on plasmid pC865 (53; generously provided by Maynard Olson), was marked by inserting complementary oligonucleotides RMO14 and RMO15 (5′ AGAAATAAAGAGTACCACGTCATTCGCGACCTTGC AGCACCT 3′) into the *Sma*I site at +83/84 relative to the tRNA start site, with RMO15 reading 5′ to 3′ with respect to the tRNA gene, to create pC865ins. The 230-bp *Bam*HI fragment bearing the marked gene was then excised and inserted into the *Bgl*II site at 852 map units (m.u.) of TRP1ARS1 in pRS104 in the orientation shown in Fig. 1 to create pRS104t. Plasmid pRS104 was made by cloning the *Hin*dIII-*Sst*I fragment of TRP1ARS1 containing the ARS, modified by insertion of a *Bam*HI site at the *Nae*I site (1067 m.u.), into pUC18. The complementary *Sst*I-*Hin*dIII fragment of pTA22 (58) was also cloned into pUC18, and the *Bam*HI-*Hin*dIII fragment of TRP1ARS1 carrying the entire ARS1 element was inserted into the *Bam*HI site adjacent to the triose phosphate isomerase terminator to create pRS110 (58). Digestion of pRS104t and pRS110 with *Hin*dIII and *Sst*I, followed by ligation, yielded TAt, which was introduced into *S. cerevisiae* SC3 (*MATα ura3-52 his3-1 trp1 gal2 gal10* [cir^0]) (56) by lithium acetate transformation (28). This plasmid and its derivatives were maintained at 10 to 20 copies per cell (data not shown). Map units in TAt and derivatives are given as corresponding map units in TRP1ARS1; map units for the tRNA insert are given relative to the tRNA start site (e.g., the oligonucleotide insert begins at +83t).

Plasmids pRS104t-100, pRS104tΔA, pRS104t-100ΔA, and pRS104t-100mutB were made from pRS104t by using the polymerase chain reaction (PCR) (25). Deletions encompassed nucleotides 8 to 19 in the tRNA gene (ΔA) (21) and from −15t to 951 m.u. in TRP1ARS1 (−100). The mutB construct was made by using PCR to convert the sequence GGCGTTCGAC (+66 to +75 in the numbering system of Goodman et al. [21]) to GGAGATGCAC. Ligation of the *Hin*dIII-*Sst*I fragments of these four plasmids bearing TRP1ARS1 sequences with the complementary part of pRS110 yielded TAt-100, TAtΔA, TAt-100ΔA, and TAt-100mutB, respectively.

To construct TALSt, *Sst*I sites were introduced by PCR at −18t (53) and +129t (21) in pC865ins. The marked tRNA gene was then excised with *Sst*I and inserted into the *Sst*I site of TALS (borne in the *Hin*dIII site of pUC19) (47) after first deleting the *Sst*I site from the pUC19 polylinker. The orientation of the tRNA gene in TALSt is as shown in Fig. 7A. TALSt was separated from pUC19 sequences by *Hin*dIII digestion, ligated, and introduced into *S. cerevisiae* YNN282 (*MATα trp1-Δ his3-Δ200 ura3-52 lys-801^a ade2-1^o gal*) and the isogenic *MAT***a** strain YNN281 by lithium acetate transformation (28). TALSt was maintained at 100 to 200 copies per cell (data not shown). Map units are based on map units for TALS (47) and the tRNA gene, as for TAt and derivatives. The sequence of the α2-MCM1 site of TALSt in YNN282 was verified by primer extension sequencing of DNA isolated from transformed clones (8). TALStΔA and TALStmutB were made by PCR as described above.

For the experiments using CEN-containing plasmids, the *Kpn*I-*Hin*dIII fragments of pRS104t and pRS104t-100 and the *Eco*RI-*Sal*I fragment from TALSt, each bearing the tRNA gene, were ligated with the CEN-containing shuttle vector pRS314 (57) cut with the same enzymes. The resulting constructs were introduced into *S. cerevisiae* as described above.

Isolation and analysis of RNA. RNA was prepared from 15-ml cultures of yeast cells, grown overnight at 30°C in 2% glucose–0.67% nitrogen base without amino acids supplemented for auxotrophy (46) to an optical density at 600 nm of 0.5 to 1.5, as described previously (26). RNA from 10^7 to 10^8 cells was taken up in formamide, denatured, and electrophoresed through 8 or 10% polyacrylamide–7 M urea gels along with ^{32}P-labeled, *Hae*III-digested ϕX174 DNA. RNA and DNA from the gels were transferred to nylon membranes by electroblotting for 1.5 h at 14 V (400 mA) in 0.3× Tris-acetate-EDTA buffer (50) in an E-C Electroblot apparatus. Following immobilization by UV cross-linking, blots were hybridized overnight at 65°C and washed as described previously (13). Probes were prepared by random primer labeling (50) of the *Bam*HI fragment from pC865ins containing the tRNA maxigene and a fragment containing the yeast 5S RNA gene made by PCR amplification from pSc5S-FM (65), generously provided by J. Segall.

Isolation and analysis of plasmid chromatin. Chromatin was prepared from 1-liter cultures of yeast cells grown as described above to an optical density at 600 nm of 0.8 to 1.8 and digested with micrococcal nuclease as described previously (47, 66). Prior to restriction for indirect end-label analysis (42, 76), purified DNA was eluted from 1-ml Sephadex G50 spun columns (50). Samples were electrophoresed on 1.2 or 1.8% agarose gels at 4 V/cm for 5 to 6 h and transferred to nylon membranes by capillary action (50). Bromophenol blue was found to interfere with transfer and was therefore omitted from the sample buffer. Hybridization with random-primer-labeled probes and washing of blots was carried out as described previously (13). Fragment sizes were determined by comparison with *Hae*III-digested ϕX174 DNA on the same gel.

Topoisomer analysis (39) and in vivo footprinting with dimethyl sulfate (48) were performed as described previously.

RESULTS

Competition between tRNA transcription and nucleosome positioning by the ARS C domain. The transcription unit chosen for competition with a nucleosome positioning signal in this work was based on the *sup4-o* $tRNA^{Tyr}$ gene (21). This gene, like other tRNA genes (20), has an internal promoter, which allows a nucleosome positioning signal to be placed in close proximity to the gene with the introduced upstream sequences having little effect on promoter function (53). The gene can be inactivated by small internal deletions or mutations, allowing the effects on nucleosome positioning due to sequences present in the tRNA gene to be separated from the effects due to transcriptional competence.

To see whether a positioned nucleosome could affect transcription in vivo, a 230-bp fragment bearing the *sup4-o* $tRNA^{Tyr}$ gene was inserted into the *Bgl*II site of the TRP1ARS1 plasmid, immediately adjacent to the ARS A element (Fig. 1). The ARS1 element can be divided into three principal subdomains designated A, B, and C. The A

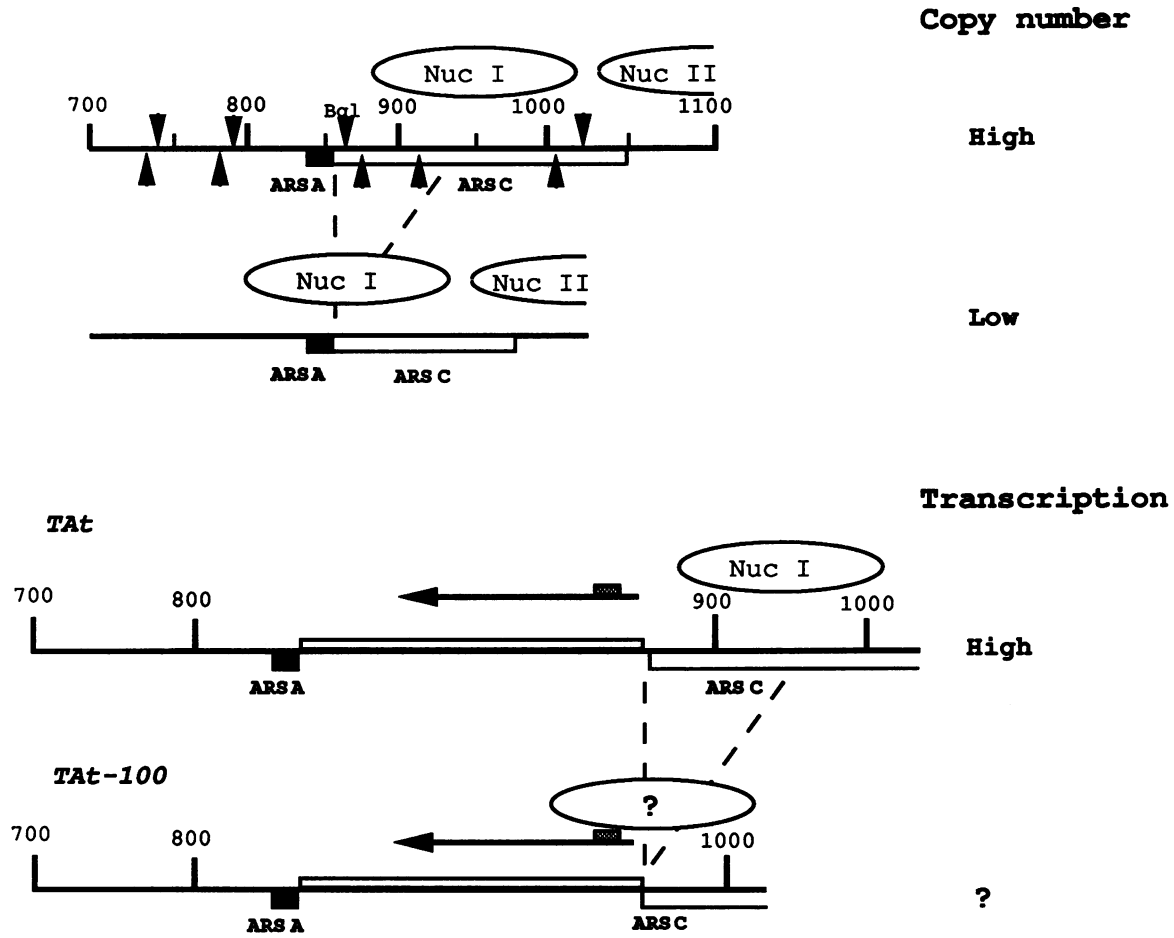

FIG. 1. Effect of the ARS C nucleosome positioning signal on transcription of a tRNA gene: Experimental strategy. The region of the TRP1ARS1 plasmid near the ARS A element, from 700 to 1100 map units on the TRP1ARS1 map, is depicted. Micrococcal nuclease cleavage sites in DNA and chromatin are shown by arrowheads below and above the line, respectively. These cleavage sites lead to the inferred positions of nucleosomes (Nuc) I and II indicated. Deletion of 60 to 80 bp to the right of the ARS A element leads to repositioning of nucleosome I as shown and concomitant inhibition of ARS function (58). The *sup4-o* $tRNA^{Tyr}$ gene represented by the black arrow was inserted into the *Bgl*II site next to the ARS A element to create the TAt plasmid (see Materials and Methods); deletion of 100 bp immediately upstream of the tRNA gene (from 951 to 852 m.u.) places the ARS C nucleosome positioning sequences in its immediate proximity and creates the TAt-100 plasmid. The small box on the arrow represents the essential A-box element of the internal promoter.

domain is an 11-bp sequence absolutely required for ARS function. The C domain contains sequences which position a nucleosome in vivo; deletion of sequences between the A and C domains causes the A element to be incorporated near the center of this nucleosome, thereby inhibiting ARS function (58). Insertion of the tRNA gene adjacent to the ARS A element places it in the same context relative to the C domain, allowing the effect of the nucleosome positioned by the C domain on the function of the tRNA gene to be assayed. A second copy of the ARS1 element was placed elsewhere on the plasmid to ensure replication to high copy number in yeast cells (see Materials and Methods).

To allow detection of the *sup4-o* $tRNA^{Tyr}$ transcripts apart from cross-hybridizing endogenous tRNA species, the $tRNA^{Tyr}$ gene was marked by insertion of a 42-bp oligonucleotide 83 bp downstream from the transcription start site. The parent plasmid (TAt) and a derivative (TAt-100) in which a 100-bp sequence between the tRNA gene and the presumptive nucleosome positioning sequences in the C domain has been deleted (Fig. 1) were introduced into *trp* mutant yeast cells. RNA was isolated from the transformed cells and analyzed by Northern (RNA) blotting (Fig. 2). Surprisingly, both the TAt and TAt-100 plasmids gave rise to an abundant marked transcript. Cells containing TAtΔA, a plasmid in which the essential A-box element of the tRNA promoter had been deleted lacked this transcript. The increased amount of transcription (threefold as measured by densitometry) seen from TAt-100 compared with that from TAt is probably due to removal of an inhibitory *Bam*HI recognition sequence 15 bp upstream of the tRNA start site (36). Similar results were obtained using single-copy centromere-containing plasmids (data not shown). Thus, the juxtaposition of the ARS C nucleosome positioning signal to a tRNA gene in vivo did not inhibit transcription.

Transcription could have taken place in spite of the presence of a positioned nucleosome partly incorporating the tRNA gene, or the active gene could have disrupted or prevented the assembly of sequences into a positioned nucleosome. Chromatin mapping was undertaken to distinguish between these possibilities. Plasmid chromatin was isolated (49, 66) and digested with micrococcal nuclease, and the cleavage sites were mapped relative to unique restriction

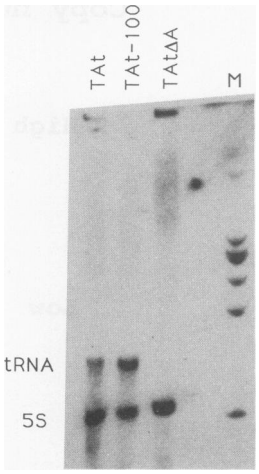

FIG. 2. Northern analysis of RNA from cells harboring TAt, TAt-100, and TAtΔA, which lacks the tRNA A box essential for transcription. The lane labeled M contains labeled *Hae*III-digested ϕX174 DNA.

sites (42, 76). Protected regions of 140 to 160 bp flanked by nuclease cleavage sites are assumed to be due to positioned nucleosomes, on the basis of previous work (47, 48, 55, 58, 66–68). Figure 3 shows that insertion of the 230-bp fragment bearing the marked tRNA gene in its active (TAt) or inactive (TAtΔA) form into the *Bgl*II site at 852 m.u. of TRP1ARS1 leaves the chromatin structure of the adjacent ARS C domain unchanged (58, 67, 68). In particular, the site cut in naked DNA at 930 m.u. is protected in chromatin, whereas the site at 1020 m.u. is strongly cut. (The accuracy with which these sites are mapped should be taken as ±20 bp [47].) This pattern is consistent with positioning of nucleosome I in this region of the ARS C domain. This was expected, since the tRNA gene was inserted between the edge of nucleosome I and the nuclease-sensitive ARS A domain, as defined in the original TRP1ARS1 plasmid (67). The site at 870 m.u., which appears more strongly cut in TAt than in TAtΔA, may correspond to the DNase I-hypersensitive site found upstream of another active tRNA gene in yeast cells (27).

Micrococcal nuclease cleavage sites for the TAt-100 and TAt-100ΔA plasmids are mapped relative to the *Eco*RV site in Fig. 4. Sites of enhanced and protected cleavage in chromatin compared with those in DNA are mostly preserved from TAt and TAtΔA, indicating that the 100-bp deletion has not caused a gross rearrangement in the chromatin structure of these plasmids. The cleavage patterns for TAt-100 and TAt-100ΔA chromatin appear identical, except for a site close to the tRNA start site at −10t (that is, 10 bp upstream of the tRNA start site; see the arrowhead in Fig. 4), which is cut in TAt-100 but not in TAt-100ΔA. This can be seen more clearly in Fig. 5, in which micrococcal nuclease cleavage sites are mapped from the *Sst*I site. The site at −5t is near the center of the position predicted for nucleosome I (about 1020 m.u. to +50t) on the basis of its position in constructs in previous work (58). The presence of this site in naked DNA (Fig. 4, lane 10; Fig. 5, lane 8) and in TAt-100 chromatin (Fig. 4, lanes 3 and 4; Fig. 5, lanes 1 to 3) and its absence in TAt-100ΔA suggest that nucleosome I occupies its predicted position in the presence of the inactive tRNA gene but is absent in the presence of the active, overlapping tRNA gene.

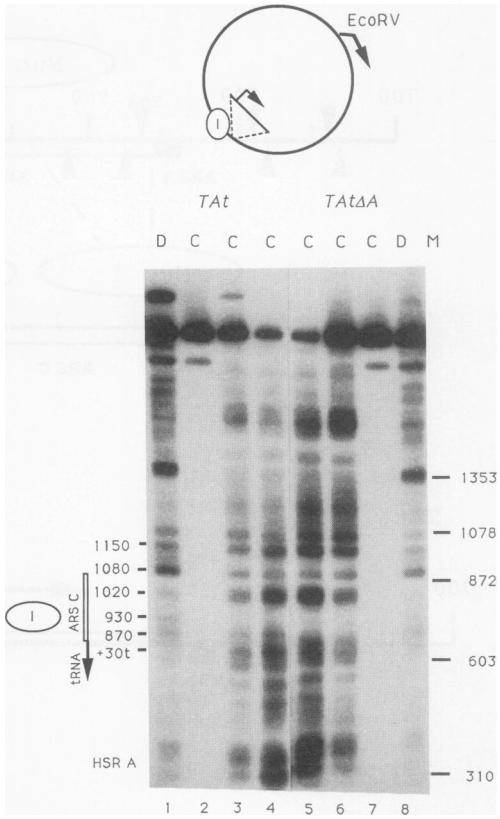

FIG. 3. Chromatin structure of TAt and TAtΔA. Micrococcal nuclease cleavage sites in chromatin (lanes C) and DNA (D lanes) in TAt (lanes 1 to 4) and TAtΔA (lanes 5 to 8) were mapped relative to the *Eco*RV site. Locations of nucleosome I, the ARS C domain, and the tRNA gene are indicated to the left of the gel and on the plasmid map at the top of the figure. Map units shown to the left of the gel correspond to TRP1ARS1 map units or to the tRNA gene insert (i.e., +30t; see Materials and Methods), and HSR A refers to the hypersensitive region centered near 800 m.u. on the TRP1ARS1 map (47). The positions of marker (M) bands are given in base pairs to the right of the gel. Samples were treated with micrococcal nuclease at 5 U/ml at 37°C for 5 min (lanes 1 and 8) or for 10 min at 37°C with 0 (lanes 2 and 7), 2 (lanes 3 and 6), or 10 (lanes 4 and 5) U/ml. Different exposures of the same blot were used for lanes 1 to 4 and lanes 5 to 8.

The chromatin structure of a version of TAt-100 with its tRNA gene inactivated by a different mutation was also examined. This plasmid, called TAt-100mutB, was constructed by mutating nucleotides at positions +68, 70, 72, and 73 in the tRNA gene (see Materials and Methods). These mutations lie near the border of the predicted position of nucleosome I. As expected (2), these alterations completely inactivate the tRNA gene (data not shown). Micrococcal nuclease cleavage sites for this plasmid are mapped relative to the *Eco*RV site in Fig. 6. The digestion pattern is similar to that seen for TAt-100ΔA. The region from −5t to +40t, which is cut in naked DNA (Fig. 6, lanes 4 and 5), is clearly protected in chromatin (lanes 1 to 3). Moreover, the band at 1000 m.u. appears hypersensitive in chromatin, as seen (at 1020 m.u. [Fig. 3]) for TAt and TAtΔA, indicating that nucleosome I is positioned, as expected, by the ARS C sequence in TAt-100mutB.

Competition between tRNA transcription and nucleosome positioning by the α2-MCM1 operator in yeast α cells. Nucle-

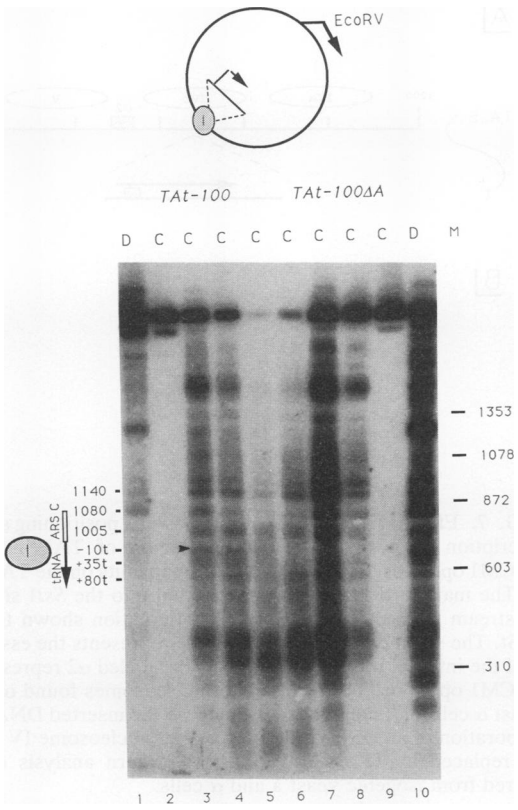

FIG. 4. Chromatin structure of TAt-100 and TAt-100ΔA. Micrococcal nuclease cleavage sites in chromatin (lanes C) and DNA (D lanes) in TAt-100 (lanes 1 to 5) and TAt-100ΔA (lanes 6 to 10) were mapped relative to the *Eco*RV site at 385 m.u. Map units shown to the left of the gel correspond to TRP1ARS1 map units or to the tRNA gene insert (e.g., −10t; see Materials and Methods). The positions of marker (M) bands are given in base pairs to the right of the gel. Note that the 100-bp deletion removed sequences from 951 to 852 m.u. present in TAt and TAtΔA (see Materials and Methods). Samples were treated for 5 min at 37°C with micrococcal nuclease at 0 (lanes 2 and 9), 2 (lanes 3 and 8), 5 (lanes 1, 4, 7, and 10), or 20 (lanes 5 and 6) U/ml. The cleavage pattern for TAt-100 DNA, seen only faintly here (lane 1), is essentially identical to that for TAt-100ΔA.

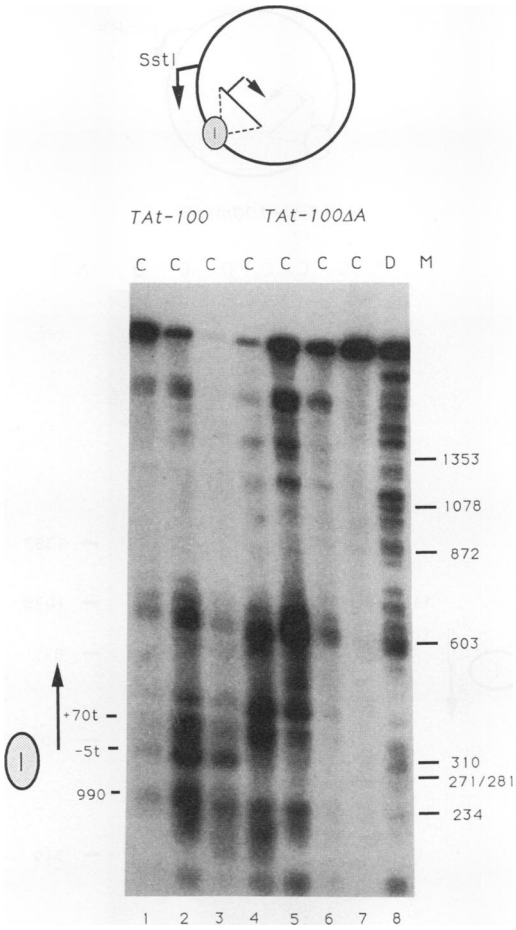

FIG. 5. Chromatin structure of TAt-100 and TAt-100ΔA. Micrococcal nuclease cleavage sites in chromatin (lanes C) and DNA (lane D) in TAt-100 (lanes 1 to 3) and TAt-100ΔA (lanes 4 to 8) were mapped relative to the *Sst*I site (at 1225 on the TRP1ARS1 map). Map units shown to the left of the gel correspond to TRP1ARS1 map units or to the tRNA gene insert (e.g., +70t; see Materials and Methods). The positions of marker (M) bands are given in base pairs to the right of the gel. Note that the 100-bp deletion removed sequences from 951 to 852 m.u. present in TAt and TAtΔA (see Materials and Methods). Samples were treated for 5 min at 37°C with micrococcal nuclease at 0 (lane 7), 2 (lanes 1 and 6), 5 (lanes 5 and 8), 10 (lane 2), 20 (lane 4), or 50 (lane 3) U/ml. No DNA control is shown for TAt-100, since the cleavage pattern is essentially identical to that for TAt-100ΔA.

osome I in TRP1ARS1 and its derivatives appears to be positioned by the incorporated DNA sequences (58, 68). Nucleosomes can also be positioned in vivo by DNA-binding proteins, which may act as passive "bookends" (17, 31) or may actively position nucleosomes (47, 48, 58).

The α2 repressor protein in yeast α cells positions nucleosomes adjacent to the α2-MCM1 binding site with great precision (47, 55). For a second, independent examination of competition between a transcription complex on a tRNA gene and a nucleosome positioning signal, the marked $tRNA^{Tyr}$ gene was therefore inserted into the yeast episome TALS (47) such that the tRNA gene start site was 90 bp from the proximal border of the *STE6* α2-MCM1 binding site (Fig. 7A). This construct, TALSt, a derivative (TALStmutB) with four point mutations in the essential B box of the tRNA gene promoter (2, 20), and TALStΔA, a derivative lacking the tRNA A box, were introduced into isogenic yeast **a** and α cells. RNA was isolated from cells transformed with TALSt and analyzed by Northern blotting (Fig. 7B). The marked tRNA transcript was equally abundant in **a** and α cells, indicating that the α2-MCM1 operator did not repress the tRNA gene in α cells. Equivalent results were obtained by using a single-copy centromere-containing plasmid, while as expected, cells carrying the plasmids bearing the mutated tRNA genes lacked the marked tRNA transcript (data not shown).

In previous studies, nucleosome positioning adjacent to the α2-MCM1 operator in α cells was revealed by chromatin mapping of plasmid episomes from **a** and α cells (47, 55). To determine whether similar cell type-dependent nucleosome positioning could be observed over the inactive tRNA gene in TALStΔA and TALStmutB, micrococcal nuclease cleavage sites in plasmid chromatin and naked DNA were mapped relative to the *Eco*RV site in **a** and α cells (Fig. 8 and 9). The results suggest that several nucleosomes are positioned in

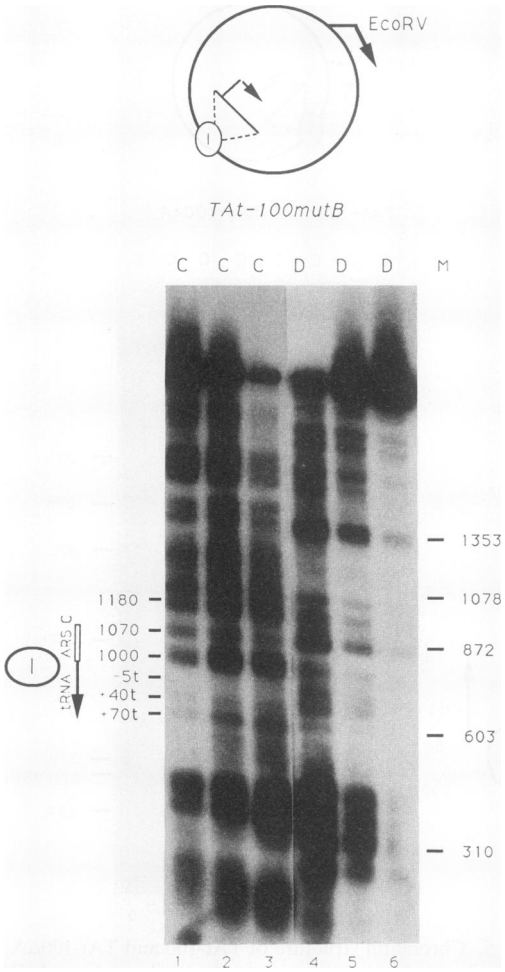

FIG. 6. Chromatin structure of TAt-100mutB. Micrococcal nuclease cleavage sites in chromatin (lanes C) and DNA (lanes D) were mapped relative to the *Eco*RV site at 385 m.u. Map units shown to the left of the gel correspond to TRP1ARS1 map units or to the tRNA gene insert (e.g., +40t; see Materials and Methods). The positions of marker (M) bands are given in base pairs to the right of the gel. Samples were treated for 10 min at 37°C with micrococcal nuclease at 2 (lane 1), 5 (lane 2), 20 (lane 3), 1 (lane 6), 4 (lane 5), or 10 (lane 4) U/ml. Different exposures of the same autoradiogram were used for lanes 1 to 3 and lanes 4 to 6.

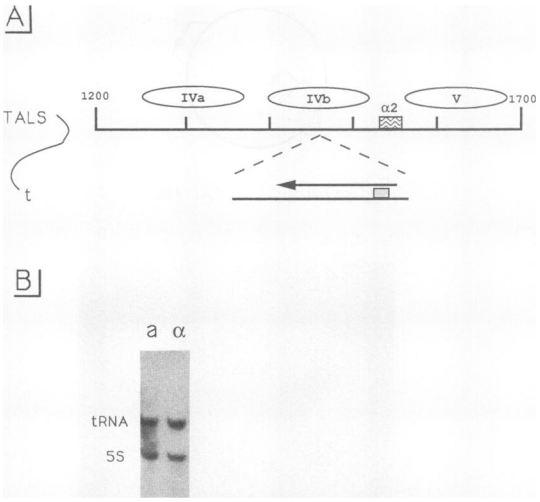

FIG. 7. Effect of the α2-MCM1 nucleosome positioning signal on transcription of a tRNA gene. (A) Region of TALS near the α2-MCM1 operator, from 1,200 to 1,700 map units on the TALS map (47). The marked tRNA gene was inserted into the *Sst*I site 75 bp downstream from the operator in the orientation shown to create TALSt. The small box on the tRNA gene represents the essential A box of the internal promoter, and the box labeled α2 represents the α2-MCM1 operator. The positions of nucleosomes found on TALS in yeast α cells (47) are indicated. Because the inserted DNA allows incorporation of an additional nucleosome, nucleosome IV (47) has been replaced by IVa and IVb. (B) Northern analysis of RNA prepared from isogenic yeast **a** and α cells.

both cell types (e.g., nucleosome V; note also the different cleavage patterns for DNA and chromatin in the higher-molecular-weight range). Adjacent to the α2-MCM1 operator, however, sites at about 1500 m.u., +20t, and 1400 m.u., which are cut in **a** cells, are partially or completely protected in α cells, suggesting the presence of positioned nucleosomes (IVa and IVb in Fig. 8 and 9) over these sequences in α cells. Thus, the tRNA sequences themselves, in the absence of transcription, do not inhibit the ability of the α2-MCM1 operator to position nucleosomes in yeast α cells.

A different result was obtained when the chromatin structure of the transcriptionally active TALSt plasmid was examined in **a** and α cells (Fig. 10). Cleavage sites protected by nucleosome IVb in TALStΔA and TALStmutB in α cells are cut in TALSt in α cells. Specifically, bands at 1500 m.u. and +1t are no longer protected, indicating loss of the positioned nucleosome adjacent to the α2-MCM1 operator in the presence of the active tRNA gene. This can be seen clearly in Fig. 11, in which micrococcal nuclease cleavage site patterns are compared for plasmids TALSt and TALSt mutB in α cells. Changes in the cleavage patterns at higher-molecular-weight range, corresponding to the region of TRP1ARS1 normally occupied by nucleosomes I, II, and III (47, 67) are also evident. Sequencing of the plasmid DNA from individual transformants indicates that the α2-MCM1 operator is intact, and dimethyl sulfate footprinting indicates at least partial occupancy of the site by the α2-MCM1 complex (data not shown). We therefore conclude that the presence of the active tRNA gene overrides the ability of the α2-MCM1 complex to position a nucleosome adjacent to its operator.

DISCUSSION

In this work, we have attempted to learn something about the role that nucleosome positioning may play in gene regulation in vivo by studying nucleosome positioning signals engineered to be in competition with a tRNA transcription unit. We examined two nucleosome positioning signals; neither affected transcription of a nearby tRNA gene. Chromatin mapping indicates that both nucleosome positioning signals worked as predicted when juxtaposed to the inactive tRNA gene sequences. In contrast, when these same signals abutted the active tRNA gene, micrococcal nuclease cleavage sites were observed within the sequences predicted to be incorporated within a positioned nucleosome. For the constructs in which the tRNA gene was inactivated by mutations in the B box of the internal promoter, the identical sequences that were cleaved by micrococcal nuclease in plasmid chromatin bearing the active gene were protected against digestion in plasmid chromatin bearing the inactive

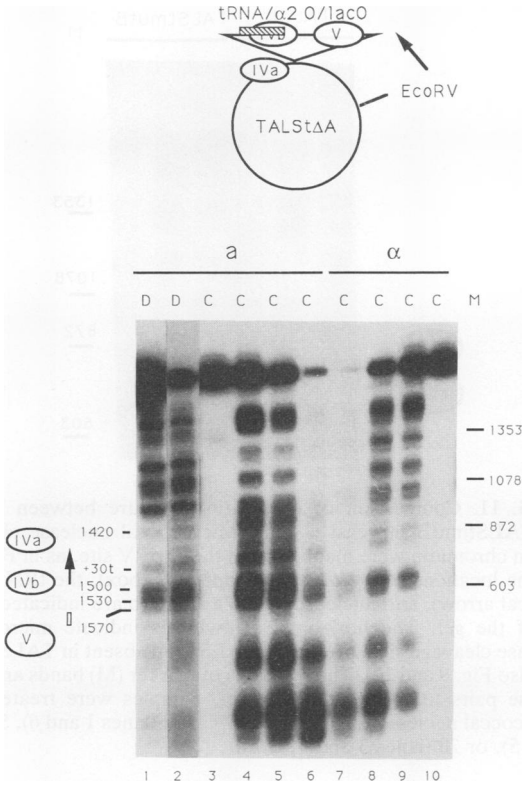

FIG. 8. Chromatin structure of TALStΔA in yeast **a** and α cells. Micrococcal nuclease cleavage sites in chromatin (lanes C) and DNA (lanes D) in TALStΔA from **a** and α cells, as indicated, were mapped relative to the *Eco*RV site. Locations of nucleosomes IVa, IVb, and V are indicated on the plasmid map at the top of the figure. Map units shown to the left of the gel correspond to TALS map units (47) or to the tRNA gene insert (e.g., +30t; see Materials and Methods). Also shown on the left are the locations of the α2-MCM1 operator (box) and the tRNA gene (arrow) and the inferred positions of nucleosomes IVa, IVb, and V. The positions of marker (M) bands are given in base pairs to the right of the gel. Samples were treated with micrococcal nuclease for 5 min at 37°C at 0 (lanes 3 and 10), 2 (lanes 1, 4, and 9), 5 (lanes 2, 5, and 8) or 20 (lanes 6 and 7) U/ml. A shorter exposure was used for lane 2 than for the other lanes.

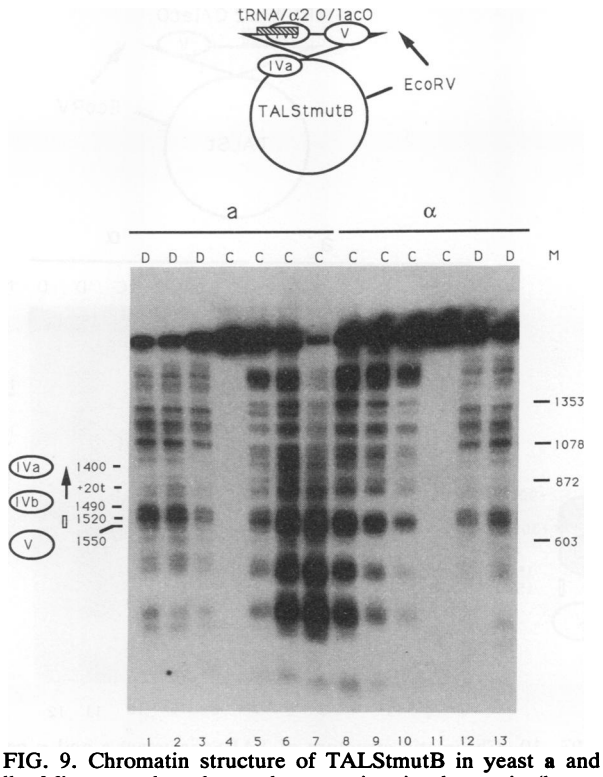

FIG. 9. Chromatin structure of TALStmutB in yeast **a** and α cells. Micrococcal nuclease cleavage sites in chromatin (lanes C) and DNA (lanes D) in TALStmutB from **a** and α cells, as indicated, were mapped relative to the *Eco*RV site. Locations of nucleosomes IVa, IVb, and V are indicated on the plasmid map at the top of the figure. Map units shown to the left of the gel correspond to TALS map units (47) or to the tRNA gene insert (e.g., +20t; see Materials and Methods). Also shown on the left are the locations of the α2-MCM1 operator (box) and the tRNA gene (arrow) and the inferred positions of nucleosomes IVa, IVb, and V. The positions of marker (M) bands are given in base pairs to the right of the gel. Samples were treated with micrococcal nuclease for 10 min at 37°C at 0 (lanes 4 and 11), 2 (lanes 5 and 10), 5 (lanes 6 and 9), 20 (lanes 7 and 8), 1 (lane 1), 4 (lanes 2 and 13), or 10 (lanes 3 and 12) U/ml.

gene. We conclude that the positioned nucleosome observed in the presence of the inactive tRNA gene is either never formed or is displaced in the presence of the active tRNA gene.

The active tRNA gene does not affect chromatin structure only in the presence of positioned nucleosomes: the pattern of micrococcal nuclease cleavage sites is also perturbed in **a** cells when the pattern for TALSt (Fig. 10) is compared with those for TALStΔA and TALStmutB (Fig. 8 and 9). We do not know whether this result is due to direct effects on nonrandom but multiply positioned nucleosomes or to folding or other poorly characterized phenomena (69). Furthermore, the digestion patterns of TALSt chromatin in **a** and α cells differ (Fig. 10), indicating that the α2 protein affects chromatin structure even in the presence of the active tRNA gene. Whether this result is due to heterogeneity (e.g., perhaps TALSt in α cells exists as a mixture of active and inactive configurations) or some other cause, it indicates that neither the α2 positioning signal nor the tRNA gene exerts a completely dominant effect on chromatin structure of the multicopy plasmid.

We have tried to assess whether the active tRNA gene causes loss of the overlapping positioned nucleosome entirely or merely induces randomization of its position, but results have been inconclusive. For example, in α cells, both TALStΔA and TALStmutB have positioned nucleosome IVa (Fig. 7) incorporating the 5′ end of the inactive tRNA gene (Fig. 8 and 9). If this nucleosome were completely absent in TALSt in α cells, TALSt should lose one corresponding supercoil compared with TALStmutB and TALSt ΔA (40). Topological measurements show no such difference (data not shown), suggesting no net loss of nucleosomes in the presence of the active tRNA gene. However, an alternative interpretation would be that nucleosome IVa is lost but that the unwinding of DNA by RNA polymerase III in TALSt compensates for this change in topology. We have also tried to examine the region occupied by nucleosome IVa by restriction enzyme accessibility (3, 61), but low and variable degrees of cutting have made these experiments hard to interpret. Moreover, even the loss of some characteristic features of nucleosome structure (e.g., protection against restriction endonuclease cleavage) does not necessarily imply the complete loss of histone-DNA contacts (12, 41). We are therefore unable at present to precisely define

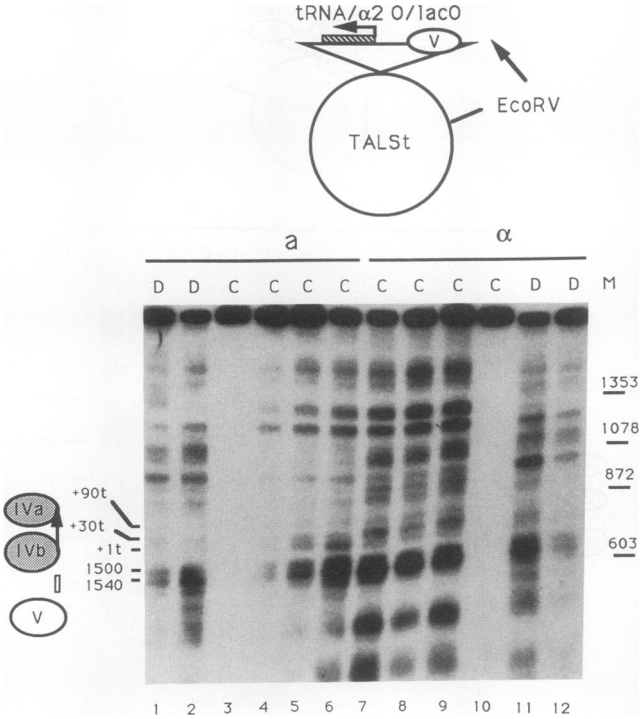

FIG. 10. Chromatin structure of TALSt in yeast **a** and α cells. Micrococcal nuclease cleavage sites in chromatin (lanes C) and DNA (lanes D) in TALSt from **a** and α cells, as indicated, were mapped relative to the *Eco*RV site. The location of nucleosome V is indicated on the plasmid map at the top of the figure. The locations indicated to the left of the gel for nucleosomes IVa and IVb correspond to those found for TALStΔA and TALStmutB in yeast α cells (Fig. 8 and 9), and the box and arrow indicate the locations of the α2-MCM1 operator and the tRNA gene, respectively. Map units shown to the left of the gel correspond to TALS map units (47) or to the tRNA gene insert (e.g., +1t; see Materials and Methods). The positions of marker (M) bands are given in base pairs to the right of the gel. Samples were treated with micrococcal nuclease for 10 min at 37°C at 4 (lanes 1 and 12), 10 (lanes 2 and 11), 0 (lanes 3 and 10), 2 (lanes 4 and 9), 5 (lanes 5 and 8), or 20 (lanes 6 and 7) U/ml.

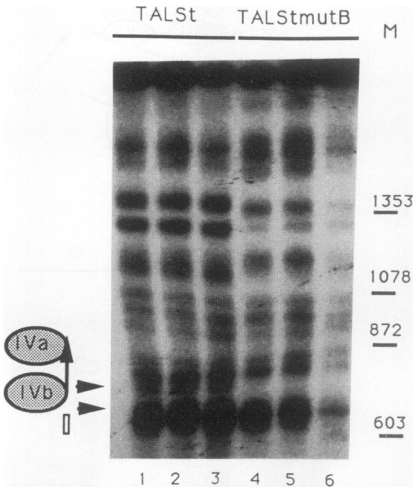

FIG. 11. Comparison of chromatin structure between TALSt and TALStmutB in yeast α cells. Micrococcal nuclease cleavage sites in chromatin were mapped from the *Eco*RV site, as in Fig. 8 to 10. The locations of the α2-MCM1 operator (box), the tRNA gene (vertical arrow), and nucleosomes IVa and IVb are indicated to the left of the gel. The horizontal arrowheads indicate micrococcal nuclease cleavage sites present in TALSt and absent in TALStmutB (see also Fig. 9 and 10). The positions of marker (M) bands are given in base pairs to the right of the gel. Samples were treated with micrococcal nuclease for 10 min at 37°C at 2 (lanes 1 and 6), 5 (lanes 2 and 5), or 20 (lanes 3 and 4) U/ml.

the changes in chromatin structure due to the active tRNA gene, other than to state that the active gene is associated with a different chromatin structure, specifically, that the active tRNA gene interferes with nucleosome positioning.

Competition between histones and *trans*-acting factors. In the competition between nucleosome positioning and *trans*-acting factor binding studied here, factor binding dominates. Two other outcomes are possible, and both have been observed. Factors and histones may be able to bind simultaneously to overlapping sequences: the competition results in a tie. This appears to be the case for GAL4 (64) and the hormone-bound glucocorticoid receptor (6, 43, 44) in vitro. The other possible outcome is for histone binding to dominate and thereby exclude *trans*-acting factors, resulting in repression of function. This has been observed in numerous in vitro studies, and in a few in vivo studies (see the introduction).

What could be the cause for different outcomes in individual instances of competition between *trans*-acting factors and histones for the same DNA sequences? One feature of the tRNA gene that could contribute to its dominating nearby nucleosome positioning signals is that its cognate transcription factors, TFIIIB and TFIIIC, may be present in much larger amounts than are whatever factors must compete with histones for the promoters of **a**-cell-type-specific genes repressed by α2. The idea that the abundance of factors at the time of replication may have a role in determining gene activity has been proposed before (63). More recently, the abundant yeast protein RAP1 has been suggested to have a role in activation of the *HIS4* promoter by binding near *cis*-acting sequences to keep them clear of histones so that the transcription factors GCN4 and BAS1/BAS2 can bind (15). Another possibility is that for genes transcribed by RNA polymerase III, such as the tRNA gene studied here, factors may initially gain a foothold at downstream sequences (i.e., the B box at about 60 bp downstream from the start site [20]) and subsequently displace the upstream nucleosome, perhaps with the aid of additional factors or RNA polymerase III itself (27, 29). The dominance of a nucleosome positioning signal over the ARS A element (58) may present a special case, as factors needed for replication might be required to bind during a different part of the cell cycle than those required for transcription; in this way, competition could be uncoupled from histone deposition, leading to a different outcome even in the presence of an abundant *trans*-acting factor.

Chromatin-mediated repression of class III genes. Previous work has shown that transcription of genes by RNA polymerase III can be inhibited by their assembly into chromatin. Transcription of the *Xenopus laevis* oocyte 5S RNA gene is repressed by histones (11, 22, 71), and histone H1 apparently plays an instrumental role in the in vivo repression of oocyte-type 5S RNA genes (51, 73). The 5S gene can also be inhibited by assembly into nucleosomes without histone H1 (4, 5, 32, 38, 54, 70).

Transcription of tRNA genes may be more resistant to nucleosome-mediated repression than are 5S RNA genes or

class II genes. Chromatin-mediated inhibition of the 5S gene takes place under conditions in which tRNA genes are transcribed (32, 73). Similarly, transcription of tRNA genes (and of rRNA genes) is not affected by partial loss of nucleosomes in yeast cells under conditions in which several class II genes are transcriptionally activated (24). Whether nucleosome positioning plays a role in this selective repression is not known; however, 5S RNA gene repression does not require a positioned nucleosome (4). In the present work, we have shown that even in the presence of nucleosome positioning signals capable of suppressing ARS function or repressing class II genes, a tRNA gene is transcribed in yeast cells. Furthermore, the tRNA sequences themselves do not exclude nucleosome formation, since the tRNA sequences could be incorporated into positioned nucleosomes when the gene was not active. This incorporation depended on an external positioning signal (e.g., it was not seen in TALStΔA or TALStmutB in **a** cells). It therefore seems unlikely that the relative resistance of tRNA genes to nucleosome-mediated repression is due to a positioning effect; more likely, it has to do with the difference in transcription factors required for its activity versus either the 5S RNA gene or class II genes. In this regard, it is noteworthy that the satellite I gene, also transcribed by RNA polymerase III and requiring only TFIIIB and TFIIIC factors for its transcription (1), is transcribed under conditions of chromatin assembly which lead to inactivation of a 5S RNA gene (4).

Although it is difficult to repress tRNA gene expression, it is not impossible. Expression of a tRNA gene can be repressed in *S. cerevisiae* by insertion into the silent mating-type locus *HMR* (52), underscoring the unusual nature of this structural component of yeast chromatin. There also exist tRNA genes whose expression is developmentally regulated in *X. laevis* (62) and whose expression is confined to specific tissues in *Bombyx mori* (60). Perhaps the apparent absence of a canonical histone H1 protein in *S. cerevisiae* (23; see also reference 34) is relevant to this point, as evidence suggests that this linker-binding histone is implicated in specific repression of other genes transcribed by RNA polymerase III in vivo (51, 73).

The ability of certain sequences to exclude nucleosomes by virtue of binding of *trans*-acting factors may have additional ramifications. The observation that Ty insertions frequently take place upstream of tRNA genes in yeast cells (16, 19) and *Dictyostelium discoideum* (35), for example, may reflect in part the presence of a nucleosome-free domain upstream of these genes. Other processes involving DNA as a template, such as recombination and repair (59), may also be facilitated for sequences close to expressed genes which are consequently not packaged into chromatin.

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