MOLECULAR AND CELLULAR BIOLOGY, Feb. 1994, p. 1266–1277
0270-7306/94/$04.00+0

Vol. 14, No. 2

tRNA Genes as Transcriptional Repressor Elements

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Received 7 September 1993/Returned for modification 29 October 1993/Accepted 8 November 1993

Eukaryotic genomes frequently contain large numbers of repetitive RNA polymerase III (pol III) promoter elements interspersed between and within RNA pol II transcription units, and in several instances a regulatory relationship between the two types of promoter has been postulated. In the budding yeast *Saccharomyces cerevisiae*, tRNA genes are the only known interspersed pol III promoter-containing repetitive elements, and we find that they strongly inhibit transcription from adjacent pol II promoters in vivo. This inhibition requires active transcription of the upstream tRNA gene but is independent of its orientation and appears not to involve simple steric blockage of the pol II upstream activator sites. Evidence is presented that different pol II promoters can be repressed by different tRNA genes placed upstream at varied distances in both orientations. To test whether this phenomenon functions in naturally occurring instances in which tRNA genes and pol II promoters are juxtaposed, we examined the sigma and Ty3 elements. This class of retrotransposons is always found integrated immediately upstream of different tRNA genes. Weakening tRNA gene transcription by means of a temperature-sensitive mutation in RNA pol III increases the pheromone-inducible expression of sigma and Ty3 elements up to 60-fold.

Many eukaryotic genomes contain families of moderately to highly repeated DNA elements containing RNA polymerase III (pol III) promoters (reviewed in references 74 and 75). Frequently these elements resemble the intragenic pol III promoter class found in tRNA and 7SL RNA genes, which consist of consensus A-box and B-box sequences downstream from the transcription start sites. These elements can be found either dispersed as individual copies or as highly reiterated tandem copies, especially in heterochromatic regions. The pol III elements are not generally transcribed into stable RNA commensurate with their copy number in vivo, although they can usually be transcribed in vitro, and there are numerous reports of condition-specific or development-specific activation in vivo (10, 27, 61, 90, 95, 97, 101). Several hypotheses have been put forward regarding possible functions for these sequences, but one particularly interesting suggestion is that dispersed RNA pol III promoters might exert either a positive or negative influence on the transcriptional activity of overlapping or nearby RNA pol II promoters (11, 12, 15, 89, 90, 96). In some cases, cryptic pol III promoter elements directly interfere with factor binding sites in the pol II promoter upstream region or with the pol II initiation site itself. In at least one report, however, repression was achieved by an *Alu* repetitive element, in which case there was no obvious steric overlap with the neighboring pol II promoter (96).

In this report, the question of whether RNA pol III promoters can exert negative transcriptional regulation on neighboring DNA has been approached by studying the budding yeast *Saccharomyces cerevisiae*. Although this yeast does not appear to have any *Alu*-type repetitive elements, the repetitive tRNA genes themselves can be considered as a dispersed family of repetitive pol III promoter elements. Yeast cells contain over 400 tRNA genes that occupy over 0.1% of the genome (32), and these genes are frequently found near the upstream control regions for genes transcribed by RNA pol II. In particular, there is a close association of tRNA genes immediately upstream of the Ty retrotransposons (5, 36, 47, 72, 79, 80). This association is precisely positioned in the Ty3 class of full retrotransposon and the more numerous free solo repeats of the sigma repetitive elements that form the Ty3 long terminal repeats. Ty3 insertion occurs with a strong preference near the tRNA gene transcription initiation site (13, 14), and it seems possible that this insertion preference developed because of a regulatory advantage conferred by the neighboring placement on the chromosome. A very similar close association with tRNA genes has been found for the DRE retrotransposons from *Dictyostelium discoideum* (69, 70).

To test whether tRNA genes affect neighboring pol II promoters in yeast cells, we placed several tRNA genes upstream of two entirely different pol II transcription units and found moderate to severe repression of RNA pol II transcription. Further, we present evidence suggesting that transcription of the neighboring tRNA genes plays a role in negatively regulating mating pheromone-responsive expression of chromosomal sigma and Ty3 elements in their normal chromosomal environment.

MATERIALS AND METHODS

Yeast strains and genetic manipulations. Unless specifically stated, plasmid expression results are reported for a strain that is wild type at *GAL4* and *GAL80*, YM607 (*MAT***a** *ura3-52 ade2-101 his3-Δ200 lys2-801 trp1-901 GAL4 GAL80*). To test the effects of *gal4* and *gal80* mutations, the related strains YM703 (*MAT***a** *ura3-52 ade2-101 his3-Δ200 lys2-801 trp1-901 tyr1-501 GAL4 gal80-Δ538*), YM708 (*MATα ura3-52 ade2-101 trp1-901 gal4-Δ542 GAL80*), and YM709 (*MAT***a** *ura3-52 ade2-101 his3-Δ200 lys2-801 trp1-901 tyr1-501 met⁻ gal4-Δ542 gal80-Δ538*) were used. Plasmid constructs were also tested for *SUP4* and *SUP53* tRNA gene and *HIS3* expression in strain YM705 (*MAT***a** *ura3-52 ade2-101 his3-Δ200 lys2-801 trp1-901 met⁻ GAL4 GAL80*), unless it is specified that the experiment was also tested in an α strain, YM2062 (*MATα ura3-52 ade2-101 his3-Δ200 lys2-801 tyr1-501 GAL4 GAL80 leu2::GAL1-lacZ*). For testing the effects

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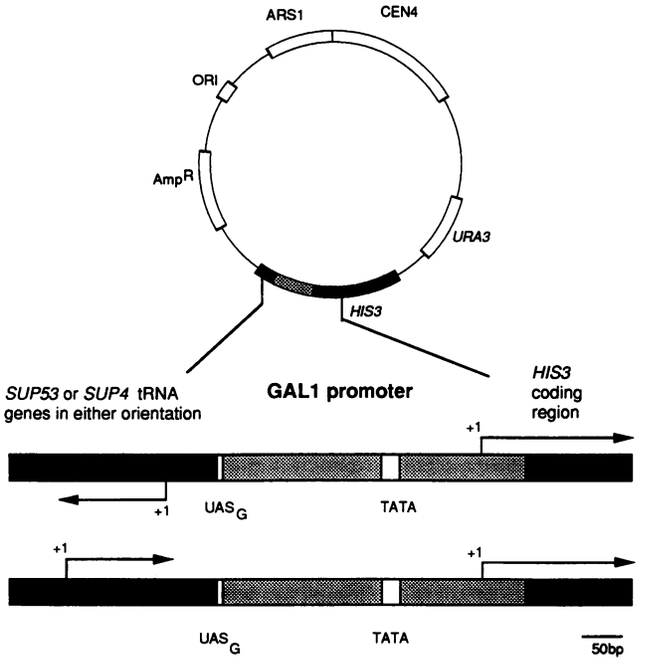

FIG. 1. Juxtaposition of tRNA genes with the UAS_{GAL}/*GAL1* promoter. Expression of *HIS3* coding sequences was put under the control of a consensus UAS_{GAL} and *GAL1* basal promoter as described in Materials and Methods. Transcription of *HIS3* sequences is induced by galactose or actively repressed by dextrose in the growth media (see Fig. 3). *SUP4* and *SUP53* tRNA gene variants were inserted upstream of the UAS_{GAL}, and their effects on transcription from the neighboring pol II promoter in yeast cells were tested by both His phenotype and *HIS3* mRNA levels (Fig. 2 and 3). The approximate extent of the primary transcript from the tRNA gene corresponds to the arrow shown over the tRNA gene.

of temperature-sensitive (ts) mutations in RNA pol III, the parental strain YNN281 (*MAT***a** *ura3-52 ade2-101 his3-Δ200 lys2-801 trp1-901*) and the same strain with a ts point mutation in the largest subunit of RNA pol III (YNN281-*rpc160-41*) were used (31, 102). Growth media were prepared and genetic manipulations performed by standard methods (46, 84).

Construction of plasmids. The general constructions of the plasmids used in this study are shown in Fig. 1 and 4. The plasmids in Fig. 1 are derived from a YCp50 variant in which small the *Eco*RI-to-*Hin*dIII fragment of YCp50 was excised and the ends were blunted and religated to remove the original *Eco*RI, *Cla*I, and *Hin*dIII sites. The *GAL1* promoter region (lacking upstream activator sites [UASs]) fused to the *HIS3* coding region was prepared from a previously described clone, p10GH (68), as a *Bam*HI-to-*Sal*I fragment and ligated into the *Bam*HI-to-*Sal*I sites of the modified YCp50. Sequencing of the insert shows that it contains a 270-bp fragment of the *GAL1* promoter (positions 546 to 816 [49]) including the major transcription start site at 760. The upstream end of the fragment was formed by exonuclease III digestion and *Eco*RI linker addition. The downstream end was created by *Xba*I cleavage and filling with DNA polymerase to blunt the site. The *HIS3* fragment was prepared by cleaving pSc2808 with *Eco*RI at −10, blunting the site for ligation to the blunted *Xba*I site of the *GAL1* promoter, and cutting with *Sal*I downstream of the *HIS3* coding sequences for ligation to the vector *Sal*I site. Upstream of the *GAL1* promoter, a single consensus UAS_{GAL} was inserted between the vector *Bam*HI site and the *Eco*RI end of the newly generated *GAL1* promoter, using a double-stranded synthetic oligodeoxynucleotide (top-strand sequence from the *Bam*HI-to-*Eco*RI sites is 5′-GGATCCGGGTGACAGCCCT CCGAAGGAATTC). This vector without tRNA genes is pBM950. tRNA genes were ligated into this *Bam*HI site upstream of the UAS_{GAL}. The *SUP4* tRNA gene variants with 70 bp of downstream sequence and variable amounts of upstream sequence in the *Bam*HI fragments were cloned in both orientations (83). *SUP53* tRNA gene variants with or without internal promoter mutations (43, 73) were amplified by PCR, using primers annealing 60 bp upstream of the start of transcription (−70 to −55; 5′-TCCTTGTTCATGTGTG) or 44 bp downstream of the transcription terminator (GAT TCTGTGCGATAGC). *Bam*HI linkers were added to the fragments, and they were cloned into the *Bam*HI site of pBM950 in both orientations. The sequences of tRNA constructs were confirmed by dideoxynucleotide chain termination sequencing from oligonucleotide primers in the vector, *GAL1*, and tRNA regions.

To make the metallothionein gene (*CUP1*) promoter constructs shown in Fig. 5, *Bam*HI fragments containing the indicated tRNA genes were excised from the pBM950 constructs and placed in either the *Bam*HI site or the *Eco*RV site (after addition of *Bam*HI linkers to the *Eco*RV site, deleting the *Bam*HI-*Eco*RV fragment of the vector) of plasmid pCLUC (gift of Dennis Thiele). pCLUC has, inserted into the *Bam*HI-to-*Eco*RI sites of YCP50, a *Bam*HI-to-*Eco*RI fragment from YIpCL containing the *CUP1* UAS/promoter fused to the β-galactosidase (*lacZ*) coding region as described previously (94). Expression of β-galactosidase activity is induced by the presence of copper in the media.

Assays for expression of the tRNA genes. *SUP4* and *SUP53* are both members of multicopy tRNA gene families (98), and RNA production is therefore difficult to assay directly. Both genes were assayed by phenotype, however. *SUP4* is an ochre suppressor that is tested by suppression of ochre mutations in *ade2* in the strains used for these studies. Constructs were also tested for suppression of the *can1-100* ochre lesion in strain W3031A (*MAT***a** *ura3-1 ade2-1 his3-11, 15 trp1-1 can1-100 GAL4 GAL80*; gift of R. Rothstein). All *SUP4* tRNA gene constructs used in this study are expressing *SUP4* by suppression phenotypes. *SUP53* genes were assayed by suppression of the *lys2-801* amber mutation in strain YM705. The *SUP53* gene without promoter mutations was expressed in all orientations and positions shown. As expected (73), all internal promoter mutations (C_{19}, G_{56}, $C_{19}G_{56}$, and $AAA_{10-12}TTT_{23-25}$; positions are denoted by subscript numbers) eliminated the suppression phenotype regardless of the strength of their effect on transcription.

Assays for *HIS3* and *lacZ* expression. Transcription of the *HIS3* gene from the UAS_{GAL}/*GAL1* promoter in the plasmid constructs was assayed qualitatively by two methods to test the effects of either *SUP4* or *SUP53* tRNA gene juxtaposition. First, His^+ phenotype was tested by the ability to grow on solid synthetic medium in the presence of galactose but in the absence of histidine. Relative growth rate (Fig. 2) was estimated by colony size with time when strains were patched or streaked side by side. All *SUP4* tRNA gene insertions shown (Fig. 3) completely prevented *HIS3* expression by this assay, whereas *SUP53* prevented expression and promoter-defective *SUP53* variants allowed different rates of growth. Growth of all constructs in the absence of histidine was tested on repressing (dextrose), noninducing (raffinose), or inducing (galactose plus raffinose) carbon

sources (shown in Fig. 3) to ensure that *HIS3* expression, when present, depended on function of the UAS_{GAL} and *GAL1* promoter. To directly test *HIS3* RNA production, cultures were grown to mid-log phase in synthetic inducing medium (galactose plus raffinose, lacking uracil). Data are shown in Fig. 2 only for the *SUP53* plasmid constructs. RNA was prepared as described previously (59), and 5 μg per lane was subjected to Northern (RNA) blot analysis (78) after separation on 1.5% agarose formaldehyde gels. Hybridization probes were 5′-end-labeled oligonucleotides complementary to the 5′ end of the chimeric *HIS3* transcript (5′-GGGCTTTCTGCTCTGTCATCTTTGCC) or the 5′ end of the *URA3* mRNA (5′-TGTAGCTTTCGACATG) as an internal control.

Expression of β-galactosidase from the *CUP1* UAS/promoter constructs (Fig. 5) was assayed in triplicate in at least two separate experiments as described previously (30, 35). Although absolute numbers of units varied between experiments, triplicate assays within an experiment were reproducible (±5%), and the degree of repression by tRNA genes was consistent between experiments. Enzyme units, shown for one representative experiment, are expressed as $200 \times (OD_{420}/OD_{600})$, where OD_{420} and OD_{600} represent optical densities at 420 and 600 nm. Cultures of 5 ml in synthetic medium lacking uracil were grown to an OD_{600} of 1.0, 100 mM cupric sulfate was added for 45 min to induce the *CUP1* promoter, and the cells were harvested for assay.

Chromosomal footprinting. Chromosomal footprinting was performed essentially as described previously (45) to detect DNase I sensitivity of the *SUP4-77s* gene and UAS_{GAL} in cellular chromatin compared with DNase I sensitivity of the naked DNA. Briefly, exponential cultures of yeast strains bearing the single-copy plasmids in selective media were harvested, washed, digested briefly with high concentrations of Zymolyase, and lysed hypotonically. Hypotonic lysates were immediately subjected to DNase I digestion for 5 min, and the DNA was purified. Cleavages were detected on both strands by annealing of ^{32}P-labeled oligodeoxynucleotides either 140 bp upstream of the tRNA gene in the plasmid vector or immediately downstream of the UAS and extension with *Taq* DNA polymerase for 15 rounds of annealing and extension. (Choice of primers was constrained by the need to overlap cloning junctions to ensure unique hybridization to only the plasmid constructs and not elsewhere in the yeast genome.) Position markers for the primer extensions were provided by dideoxynucleotide sequencing ladders produced from the same primers. Detailed chromosomal footprint analysis of the tRNA genes and interaction of Gal4 protein with the UAS_{GAL} is described elsewhere (41, 44).

Assays for sigma and Ty3 element induction. Strain YNN281 and its derivative bearing a ts mutation in the large subunit of RNA pol III (YNN281-*rpc160-41*) were grown to early log phase in synthetic complete medium at room temperature (23°C) and shifted to 37°C with prewarmed medium for the indicated length of time. At the indicated time, α mating pheromone (Sigma Chemical) was added to 10 μg/ml, and growth continued for 30 min. Cells were harvested and RNA was prepared as for Northern analysis, and the levels of sigma, Ty3, and *ADH1* RNAs were determined by primer extension on all three types of RNA simultaneously in the same reactions (62). The 5′-^{32}P-labeled primers annealed near the 5′ ends of the sigma element consensus (5′-CGAGTAATACCGGA [79]), the Ty3-1 element that is known to be expressed (5′-AGACTCATAA GATGA [16, 34]), and ADH1 (5′-CGTAGAAGATAACAC CT [4]). Primer extension products were separated by electrophoresis on DNA sequencing gels, the gels were dried under vacuum, and the radioactivity in the indicated bands (Fig. 6) was quantitated with a Betascope 603 blot analyzer. Under no conditions were significant levels of sigma or Ty3 RNA detected in the absence of α-factor induction, and only the α-factor-induced RNA is shown. Expression of sigma and Ty3 RNAs was also evaluated relative to a *URA3* internal control by Northern blot analysis (not shown), with qualitatively indistinguishable results. Primer extension results were used for quantitation because multiple sigma-specific bands were present in the small-molecular-weight range of Northern blots, presumably as a result of differences in 3′-end formation of transcripts initiated within the many sigma copies. (We estimate by Southern blot that there are 4 copies of Ty3 and >20 copies of sigma in strain YNN281.) Probes were random-primed DNA from PCR-generated entire sigma fragment or an internal *Bgl*II fragment of Ty3-1 (34).

RESULTS

Repression of pol II promoters by upstream tRNA genes. To approach the question of interference between neighboring pol II and pol III promoters, we juxtaposed tRNA genes and pol II transcription units that could be assayed phenotypically for expression. We chose two entirely distinct tRNA genes: the *SUP53* amber suppressor variant of a $tRNA^{Leu}$ gene family (1, 73) and the *SUP4* ochre suppressor of a $tRNA^{Tyr}$ gene family (28, 60, 83). These genes were inserted upstream of two different pol II-specific promoters. The first of these plasmid constructs is depicted in Fig. 1. The pol II transcription unit consists of the yeast *HIS3* coding region fused to the *GAL1* promoter, with a single consensus Gal4 protein binding site (UAS_{GAL} or UAS_G) upstream of the basal promoter. In this construct, *HIS3* expression, and therefore the ability to grow in the absence of histidine, depends on Gal4 protein. Cells were always His^- in media containing dextrose, since Gal4 protein is inactive under this condition; expression of *HIS3* was tested in medium containing galactose, in which Gal4 protein is active. Expression of the various tRNA genes inserted upstream of the UAS_G was tested phenotypically by suppression of an amber *lys2* allele in the case of *SUP53* and by suppression of ochre *ade2* and *can1* alleles in the case of *SUP4*. On growth media not selective for *HIS3*, *SUP4*, or *SUP53*, the yeast strains grew equally well regardless of which pol II or pol III promoter construct was present on the plasmid.

Figure 2 shows the results of inserting the *SUP53* tRNA gene and variants with previously characterized point mutations in the A-box and B-box internal promoters (41, 73). The effects of inserting the tRNA genes were qualitatively the same regardless of their orientation (not shown). The expressed *SUP53* gene with a wild-type promoter abolished growth in the absence of histidine (Fig. 2A) and reduced *HIS3* RNA expression (Fig. 2B). However, mutations in the tRNA gene internal promoter alleviated this repression to a degree roughly commensurate with the severity of the effect of the mutations on transcription. It is interesting that promoter mutations in both the A box and B box alleviate repression. The B-box mutations (C_{56} and $G_{19}C_{56}$) completely block binding of the initial transcription assembly factor, TFIIIC, in vitro and in vivo (41, 73), which prevents all further complex formation on the genes (41). In contrast, the A-box mutations alone decrease transcription by interfering with upstream transcription initiation complex forma-

A

UAS_G	*SUP53* tRNA gene variant[1]	tRNA gene promoter strength[2]	*HIS3* expression[3] dex[4]	gal[4]
-	-	-	-	-
+	SUP53 (WT)	100%	-	-
+	$AAA_{10-12}TTT_{23-25}$ (no A box)	10%	-	+
+	C_{19} (no A box)	10%	-	+
+	G_{56} (no B box)	5%	-	++
+	$C_{19}G_{56}$ (no A or B box)	<1%	-	++
+	-	-	-	+++

[1]-50 bp of 5' flanking sequence; same result in either orientation
[2]-from Newman *et. al.*, 1983
[3]-growth in media lacking histidine
[4]-dextrose or galactose carbon source

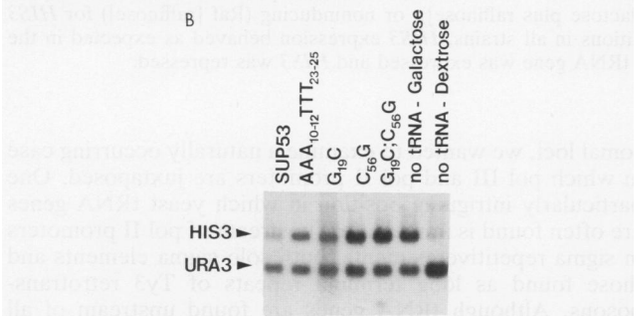

FIG. 2. Effects of the *SUP53* tRNA gene on neighboring expression of *HIS3*. The *SUP53* tRNA gene and four internal promoter variants with different degrees of residual transcription activity (73) were inserted in both orientations upstream of the UAS_{GAL} as shown in Fig. 1 and tested for their effects on *HIS3* expression. The *SUP53* gene fragments contained 60 bp upstream of the transcription initiation site and 44 bp downstream of the transcription terminator. Both orientations of a given tRNA gene variant gave the same results. (A) *HIS3* expression from the *GAL1* promoter (with or without the UAS_G) in either inducing (galactose) or repressing (dextrose) medium was determined by relative growth phenotypes in medium lacking histidine. (B) RNA from yeast cells containing the same plasmids as in panel A was subjected to Northern analysis with simultaneous probes to *HIS3* mRNA and *URA3* mRNA as an internal control. The two right-hand lanes show *HIS3* expression from the plasmid without a tRNA gene insert under inducing (galactose) and repressing (dextrose) conditions. RNAs in all other lanes are from cells grown under inducing conditions. Data are shown only for tRNA gene fragments inserted in the transcriptional orientation opposite that of the UAS_{GAL}/*GAL1* promoter.

tion but do not prevent binding of TFIIIC to the internal promoter B box. This finding suggests that it is not TFIIIC binding that results in repression of the neighboring pol II promoter. The ability of the tRNA gene to repress in both transcriptional orientations (see also Fig. 3) is also noteworthy. It suggests that the repression mechanism does not involve pol III readthrough transcription stripping bound complexes from the pol II promoter sites (see Discussion). It also makes it seem less likely that pol II repression results from direct steric interference by any particular protein components bound to either the upstream or downstream side of the tRNA gene transcription complex.

To reduce further the chances that some DNA sequences extraneous to the tRNA gene contribute to the repression phenomenon, an unrelated tRNA gene, *SUP4*, was also tested. Figure 3 summarizes phenotype assays for *SUP4* tRNA gene expression (scored by ochre suppression) and *HIS3* expression (scored by growth in medium lacking histine) in various genetic backgrounds with respect to the *GAL4* and *GAL80* transcriptional regulators. In addition, all constructs were tested under repressing, inducing, and noninducing growth conditions for Gal4 protein-mediated transcription. In the absence of a tRNA gene, the predicted phenotypes were obtained. A functional Gal4 protein was required for *HIS3* expression to be detected, and this also required induction with galactose unless negative regulation by *GAL80* was alleviated. With a *gal80* mutant, weak growth in the absence of histidine was obtained on a noninducing carbon source ($GAL4^+$ $GAL80^-$, Raf column) but not with a repressing carbon source (Dex column). In the presence of a *SUP4* tRNA gene insert, however, the tRNA gene was always expressed and *HIS3* was not expressed.

As indicated in Fig. 3, *SUP4* was inserted in both orientations upstream of the UAS_{GAL}, with various amounts of upstream flanking sequence to buffer the UAS_{GAL} from the tRNA transcription unit. Since the required yeast tRNA gene promoter sequences are contained entirely within the coding sequences, it was expected that even deletion of all 5′ sequences down to position −4 (−4*o* and −4*s* in Fig. 3) would allow expression of the tRNA gene. In the case of −4*o*, the pol III preinitiation complexes and TFIIIB binding are likely to be in direct competition with Gal4 protein binding to the UAS_{GAL}, which may have been expected to inhibit tRNA transcription (22, 23). However, for all of the other constructs shown in Fig. 3, it is unlikely that there is direct steric overlap between components of the tRNA gene transcription machinery and Gal4 protein. We have previously examined the DNase I footprints of both tRNA gene complexes and Gal4 protein complexes in chromatin (41, 43, 44, 45). The tRNA gene complexes extend from 50 bp upstream of the tRNA coding sequences to 10 bp downstream of the coding sequences. This is in good agreement with steric limits of such complexes in vitro (52, 54), and the positions of factors and RNA pol III within the complex have been assigned (see models in Fig. 7). Gal4 protein-dependent complexes lead to a much smaller protected region in chromatin footprinting (44), only about 20 bp centered on the UAS sequences.

Complexes formed in vivo on the *SUP4*-77*s* construct (Fig. 3) were probed by chromatin footprinting of both strands, and the results are shown in Fig. 4. Both the full expected footprint from the tRNA gene complex and a strong footprint over the UAS_{GAL} are clearly visible, with over 40 bp of DNase I-accessible DNA separating the two regions on both strands. Since these footprints were done under conditions in which growth in galactose failed to induce *HIS3* expression in −77*s*, the tRNA gene is probably not acting by blocking the binding of the Gal4 protein to the UAS_{GAL} but rather is acting at some subsequent step in activation (see Discussion).

To rule out the possibility that repression of pol II expression was some phenomenon peculiar to the *GAL* regulatory

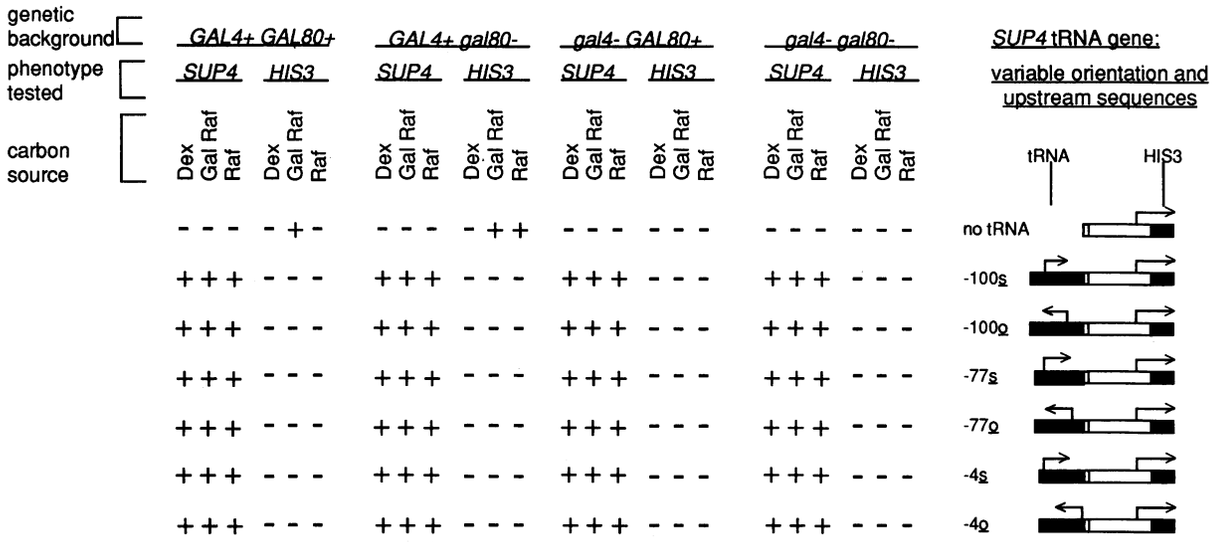

FIG. 3. Insertion of a SUP4 tRNA gene: effects of tRNA gene flanking region and *GAL* regulatory protein mutations. An ochre suppressor *SUP4* tRNA gene with 70 bp of native downstream flanking sequence and between 4 and 100 bp of upstream flanking sequence was inserted upstream of the UAS_{GAL} in the constructs shown in Fig. 1. Individual constructs are indicated at the right. tRNA gene fragments were inserted either in the same (*s*) or opposite (*o*) transcriptional orientation as the *GAL1* promoter-*HIS3* fusion gene. Expression of both the *SUP4* tRNA genes and the *HIS3* gene was assayed by growth phenotype. Expression of the tRNA gene allows growth in the absence of adenine (*SUP4* columns) by suppressing an *ade2* ochre mutation. Expression of *HIS3* allows growth in the absence of histidine (*HIS3* columns). Different combinations of mutations in the *GAL4* and *GAL80* regulatory protein genes were tested for their effects on promoter interference under conditions that were repressing (Dex [dextrose]), inducing (Gal Raf [galactose plus raffinose]), or noninducing (Raf [raffinose]) for *HIS3* expression from the UAS_{GAL}/*GAL1* promoter construct. Under all conditions in all strains, *HIS3* expression behaved as expected in the absence of a tRNA gene. In the presence of any of the tRNA genes, the tRNA gene was expressed and *HIS3* was repressed.

cascade, selected tRNA genes were also inserted upstream of an entirely unrelated UAS/promoter region, that of the *S. cerevisiae* metallothionein gene, *CUP1*. This promoter is activated by the presence of copper and other metals, which induce binding of the *ACE1* transcription factor to the UAS_{CUP1} in vivo and in vitro (25, 42). This UAS/promoter was fused to the β-galactosidase coding region to provide a reporter pol II gene product that was unrelated to *HIS3*, and tRNA genes were inserted in both orientations either 200 or 40 bp upstream of the UAS_{CUP1} control elements (Fig. 5). The result of this experiment, as judged by β-galactosidase activity induced by copper, is that the presence of either the *SUP4* or *SUP53* tRNA gene in either orientation represses expression from the *CUP1* UAS/promoter by between three- and eightfold. Internal promoter mutations in the *SUP53* tRNA gene ($C_{19}G_{56}$) alleviated this repression. Pol II expression was not as completely blocked as in the *GAL* constructs, which we take as an indication that the degree of repression may depend on the exact type of pol II promoter. Repression, although slightly variable among constructs, appeared to be of roughly the same magnitude whether the tRNA genes were inserted 40 or 200 bp from the nearest *CUP1* transcriptional regulatory site. This finding lends further support to the notion that repression is not due to simple steric blockage of the pol II UAS or promoter site. These results clearly confirm that the repressing effect is not specific for the *GAL* UAS/promoter and suggest that tRNA genes might repress surrounding chromatin in their naturally occurring chromosomal locations.

Repression by tRNA genes in native chromosomal locations. Repression by tRNA genes was documented with the *GAL1* and *CUP1* promoters because their regulation and the factors bound to their UAS sequences are well understood. To test the physiological validity these observations at chromosomal loci, we wanted to examine a naturally occurring case in which pol III and pol II promoters are juxtaposed. One particularly intriguing position in which yeast tRNA genes are often found is immediately upstream of pol II promoters in sigma repetitive elements, both solo sigma elements and those found as long terminal repeats of Ty3 retrotransposons. Although tRNA genes are found upstream of all classes of Ty retrotransposons (5), sigma and Ty3 elements are always found inserted exactly at the transcription initiation site of various tRNA genes in a head-to-head orientation. This appears to reflect a preferential Ty3 insertion mechanism (13, 14, 57), but it is possible that this insertion preference might have developed because the juxtaposition confered some advantage to either the retrotransposon or the organism. For example, one could imagine unrestrained expression of the Ty reverse transcriptase genes being detrimental to the yeast host and conditional repression by the neighboring tRNA gene providing a selective advantage. Since it is possible to induce pol II transcription of sigma and Ty3 elements with α mating pheromone, such repression could not be complete as for the *GAL* UAS/promoter. It might, however, partially repress expression or function only conditionally to prevent promiscuous Ty or sigma transcription.

To test for possible repression of all of the transcribed chromosomal Ty3 and sigma elements by neighboring pol III transcription, we used a conditional mutation in RNA pol III. The mutant strain, ts-*rpc160-41*, bears a ts point mutation in the largest subunit of RNA polymerase III that appears to cause defective enzyme assembly and inhibit pol III transcription starting 6 to 10 h after shift to 37°C (31, 62, 102). The mutant and its parental wild-type strain were shifted from the permissive temperature (23°C) to the nonpermissive temperature (37°C) for various lengths of time

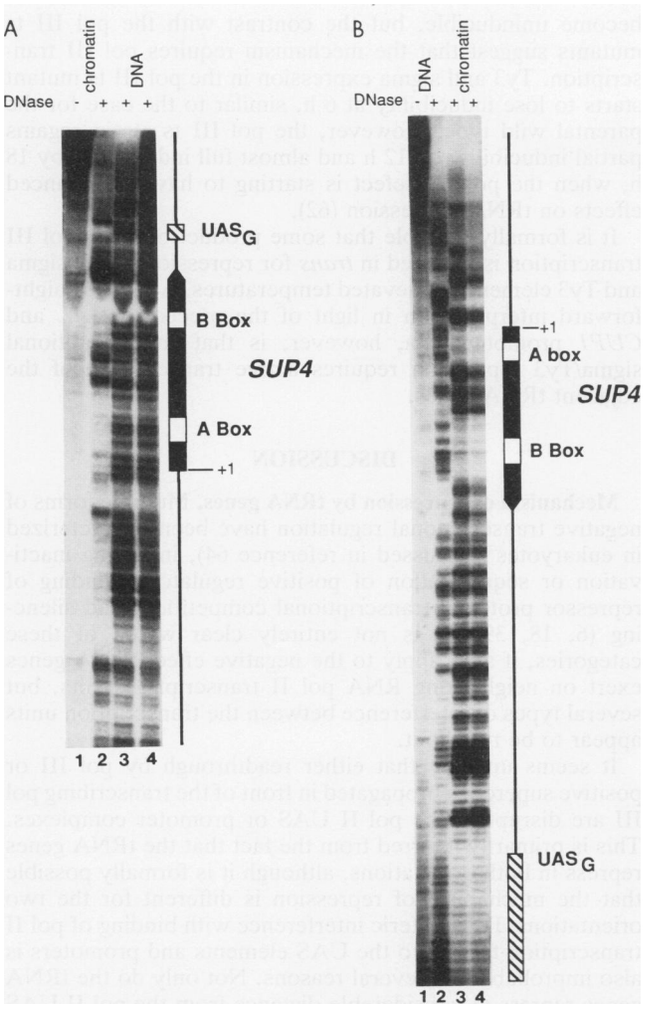

FIG. 4. Chromatin footprinting of the *SUP4* tRNA gene/UAS$_{GAL}$ region. Chromatin footprinting was used to examine complexes stably formed on the UAS$_{GAL}$ and *SUP4-77s* tRNA gene in vivo. Primer extension was used to detect DNase I-sensitive cleavages in chromatin (A, lane 2; B, lanes 3 and 4) in comparison with DNase I digestion of deproteinized DNA (A, lanes 3 and 4; B, lane 2). Primer extensions are also shown on undigested DNA (lanes 1) to indicate primer extension stops independent of cleavage. (A) Cleavage patterns of the template strand; (B) cleavage patterns on the sense strand. A schematic representation to the right of each panel indicates the positions of the tRNA coding sequences (dark arrows) with A-box and B-box internal promoters and the *UAS*$_G$ (striped box). Schematic representations of complexes on the tRNA gene and UAS are shown in Fig. 7. (Duplicate digestions of the naked DNA [A] or chromatin [B] at different DNase I concentrations are shown because the degrees of digestion between the naked DNA and chromatin did not match precisely and the duplicate digestions encompass the exactly equivalent degree of digestion.)

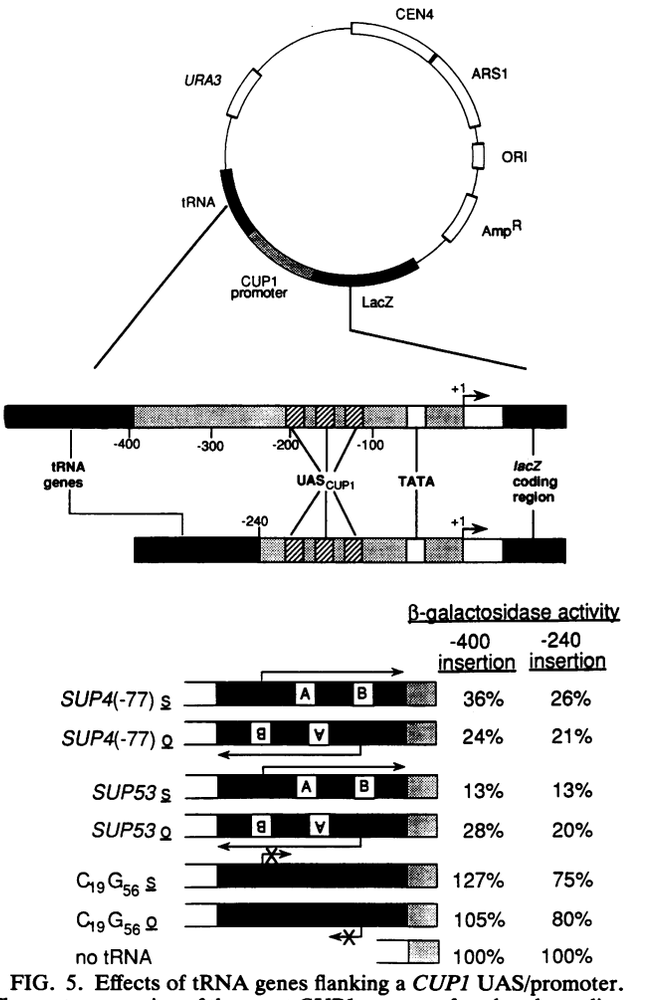

FIG. 5. Effects of tRNA genes flanking a *CUP1* UAS/promoter. The upstream region of the yeast *CUP1* gene was fused to the coding region for β-galactosidase, and various tRNA genes were inserted either 40 bp (−240 insertion) or 200 bp (−400 insertion) upstream of the most distal UAS$_{CUP1}$ transcriptional control element (41). The *CUP1* promoter was induced by addition of copper to the growth media in exponential cultures, and the induction of β-galactosidase activity was measured as described in Materials and Methods. Activity is expressed as a percentage of activity from the construct with no tRNA gene inserted. tRNA genes are the same as in those used in the *GAL* constructs (Fig. 1 to 3).

and then induced with α mating pheromone for 30 min prior to harvest and RNA primer extension analysis. The results of simultaneous primer extensions on total sigma RNAs, Ty3 RNAs, and *ADH1* RNA (internal control for pol II transcription) after α-factor induction are shown in Fig. 6 and expressed as ratios in Table 1. Uninduced sigma and Ty3 RNA levels (not shown) were low and indistinguishable between the wild-type and mutant strains. When both strains were induced after growth at the permissive temperature, induction of sigma and Ty3 RNAs was slightly (less than twofold) but reproducibly elevated in the pol III mutant relative to the wild type. This might be due to mildly defective pol III even at permissive temperatures, since the mutant strain grows slightly more slowly than the parental wild type under these conditions.

A very large difference in sigma and Ty3 expression between the wild-type and pol III ts strain was seen at the nonpermissive temperature, however, and this difference was due primarily to the fact that only the wild-type strain became uninducible by mating pheromone at 37°C. To test the full effect of the pol III mutation, a time course of temperature shift before induction was required. Unexpectedly, the parental wild-type strain consistently became uninducible by α factor, starting less than 6 h after the temperature shift and becoming almost completely uninducible by 12 h. It is not clear why the sigma and Ty3 elements

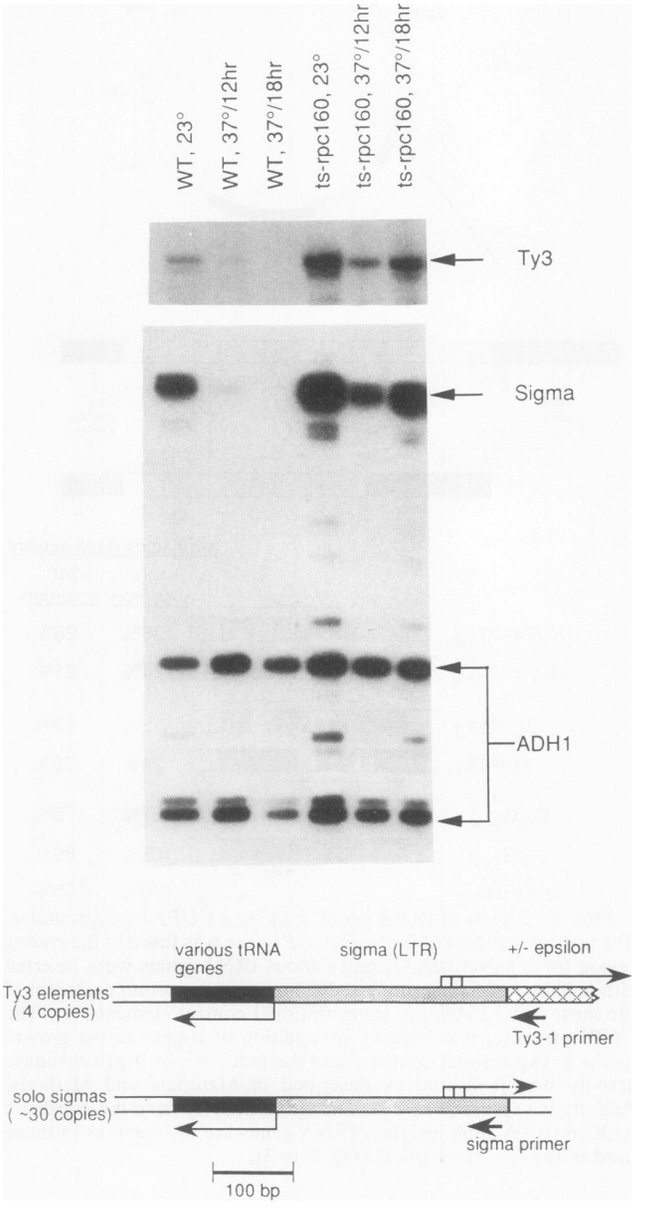

FIG. 6. Effects of a ts pol III mutation on sigma/Ty3 element induction. A yeast strain with a ts point mutation in the large subunit of RNA polymerase III (ts-rpc160) and the parental wild-type strain (WT) were initially grown to early log phase at 23°C and then shifted to 37°C to continue growing for the indicated lengths of time to allow phenotypic manifestations of the ts mutation (see Materials and Methods). Transcription from the solo sigma elements and Ty3 elements was then induced with α mating pheromone for 30 min. RNA was prepared and analyzed by primer extension using radiolabeled oligonucleotides specific for *ADH1* mRNA (internal control), sigma RNA, and Ty3 mRNA in the same primer extension reactions. The positions to which the primer probes hybridized to the consensus sigma sequence and expressed Ty3-1 sequence are indicated at the bottom on a schematic representation of the ubiquitous association of sigma and Ty3 with various tRNA genes. In the absence of α mating pheromone induction, sigma and Ty3 signals were insignificant under all growth conditions (not shown). LTR, long terminal repeat.

become uninducible, but the contrast with the pol III ts mutants suggest that the mechanism requires pol III transcription. Ty3 and sigma expression in the pol III ts mutant starts to lose inducibility at 6 h, similar to the case for the parental wild type. However, the pol III ts strain regains partial inducibility by 12 h and almost full inducibility by 18 h, when the pol III defect is starting to have pronounced effects on tRNA expression (62).

It is formally possible that some product of RNA pol III transcription is required in *trans* for repression of the sigma and Ty3 elements at elevated temperatures. A more straightforward interpretation in light of the preceding *GAL* and *CUP1* promoter data, however, is that this conditional sigma/Ty3 repression requires active transcription of the adjacent tRNA genes.

DISCUSSION

Mechanism of repression by tRNA genes. Multiple forms of negative transcriptional regulation have been characterized in eukaryotes (discussed in reference 64), including inactivation or sequestration of positive regulators, binding of repressor proteins, transcriptional competition, and silencing (6, 18, 39). It is not entirely clear which of these categories, if any, apply to the negative effect tRNA genes exert on neighboring RNA pol II transcription units, but several types of interference between the transcription units appear to be ruled out.

It seems unlikely that either readthrough by pol III or positive supercoils propagated in front of the transcribing pol III are disrupting the pol II UAS or promoter complexes. This is primarily inferred from the fact that the tRNA genes repress in both orientations, although it is formally possible that the mechanism of repression is different for the two orientations. Direct steric interference with binding of pol II transcription factors to the UAS elements and promoters is also improbable for several reasons. Not only do the tRNA genes repress at considerable distance from the pol II UAS elements, but direct examination of the chromatin showed that the UAS is occupied, presumably by Gal4 protein (44), when pol II transcription is repressed by the tRNA gene. The ability of the tRNA genes to repress in both orientations also argues against direct occlusion of the UAS, since different ends of the tRNA complex would have to be involved in the two cases.

Although derepression of Ty3/sigma expression by the pol III ts mutant suggests that the negative regulatory component might be (or act through) pol III itself, there is no direct evidence for this. Other components that interact with the polymerase and would therefore be indirectly affected by a pol III mutation should be considered. Similarities between the upstream elements of pol II and pol III promoters and the factors that help to recognize them suggest that there might be crossover interactions between pol II and pol III components (58, 65, 71, 86, 99, 100). One intriguing idea is that the TFIIIB factor, which binds immediately upstream of the pol III transcription initiation site, might be inhibiting pol II transcription by serving as a competitive ligand for components of the pol II transcription machinery. TFIIIB is composed of several subunits, including the TATA-binding protein (3, 40, 50, 53, 66, 67, 92, 103, 104). If this factor, or any other component of the pol III complex (7, 9, 17, 19, 21, 48, 51, 63, 91, 105), bound tightly to the activation domains of factors bound at the UAS elements, interactions with the pol II basal promoter complex could be competed for (Fig. 7, model A). Similarly, some component of the pol III complex

TABLE 1. Quantitation of sigma and Ty3 RNAs[a]

Construct	σ/*ADH*	Ty3/*ADH*	$(\sigma/ADH)_{mut}/(\sigma/ADH)_{WT}$	$(Ty3/ADH)_{mut}/(Ty3/ADH)_{WT}$
WT				
23°C	2.52	0.68		
37°C				
12 h	0.57	0.17		
18 h	0.07	0.07		
ts-*rpc160*				
23°C	5.11	0.88		
37°C				
12 h	0.74	0.20		
18 h	4.23	1.00		
ts-*rpc160*/WT				
23°C			2.01	1.29
37°C				
12 h			1.30	1.18
18 h			60.4	14.3

[a] The amount of radiolabel in the sigma and Ty3 primer extension bands in Fig. 6 is expressed as a ratio to the *ADH1* internal control for the wild-type parent (WT) and mutant ts-*rpc160*. The derepression of sigma and Ty3 RNA signals by the pol III ts lesion is also expressed as a ratio of the mutant (mut) signal to the wild-type signal.

might directly interact with the pol II basal promoter elements and either prevent productive interactions with upstream factors bound at the UAS (model B) or sterically block some aspect of assembly of the pol II complex (model C). The pol III complex might also attract the binding of an abundant general repressor (56) that is not yet apparent in our footprinting studies.

A last major category of repression mechanism might be modification of chromatin structure by the tRNA gene transcription complex (model D) (8) and could be thought of more generally as attracting unknown repressors to the region of DNA. While we currently have no evidence that tRNA genes order local chromatin structure, phased nucleosomes have been found associated with chromosomal tRNA and 5S rRNA genes (20, 24, 93). Such nucleosome arrays could specifically obscure pol II transcription signals bidirectionally at considerable distance from the tRNA gene, making the tRNA gene seem like a silencer element. Silencers, like enhancers, are defined as being orientation independent and reasonably distance independent, whereas repressor action is normally defined as position-specific binding that sterically interferes with some step in initiation complex assembly. Silencing at the two silent mating-type loci in *S. cerevisiae* has been studied extensively and requires both sequence-specific DNA-binding proteins and modulation of local chromatin structure (55; reviewed in reference 37). Repression of transcription near telomeres and at specific promoters (e.g., *PHO5*) has also been observed to involve chromatin structure in yeast cells (2, 26, 29, 33, 38, 76, 77, 81, 85, 87, 88), although the mechanisms by which these effects occur and their relation to silencing are not yet known. We have avoided the use of the term "silencer" in referring to repression caused by tRNA genes in yeast cells, at least until more is known of the mechanisms in both cases. In considering the relationship between the two, it is worth noting that the silencer at a yeast silent mating-type locus is dominant over (represses) transcription of a tRNA gene at the locus (82).

Possible roles for repression by tRNA genes. It seems certain that tRNA genes did not originally evolve to function as pol II transcriptional repressor elements, yet it is not unreasonable that the cell might use the repressing properties of a pol III promoter as one regulatory aspect of the chromosomal context of a pol II transcription unit. Many tRNA genes in yeast cells are repetitive, and eukaryotes with larger genomes tend to have both dispersed repetitive elements with tRNA-like promoters and cryptic pol III promoter elements distributed in and around pol III transcription units. Whatever forces gave rise to these repeated pol III elements and determined their positioning in chromosomes, there have been suggestions in the literature that they affect nearby pol II transcription. Our current data show that the presence of a tRNA gene can strongly repress nearby pol II transcription but that the degree of repression and conditions under which it manifests are variable.

The *GAL1* and *CUP1* promoters, controlled by quite different UAS-binding proteins, are both subject to this repression under all growth conditions tested, but it is not clear that the chromosomal sigma and Ty3 element promoters are as strongly affected by their associated tRNA genes at moderate growth temperatures. We are able to demonstrate complete repression of α-factor-induced sigma/Ty3 transcription only when cells are grown at elevated temperature. The fact that this repression is alleviated by inactivation of RNA pol III suggests the neighboring tRNA gene is involved in the repression mechanism. There are also many other instances in which yeast tRNA genes naturally occur upstream of pol II transcription units that can clearly be expressed (e.g., the *LEU2* gene [1]). It is not known, however, whether the tRNA genes contribute to the regulation of pol II transcription units in a more subtle fashion than the constructs tested here do. As noted above, both the type of pol II promoter and other determinants of the chromatin context could be dominant over the tRNA effects.

It is not clear what relationship the observed transcriptional repression by yeast tRNA genes might have to tRNA-like promoter elements distributed throughout the genomes of larger organisms. It seems unlikely that the pol III promoters act as repressors in all or even most instances, especially since they appear transcriptionally inert in most tissues examined. However, these repeated sequences have the capacity to bind pol III transcription factors and be transcribed in vitro, and a subset of these sequences might be activated in vivo, whether or not stable RNA products accumulate (10, 27, 61, 90, 95, 101). Given this potential, it would not be surprising to find that the pol III promoters contribute to the transcriptional regulation of the surrounding chromatin in many different contexts.

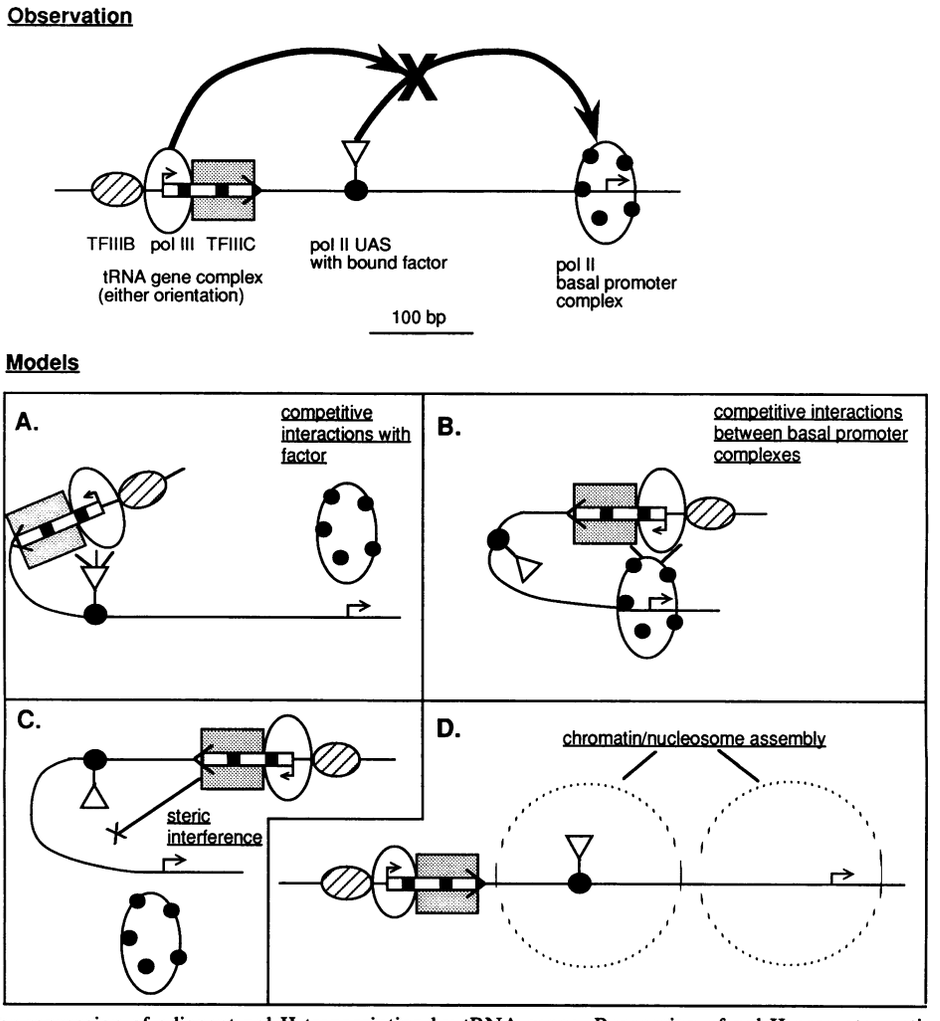

FIG. 7. Models for repression of adjacent pol II transcription by tRNA genes. Repression of pol II promoter activation is represented schematically at the top, with the tRNA gene complexes (in either orientation) interfering with activation by bound Gal4 protein. Four possible models for this repression are presented for discussion. In model A, components of the pol III complex interact with activation domains in the UAS complex to give competitive inhibition. In model B, components of the pol III complex interact with components of the pol II basal promoter machinery to block productive interactions with the UAS activation domains. In model C, the bulky pol III complex sterically blocks the ability of pol II and its basal transcription factors from interacting simultaneously with the UAS and the basal promoter. In model D, the pol III transcription complex attracts some type of repressor or repressing phased nucleosomes that block access by pol II transcription components.

ACKNOWLEDGMENTS

We are indebted to Danny Gars, Maynard Olson, Dennis Thiele, Pierre Thuriaux, and Andre Sentenac for the gifts of DNA and yeast strains, to Lisa Molony for technical assistance, and to Maynard Olson and David Burke for helpful discussions.

This work was supported by National Science Foundation grant DMB-891559 to D.R.E. and NIH grant GM32540 to M.J., and benefited from computer facilities supported by NIH grant MO1-RR00042 to the General Clinical Research Center of the University of Michigan. Oligonucleotide synthesis was partially subsidized by NIH grant P30CA46592 to the University of Michigan Cancer Center. M.W.H. was a participant in the Molecular and Cellular Biology Predoctoral Training Program at the University of Michigan.

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