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Adjacent pol II and pol III promoters: transcription of the yeast retrotransposon Ty3 and a target tRNA gene

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ABSTRACT

The *Saccharomyces cerevisiae* retrotransposon Ty3 integrates 16 to 19 basepairs upstream of tRNA genes in a region where sequences have been shown to affect the expression of tRNA genes *in vivo* and *in vitro*. Sigma, the isolated long terminal repeat of Ty3, is also found in this region. The purpose of these experiments was to elucidate the effects of Ty3 and sigma expression on that of an associated *SUP2* tRNA^{Tyr} gene *in vivo*. *SUP2* pre-tRNA levels were moderately increased when *SUP2* was associated with Ty3 or sigma in either orientation. These increases were independent of Ty3 or sigma promoter activity. The presence of Ty3 or sigma also increased the usage of a minor *SUP2* transcription initiation site 2 basepairs upstream of the major initiation site and within the 5 basepair direct repeat flanking Ty3 and sigma. Transcription from an isolated sigma directed toward the tRNA gene was observed to extend through the tRNA gene. In contrast to the lack of an effect of sigma induction on pre-tRNA^{Tyr} levels, levels of this sigma transcript were increased when the *SUP2* promoter was inactivated by a single basepair mutation.

INTRODUCTION

Ty3 is one of four classes of *Saccharomyces cerevisiae* retrotransposons (1,2). Nevertheless, it is more similar in sequence and organization to *Drosophila melanogaster* gypsy-like elements and to animal retroviruses than to the other yeast retrotransposons (3). Ty3 is composed of a 4.7 kilo basepair (kbp) internal domain flanked by directly repeated 340 basepair (bp) long terminal repeats (LTRs) or sigma elements (2). The low level of transcripts produced from the sigma promoter in *MAT*a cells is increased 20 to 50-fold in the presence of the mating pheromone, α-factor (2,4). In *MAT*a/α cells, the sigma promoter is repressed by mating-type control (V. Bilanchone, pers. comm.). Sequences that mediate these effects are present within the sigma element. Ty3 is distinguished from other retrotransposons and from retroviruses by its unique integration specificity. Comparison of a large number of isolated sigma elements and examination of two naturally-occurring Ty3 elements showed that they occurred in one of the two possible orientations 16 to 19 basepairs (bp) upstream of mature tRNA-coding sequences (5).

The integration of transposable elements has been shown to alter the expression of nearby genes. Ty1 and Ty2 insertions near polymerase II-transcribed genes can activate, vary the regulation of or inactivate their transcription (reviewed in 1). Altered gene expression results either directly, from the substitution of Ty sequences for upstream regulatory sequences, or indirectly, from interference with target gene transcription by the transcriptional activity of the inserted element. The extent of disruption of adjacent gene expression can therefore vary as a function of the expression of the inserted element. We hypothesized that Ty3 insertion could affect expression of the target tRNA gene by either of these mechanisms.

RNA polymerase III transcription of tRNA genes initiates in the 5'-flanking region and is directed by transcription factors, TFIIIB and TFIIIC, bound upstream and downstream of the initiation site, respectively (6–8). Although Ty3 insertions do not disrupt the internal promoter elements of the tRNA genes, *per se*, they do replace sequences in the 5'-flanking region, a region which has been shown to affect the expression of tRNA genes *in vitro* and *in vivo* (9–13). Due to the highly-regulated nature of the sigma promoter, the existence of Ty3 and sigma element insertions offered a unique opportunity to study the effects of altering the 5'-flanking sequences of the tRNA gene, as well as potential interactions of polymerase II and III transcription units. A single yeast tRNA gene, *SUP2*, from the well-characterized tRNA^{Tyr} gene family (14–18), was used to analyze the effects of Ty3 on tRNA gene expression *in vivo*.

MATERIALS AND METHODS

Yeast and bacterial strains and culture conditions

Methodology for manipulation of yeast and bacterial strains was essentially as described by Ausubel et al. (19). The *S. cerevisiae* strains used in this study were yVB110 (*MAT*a *Δtrp1-901 ura3-52 his3-200 ade2-101 lys2-1 leu1-12 can1-100* ΔTy3). yVB114 and yVB115 are *MAT*α and *MAT*a/α diploid strains, respectively, that are otherwise isogenic with yVB110 (V. Bilanchone, pers. comm.). Variants of these parental strains are described later.

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Synthetic medium (SD), minus uracil was used to maintain plasmids in transformed cells. For experiments involving α-factor induction, *MAT***a** cells were shifted to SD minus-uracil medium containing 0.35 μM α-factor and incubated for 20 min at 30°C prior to harvest (4).

Plasmids were maintained in *Escherichia coli* strain HB101 (*F⁻ hsdS20* [$^{r}B^{-}$, $^{m}B^{-}$] *recA13 leuB6 ara-14 proA2 lacY1 galK2 rpsL20* [*Sm*^{r}] *xyl-5 mtl-1 supE44* λ⁻). Single-stranded DNA used for oligonucleotide mutagenesis and nucleotide sequencing was prepared from *E. coli* strain RZ1032 (*lysA* [*61-62*] *thi-1 relA1 spoT1 dut-1 ung-1* [*Tet*^{r}] *supE44*) and NM322 (Δ [*lac-proAB*] *thi* Δ*hsd-5 sup-5 supE* [F′ *proAB lacI*^{q}*Z* Δ*M15*]), respectively, as described by International Biotechnologies Inc.

Recombinant DNA manipulations

Recombinant DNA manipulations, hybridization, and sequence analysis were performed essentially as described by Ausubel et al. (19). A set of constructs was created which represented all naturally-occurring Ty3-tRNA gene and sigma-tRNA gene configurations. These are displayed in Figure 1. Cloning of the sigma-plus and -minus *SUP2* alleles on which these constructs are based was described previously (20) (Fig. 1B). For ease of manipulation, the alleles were first subcloned into the multipurpose cloning vector pIBI21 (International Biotechnologies Inc.), on EcoRI-PvuII fragments. Each *SUP2* gene was marked by the insertion of 6 bp into the intervening sequence. This was performed using the site-directed mutagenesis procedure of Kunkel (21) with the oligonucleotide 5′-GATTTCGTAGGTTA-CCTGATAAATTACAG-3′ (Operon Technologies Inc., Alameda, CA.). This mutation introduced a BstEII restriction site (see Fig. 1A) and using the mutating oligonucleotide as a probe, allowed specific detection of pre-tRNA^{Tyr} produced from the constructs. The BstEII-marked tRNA^{Tyr} gene is referred to as *sup2+b*. The 5.1 kbp-XhoI fragment containing the internal domain of Ty3-1 and an interrupted copy of sigma was cloned into the XhoI site of the sigma element associated with *sup2+b*, thereby creating a *sup2+b*-associated Ty3 element. EcoRI-HindIII fragments containing the *sup2+b* alleles were cloned into the EcoRI-HindIII site of the shuttle vector YCp50 (22). In this set of constructions, referred to as the 1-series plasmids, transcription from the sigma promoter proceeds away from the tRNA gene.

The 2-series constructs, in which Ty3 and sigma transcription proceeds toward the tRNA gene, were constructed as follows. Oligonucleotide mutagenesis was carried out on the two cloned, full-length Ty3 elements: Ty3-1 and Ty3-2 (3,23), and on the BstEII-marked *sup2+b* gene present on pIBI21 plasmids. EcoRI restriction sites were introduced at the 5′ end of Ty3-1, the 3′ end of Ty3-2, and at the 5′ end of *sup2+b*. A three-fragment ligation was used to combine an EcoRI-SalI fragment containing the 5′ half of Ty3-1 and a SalI-EcoRI fragment containing the 3′ half of Ty3-2 into the EcoRI site of pIBI211, a derivative of pIBI21. The 5.1-kbp XhoI fragment, containing the internal domain and portions of the sigma elements on each end, was deleted, leaving a solo sigma element flanked by EcoRI sites. The internal domain of Ty3-1 and Ty3-2 are 99.2% identical at the nucleotide level and the composite sigma differs from that found naturally at the *SUP2* locus at 10 of 340 nucleotide positions; none of which are coincident with putative regulatory or known transcription initiation or termination sites. The resulting Ty3 and sigma elements on EcoRI fragments were cloned into the engineered EcoRI site in the 5′ flank of *sup2+b*. The spacing between the Ty3 and sigma elements and the *sup2+b* gene of these constructs and the orientation within YCp50 was the same as in the 1-series constructions.

The plasmids used for genomic integration of the *sup2+b* loci (see below) were based on the plasmid YIp21U, a pIBI21 plasmid containing the yeast *URA3* gene on a 1.1 kbp HindIII fragment, inserted by blunt-end ligation into the HincII site of the polylinker. The 1-series *sup2+b*-containing fragments were cloned into the EcoRI-BamHI sites of YIp21U and the 2-series *sup2+b* fragments were cloned into the BamHI site of YIp21U. Orientations of the inserts, relative to plasmid sequences, were maintained as in the 1- and 2-series YCp50-based constructs.

Yeast strain construction

Yeast strains containing integrated copies of the *sup2+b* loci were derived from yVB110 by integration of linearized *sup2+b*-containing plasmids. The integrating plasmids were linearized by digestion of one of the two or three SmaI sites present in the *URA3*, *SUP2* and vector sequence of the 1-series plasmids. Strains containing single integrations at *SUP2* were identified by Southern analysis of genomic DNAs digested with EcoRI and analyzed with *URA3*-, *SUP2*-, and *sup2+b*-specific probes (data not shown). The *MAT***a** strains containing integrated copies of the various constructs were designated ND1 and are differentiated by the specific *sup2+b* allele inserted (eg. ND1-1σt and ND1-2Ty3t). Diploid strains containing the *sup2+b* loci were constructed by mating the ND1-series strains to yVB114. These strains were designated ND3. ND strains were maintained on SD, minus-uracil medium to select for cells that have not undergone recombination between the duplicated *SUP2* sequences that flank the *URA3* gene of the integrating vector.

RNA isolation and northern analysis

RNA was isolated from transformed cells, or cells containing the integrated forms of *sup2+b*, that were grown to an OD_{600} between 0.5 and 0.7 in SD minus-uracil medium. For α-factor induction experiments, RNA was prepared from cells 20 min after induction. In strain yVB110, Northern blot analysis has shown that the rate of increase in levels of Ty3 transcripts diminishes by about 20 min (V. Bilanchone, pers. comm., unpublished data). Total RNA was extracted, denatured by glyoxylation, and fractionated by agarose gel electrophoresis as previously described (2). The RNA was transferred to nitrocellulose for Northern blot analysis. Loading and transfer of total RNA samples was normalized using a *URA3* probe (data not shown). Radioactive filters were exposed to XAR-5 film (Kodak) at −70°C with a Cronex Quanta III intensifying screen (DuPont) for 24−48 hours.

For quantitation of pre-tRNA species, low molecular weight RNA was isolated as described by Knapp (24). These RNA samples were fractionated by polyacrylamide gel electrophoresis, and stained in ethidium bromide to examine the integrity and relative concentrations of the RNA. The RNA was then electrophoretically transferred to a Gene Screen Plus filter as described by the manufacturer (DuPont). After transfer, the membrane was washed for 15 min in transfer buffer, dried and baked at 80°C for 1.5 hr. The filter-bound RNAs were hybridized to the ^{32}P-labeled *sup2+b*-specific oligonucleotide at 42°C for 12−16 hr according to manufacturer's instructions. Quantitation of levels of *sup2+b* pre-tRNA from low molecular weight RNA samples was by analysis of slot blot hybridizations performed as described (19). *Sup2+b* pre-tRNA^{Tyr} was detected in hybridizations carried-out in 15% formamide whereas wild-type

pre-tRNA^{Tyr} was only detectable in the absence of formamide (see Fig 3A). After hybridization, filters were washed and prepared for autoradiography as described for total RNA, except that the filters hybridized in the absence of formamide were washed at room temperature.

Relative plasmid copy number

The relative copy number of the various *sup2+b* plasmids was determined by slot blot analysis with pBR322- and yeast *LEU2*-specific probes used to detect plasmid and yeast genomic DNA, respectively. Total DNA was isolated by the method of Boeke (25) from aliquots of cell cultures used for RNA isolation. The DNA was denatured and transferred to nitrocellulose in a slot blot apparatus. Each culture was represented in duplicate slots and duplicate filters were incubated with the different probes. Hybridization was quantitated by scanning laser densitometry. The ratio of pBR322 to *LEU2* hybridization of different samples provided a measure of the relative plasmid copy number present in different transformed populations. These ratios were used to normalize the measurements of pre-tRNA for gene dosage.

Primer extension analysis

Primer extension analysis was performed on low molecular weight RNA and total RNA samples by the method of Carey et al. (26). Ten μg of low molecular weight RNA or 20 μg of total RNA was used. Primer extension reactions were titrated with template to demonstrate that the extension products were linearly proportional to template concentration and that the primer was therefore in excess under our assay conditions (data not shown). The ^{32}P-labeled *sup2+b*-specific oligonucleotide was used as the extension primer. DNA sequence ladders used to map the extension products were produced using a single-stranded DNA template and the end-labeled *sup2+b*-specific primer in reactions with dideoxynucleotides and Sequenase (United States Biochemical Corp.).

RESULTS

Recombinant constructs are correctly regulated

In order to analyze the effects of Ty3 and sigma on the expression of an associated tRNA gene, naturally-occurring alleles (20) were used to generate a set of constructs in which a biochemically distinct tRNA^{Tyr} gene was associated with sigma or Ty3 elements in both orientations (see Fig. 1B). *SUP2* was marked by the insertion of 6 bp into its 14 bp intron, thereby allowing the pre-tRNA from this gene to be distinguished from that of the endogenous wild-type tRNA^{Tyr} genes in Northern blot analysis. The 6-nucleotide insertion into *SUP2* created a BstEII restriction site and the marked *SUP2* gene was designated *sup2+b* (Fig. 1A). *In vivo* levels of these pre-tRNAs were monitored by Northern and slot blot analysis using a *sup2+b*-specific oligonucleotide probe. Plasmids were transformed into Ty3 null strains, yVB110 and yVB115, *MAT***a** and *MAT***a**/α strains, respectively, for measurement of *sup2+b* pre-tRNA levels. The *sup2+b* genes and associated elements were also integrated into yVB110 to create the ND strains.

Transcription from the Ty3 promoter is at a basal level in *MAT***a** cells, but is inducible by α-factor. Ty3 transcription is under mating-type control in *MAT***a**/α cells (2) (V. Bilanchone, pers. comm.). The recombinant Ty3 elements were shown by Northern blot analysis, using a Ty3-specific probe, to be regulated similarly

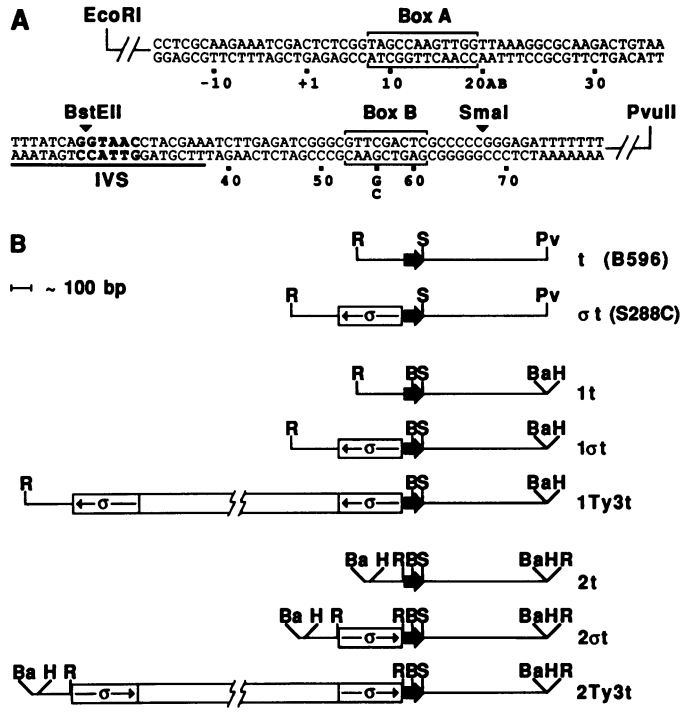

Figure 1. A. The nucleotide sequence of the *SUP2* tRNA^{Tyr} gene (this work and S.H.Goh and M.Smith, pers. comm.) shown containing the 6-bp BstEII site insertion. The BstEII insertion is shown in boldface type. **B.** *Sup2+b* alleles used in this study. All fragments depicted were cloned into the low-copy shuttle vector YCp50. Ty3 and sigma elements are indicated by open boxes; *SUP2* alleles are indicated by solid arrows. Direction of Ty3, sigma, and tRNA gene transcription is indicated by arrows. Restriction sites are indicated as follows: B, BstEII; H, HindIII; R, EcoRI; S, SmaI; and Ba, BamHI.

to wild-type elements under these three conditions (data not shown).

tRNA gene transcription initiates at the target site of Ty3 integration

Ty3 integrates into a region which can affect the site of transcription initiation as well as the expression of the tRNA gene (9–13). Primer extension analysis of low molecular weight RNA was performed in order to determine the transcription initiation site of *sup2+b* relative to the position of Ty3 insertion and to determine any effects of insertion on the position of initiation.

Low molecular weight RNA isolated from cells harboring the various *sup2+b*-containing plasmids was used as the extension template. The primer was a ^{32}P end-labeled oligonucleotide complementary to the unspliced *sup2+b* pre-tRNA. The products of the reaction are shown in Figure 2, relative to a ladder generated in a parallel reaction using a DNA template from the *sup2+b* region. Lanes 6, 7 and 8 represent the extension products of *sup2+b* tRNA from cells containing the 1-series plasmids. The extension products in lane 6 indicated that transcription of the *sup2+b* gene begins at position −10(A), relative to the mature tRNA-coding sequence. Lanes 7 and 8 show an additional initiation site, at position −12(G), when sigma or Ty3 sequences are upstream of the tRNA gene. Longer exposure of the autoradiograph revealed an extension product mapping to position −12(G) in lane 6 as well (P.T.K. and S.B.S., unpublished observations). Therefore the presence of Ty3 or sigma increases usage of a minor transcriptional initiation site for the *sup2+b* tRNA^{Tyr} gene at position −12. Similar results are shown for the

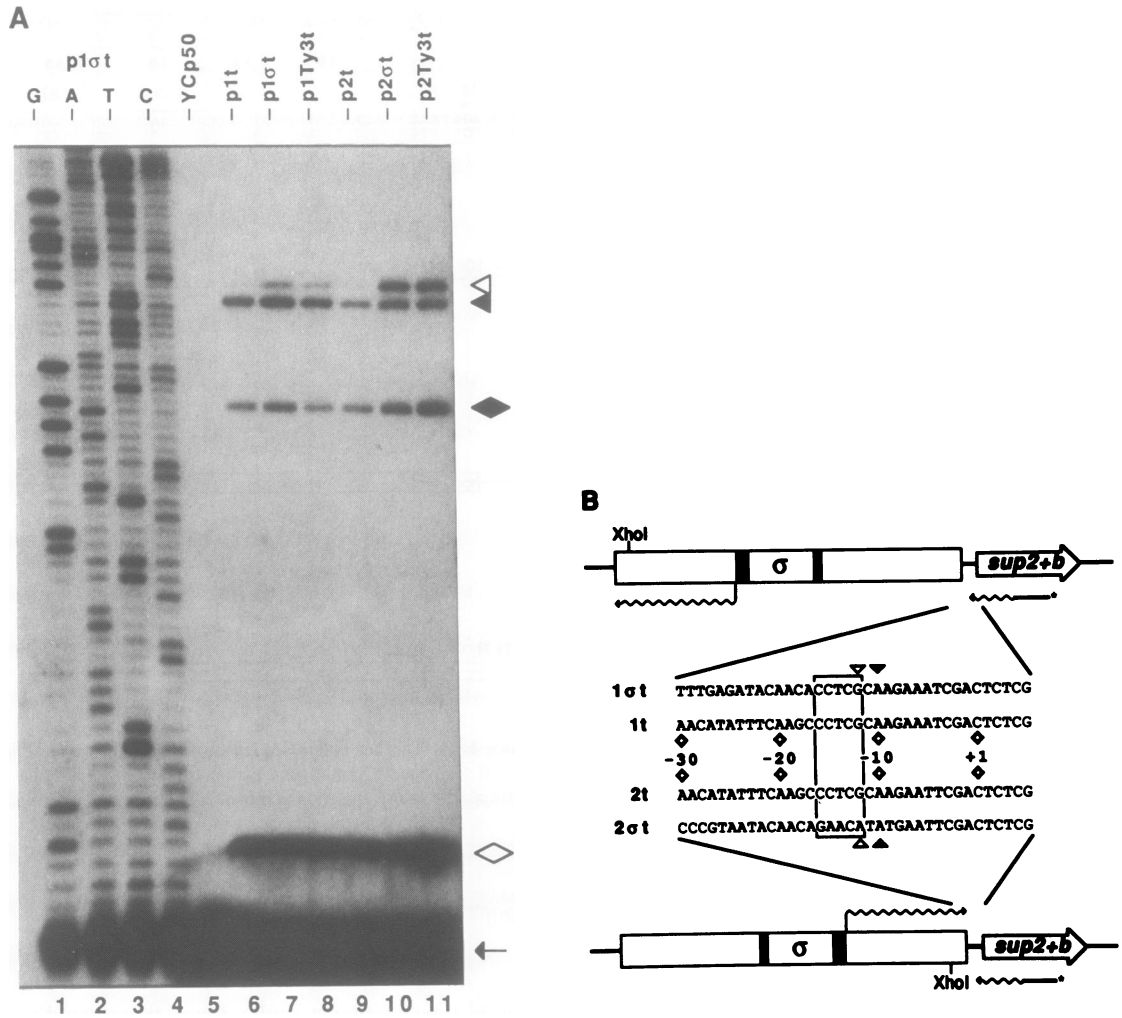

Figure 2. *Sup2+b* pre-tRNA 5′-end analysis. **A.** Low-molecular weight RNA samples from *MAT***a** cells were used as templates for primer extension. Reactions were primed with the ^{32}P-labeled *sup2+b*-specific oligonucleotide. The nucleotide sequence is of p1σt, generated using single-stranded p1σt DNA and the same end-labeled primer as in the extension reactions. Symbols indicate: (◀) major initiation site; (◁) minor initiation site; (◆) mature 5′ end of the tRNA; (◇) strong stop product of the D-loop stem; (←) excess primer. **B.** Schematic representation of the *sup2+b* loci. The nucleotide sequences of the intergenic regions of the 1t, 1σt, 2t and 2σt loci are shown between the schematic diagrams. The 5-bp duplication found flanking the *SUP2*-associated sigma element is boxed. +1 indicates the first structural nucleotide of the tRNA. The transcription initiation sites at position −10(A) and −12(G/A) are shown in closed and open triangles, respectively. Potential sigma element TATA sequences are shown as solid boxes.

2-series clones in lanes 9, 10 and 11, although as can be seen in Figure 2B, the purine at position −12 in the p2σt and p2Ty3t construct is adenine, rather than the wild-type guanine.

The extension products represented in Figure 2 were generated from RNA templates isolated from *MAT***a** cells containing the test plasmids. Primer extension analyses of RNA samples isolated from *MAT***a** cells treated with α-factor and *MAT***a**/α cells gave the same pattern of extension products within each series of plasmids (data not shown). The increased level of pre-tRNA initiated at position −12 can most simply be explained by the change in nucleotide sequence upstream of the tRNA gene resulting from insertion of a Ty3 element. The different usage of the −12 start site relative to the −10 start site between the 1- and 2-series constructs may be attributable to changes in the *sup2+b* 5′-flanking sequences (see Fig. 2B) introduced in construction of the 2-series plasmids. Interestingly, the position of the −12 transcription initiation site is within the 5-bp direct repeat flanking the *SUP2*-associated sigma element that is introduced in the course of integration and underscores the proximity of transcription initiation and Ty3 integration.

Steady state pre-tRNA levels are unaffected by induction of Ty3 transcription

In order to determine the effect of different levels of sigma promoter activity on *sup2+b* expression, low molecular weight RNAs isolated from *MAT***a** cells, *MAT***a** cells treated with α-factor, and diploid cells carrying plasmid constructs were subjected to Northern blot analysis. Hybridization conditions were determined that allowed the specific detection of the *sup2+b* pre-tRNA and end-processed forms, but not wild-type pre-tRNA^{Tyr} (see Fig 3A). In order to improve reproducibility, RNA was quantitated by slot blot analyses. The results of two independent experiments are shown in Figure 3. The pre-tRNA^{Tyr} levels in cells containing the 1- and 2-series sigma and Ty3 plasmids are expressed relative to levels in cells containing the p1t and p2t

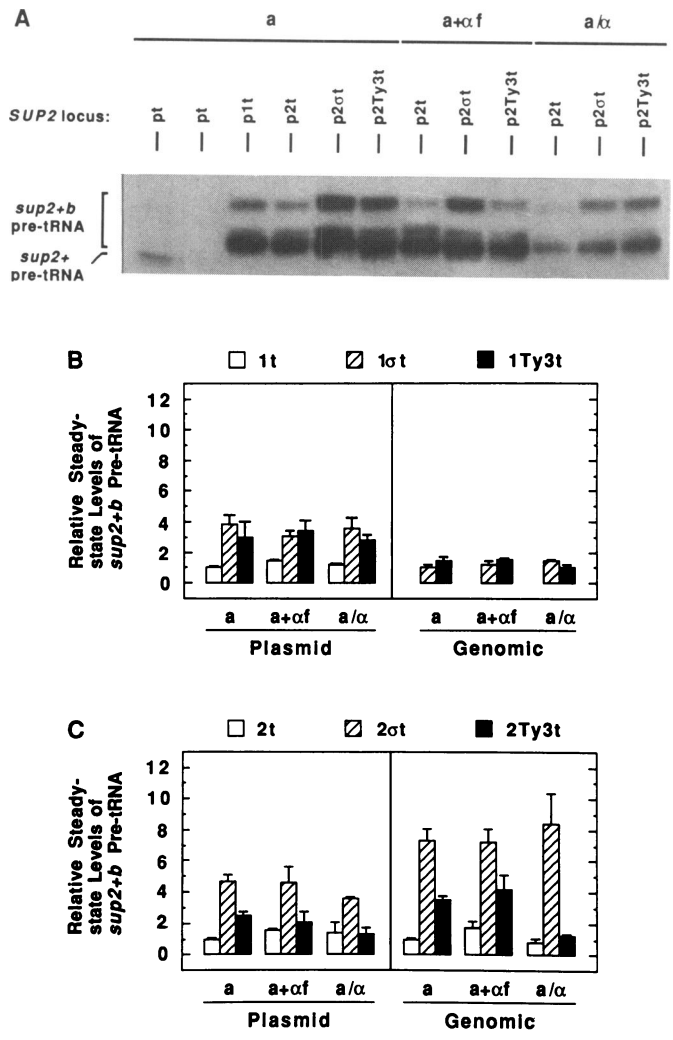

Figure 3. Analysis of *sup2+b* pre-tRNA. A. Autoradiograph of RNA hybridized with a ^{32}P-labeled *sup2+b* oligonucleotide probe. Lanes 1 and 2 are RNA samples from YCp50-transformed cells. Lanes 3–12 are RNA samples from cells containing the various *sup2+b* constructs. Lane 1 was hybridized under conditions allowing detection of wild-type pre-tRNA^{Tyr}. Lanes 2–12 were hybridized under more stringent conditions allowing specific detection of the *sup2+b* pre-tRNA. **B.** and **C.** Histograms showing the combined results of two independent slot blot or two independent Northern blot analyses of RNA from cells carrying plasmid-borne or genomically integrated 1- and 2-series *sup2+b* constructs, respectively. Pre-tRNA levels from cells in which *sup2+b* alleles were present on plasmids are corrected for relative plasmid copy number (see Materials and Methods). This copy number varied over a five-fold range. Error bars are shown above the columns and represent one-half of a standard deviation. Integrated 1t constructs were not available for analysis. Therefore, the 1-series steady-state levels are expressed relative to levels from the ND1-1σt strain.

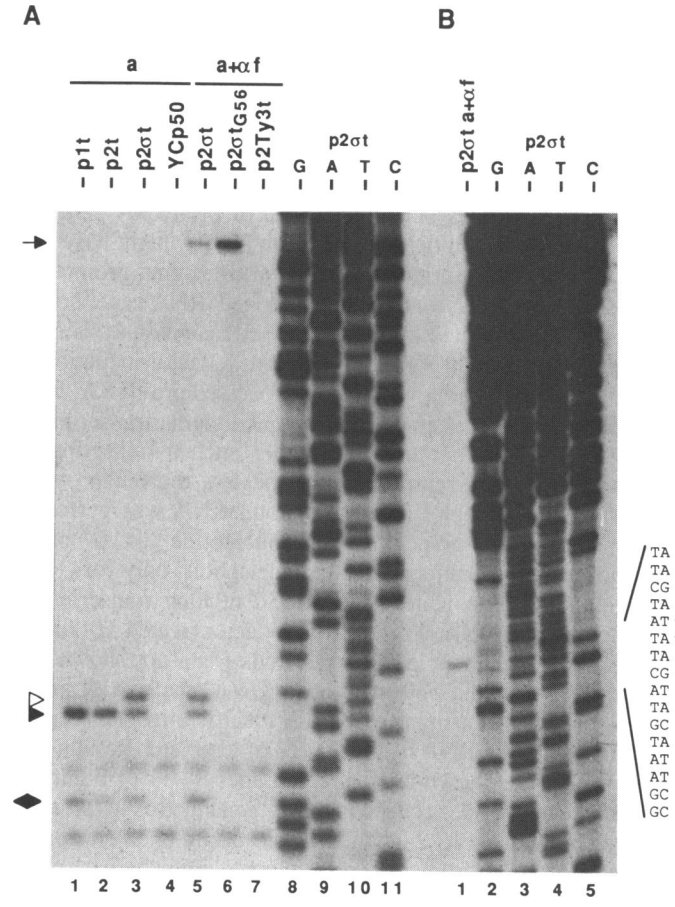

Figure 4. 5′-end analysis of the p2σt sigma transcript **A.** Extension products of total RNA were primed with the ^{32}P-labeled *sup2+b*-specific oligonucleotide and are shown in lanes 1–7. The nucleotide sequence of p2σt, primed with the same end-labeled oligo used in the extension analysis is shown in lanes 8–11. (▶ and ▷) indicate the major and minor *sup2+b* transcriptional initiation sites respectively. (◆) indicates the mature 5′ end of the pre-tRNA. The arrows point to the extension products that terminate within the sigma sequences. The extension products seen bracketing the mature-tRNA 5′-end extension products in all lanes were specific to this preparation of reverse transcriptase. **B.** Further separation of the extension products of total RNA isolated from α-factor treated *MAT***a** cells containing the p2σt construct. The asterisks adjacent to the nucleotide sequence indicate the extension products that map to the major transcriptional initiation site of sigma [position 223(T)] and position 224(A).

control plasmids, respectively. The steady-state level of *sup2+b* pre-tRNA is increased approximately 3 to 4 fold in cells containing the p1σt and p1Ty3t plasmids versus the p1t control plasmid (Fig. 3B). The increase in pre-tRNA levels was independent of the level of Ty3 or sigma promoter activity. Therefore, the increased levels of pre-tRNA may be attributable to the change in 5′-flanking sequence of the tRNA genes associated with each of the elements. Slot blot analyses of RNA isolated from cells containing the 2-series plasmids showed that steady-state *sup2+b* pre-tRNA levels were also unaffected by induction of the sigma promoter on either the p2σt or p2Ty3t plasmids. Steady-state levels of *sup2+b* pre-tRNA were increased 3.5 to 4.5 fold in cells containing p2σt, and up to 2.5 fold in cells containing p2Ty3t, compared to cells containing the p2t control plasmid (Fig. 3C). In diploid cells, pre-tRNA levels are unchanged from control levels when transcribed from p2Ty3t.

The effects of Ty3 and sigma association on tRNA gene expression were also tested using the genomic integrants. The cloned *sup2+b* genes and associated elements were integrated into the *SUP2* locus of yVB110 to produce the ND set of strains (see Materials and Methods). Total RNA was isolated from these strains and subjected to Northern blot analysis using the *URA3*- and *sup2+b*-specific probe and hybridization conditions described earlier. Results from these analyses are also presented in Figure 3. As with the 1-series plasmid data, steady-state pre-tRNA levels were unaffected by sigma or Ty3 promoter activity. The relative increase in steady-state pre-tRNA levels was different between the cells containing the 2-series *sup2+b* plasmids and genomic insertions (by approximately 2-fold), but the pre-tRNA levels in both were unaffected by induction of Ty3 or sigma transcription.

As was the case for the plasmid-borne *sup2+b*, pre-tRNA levels in diploid cells carrying the integrated 2Ty3t were equal to control levels.

A transcriptionally active tRNA gene interferes with expression from an upstream sigma promoter

The results described above suggested that transcription from the sigma promoter does not affect levels of pre-tRNA generated from the downstream tRNA gene. Although it might have been expected that a transcript originating at the sigma promoter in the 2-series constructs would extend into the tRNA gene, no such transcript was detected by the primer extension analysis of the low molecular weight RNA. In addition, α-factor induction did not result in increased levels of end-processed pre-tRNA, which argues against processing of an extended sigma transcript into a pre-tRNA. In order to test whether such a transcript was produced, but not represented in the low-molecular weight fraction, Northern blot analysis of total RNA was performed using the *sup2+b*-specific oligonucleotide as a probe. Hybridization conditions allowed detection only of RNAs containing *sup2+b* sequences. A 530 nt long transcript was detected in total RNA isolated from α-factor treated *MAT***a** cells containing the p2σt or p2Ty3t plasmids (data not shown).

The 5′ end of the 530 nt transcript was mapped by primer extension analysis using the *sup2+b*-specific oligonucleotide as a primer and total RNA as the source of template. Results from this analysis are shown in Figure 4. Lanes 8−11 of Figure 4A present the nucleotide sequence of p2σt, generated as in the earlier analysis. In lanes 1, 2 and 3, *sup2+b* pre-tRNA extension products of RNA from *MAT***a** cells containing the p1t, p2t or p2σt plasmids are shown. Lane 5 shows the extension products from total RNA of *MAT***a** cells containing the p2σt plasmid that were treated with α-factor. In addition to pre-tRNA species generated from the low molecular weight RNA template, an additional, higher molecular weight extension product is apparent that was separated into two species by further electrophoresis (see Fig. 4B lanes 1−5). The 5′ ends of these products mapped to positions 223 and 224 of the sigma element. The former was previously characterized as the major transcriptional initiation site for the sigma promoter (4,2). The absence of this product in lane 3 reflects the low basal level of this sigma transcript in haploid cells. Thus, α-factor induced transcription, initiating within sigma, extends through the tRNA gene. If this RNA is not processed, its size, together with the position of the 5′ end would place the 3′ end approximately 250 nts downstream of the tRNA gene.

The sigma and tRNA gene transcripts of p2σt were further examined to determine whether tRNA gene transcriptional activity interfered with production of the 530 nt sigma transcript. The C at position 56 of yeast tRNA genes is absolutely conserved and is within the box B promoter element (see Fig. 1A). Mutation of this base to a G, has been shown to decrease *in vitro* expression of the *SUP4* $tRNA^{Tyr}$ gene to 5% of its wild-type activity (17). This mutation was introduced into the *sup2+b* gene on the p2σt plasmid (see Materials and Methods and Fig. 1A). Total RNA isolated from cells bearing the mutated tRNA gene construct was subjected to primer extension analysis and results are presented in Figure 4. The level of the 530 nt transcript from the sigma element was higher in cells containing the mutated *sup2+b* gene than in control cells containing the wild-type *sup2+b* gene (compare the bands near the top of the autoradiograph in lanes 5 and 6). As expected, no *sup2+b*-specific pre-tRNA was detectable in these RNA samples.

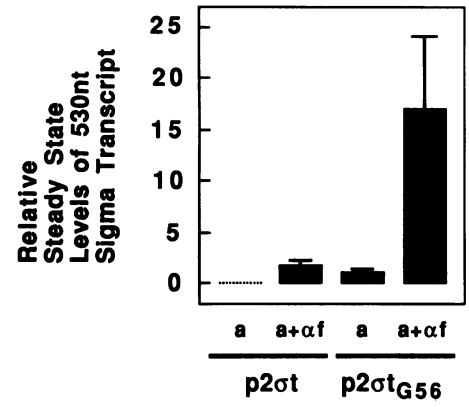

Figure 5. Northern analysis of the 2-series sigma element. The histograph represents cumulative results from three independent experiments that have been adjusted for relative plasmid copy number and total RNA. The relative plasmid copy number varied over a 3-fold range in these experiments. The 530 nt transcript from the p2σ1t plasmid in *MAT***a** cells could not be detected and is represented by a dashed line. The level of transcript from the $p2\sigma t_{G56}$ plasmid in *MAT***a** cells was assigned a value of one. Error bars represent one-half of a standard deviation.

Total RNA isolated from cells containing the mutant gene construct was subsequently examined quantitatively by Northern blot analysis using the *URA3*- and *sup2+b*-specific probes as before. The results of these experiments are summarized in Figure 5. The 530 nt transcript could not be detected in total RNA in *MAT***a** cells containing the p2σt plasmid and is represented by the dashed line. The level of the 530 nt transcript in *MAT***a** cells containing $p2\sigma tG_{56}$ was estimated by laser densitometry to be approximately half of that from *MAT***a** cells containing p2σt and treated with α-factor, and was assigned a value of one (see column 3). By comparing the levels of sigma transcripts from the p2σt and $p2\sigma tG_{56}$ plasmids in *MAT***a** cells treated with α-factor, the effect of the tRNA gene transcription complex on expression of the sigma transcript could be deduced. In cells containing the sigma promoter associated with a transcriptionally inactive tRNA gene (compare columns 2 and 4) the steady-state level of the sigma transcript observed was approximately 9-fold higher.

DISCUSSION

Constructs representing all naturally occurring permutations of tRNA genes with sigma and Ty3 elements were investigated in this study under conditions of different sigma promoter activity to determine the effects of these insertions on the expression of an adjacent tRNA gene *in vivo*. The spatial linkage between the sites of tRNA gene transcription initiation and Ty3 integration shown here suggests that the integration site may be dictated by features which influence initiation or that the transcription complex may be involved in targeting Ty3. Primer-extension mapping of the initiation site of the *sup2+b* with no insertion showed that initiation occurs within a few basepairs of the Ty3 target site. The presence of sigma or Ty3 in either orientation upstream of *sup2+b* resulted in increased usage of a minor tRNA transcription initiation site and increased levels of total *sup2+b* pre-tRNA. In cells bearing the 1-series constructs, higher levels of pre-tRNAs were observed for the sigma- and Ty3-associated tRNA genes than for the isolated tRNA gene. In the 2-series,

the pre-tRNA from the sigma-associated gene was present at higher levels that the pre-tRNA from the Ty3-associated, gene and pre-tRNAs from both were present at higher levels than from the isolated gene, except in the case of the Ty3-associated gene in diploid cells. These relationships were most dramatic in the genomic series. In diploid cells, pre-tRNA derived from the Ty3-associated gene was present at about the same levels as that from the isolated tRNA gene. While it is tempting to speculate that this is in some way related to mating-type control, the absence of a general positive or negative correlation between sigma/Ty3 promoter activity and pre-tRNA levels confounds most models. Indeed, the most striking finding of this study is that pre-tRNA levels were resistant to extreme modulation of sigma promoter expression and in fact, that tRNA transcription may interfere with initiation or elongation of sigma transcripts.

Because the steady-state level of the pre-tRNA species reflects the rates of both transcription and processing, one or both of these activities could be affected by Ty3 insertion near the tRNA gene. Nevertheless the only structural difference between pre-tRNAs generated from the sigma- or Ty3-associated *sup2+b* genes and the 'wild-type' *sup2+b* pre-tRNA is 2 additional nucleotides at the 5′ terminus in some transcripts. Sequences which affect 5′-terminus maturation have been identified within the mature-tRNA coding region (27,28), but not in the flanking region which differs among tRNA genes as a whole. Differences in processing therefore probably did not contribute to the observed differences in levels of the pre-tRNAs generated from different *sup2+b* constructs. The results of primer extension analysis are consistent with increased transcription from both the −12 and −10 initiation sites.

No change in pre-tRNA levels resulted from the activation of Ty3 or sigma transcription. Detection of an effect would depend on the contribution of *sup2+b* pre-tRNA synthesized during the period in which Ty3 or sigma transcription was induced to the total amount of *sup2+b* pre-tRNA at the time the RNA was extracted. Although the half-life of pre-tRNA has not been directly determined, pulse-labeling experiments (29) suggest that the half-life is less than 10 minutes. If this is the case, greater than 75% of the pre-tRNA pool we measured would have been synthesized during the α-factor induction period and significant changes in tRNA gene expression should have been detectable. Furthermore, *sup2+b* pre-tRNA levels in cells containing the integrated 2σt construct were unchanged throughout a 2-hr time period after induction of the sigma promoter (data not shown). Formally, an increase in the synthesis of pre-tRNA could go undetected, if compensated by the titration of a stabilizing activity. However, this explanation seems unlikely in view of the low percent of total pre-tRNA represented by the *sup2+b* pre-tRNA species. The most simple interpretation of these data is rather that sigma promoter induction has no significant positive or negative effect on the rate of *sup2+b* transcription.

The insensitivity of tRNA gene expression to transcription from an upstream promoter is in contrast to observations with some tandem promoters transcribed by RNA polymerases I or II. In tandem arrangements of polymerase I promoters from *Acanthamoeba castellanii*, transcription from the upstream promoter predominates over the downstream promoter by occluding binding of the transcription initiation factor to the downstream promoter (30). Transcriptional interference of an upstream polymerase II promoter with downstream promoter activity has been demonstrated for tandemly arranged α-globin genes (31) and the tandem promoters (LTRs) of the avian leukosis retrovirus (32). However, transcription from downstream promoters can be dominant, and in these cases, relative promoter strength may be a factor in determining which promoter is used (33). Epigenetic suppression of the upstream promoter can also occur upon selection for expression of the downstream transcriptional unit (34). Polymerases II and III have some common subunits (reviewed in 6 and 7) and may share transcription factors (35,36). Thus, whether the tRNA gene promoter is likely to compete with or suppress sigma promoter transcription or interfere with elongation is not known.

The lack of an effect of increased sigma transcription directed towards the tRNA gene on expression of the tRNA gene could be explained by one of the following models: 1) transcription by polymerase II through the tRNA gene does not affect polymerase III transcription; 2) different populations of templates are transcribed by RNA polymerases II and III; or 3) the polymerase III promoter disrupts either initiation or elongation by polymerase II. Wolffe and Brown (37) showed that *in vitro*, DNase I protection of the 5S gene by transcription factor TFIIIA could be maintained despite demonstrated readthrough of the 5S gene by T7 RNA polymerase. This would be consistent with model 1. According to model 2, the absence of interference with *sup2+b* transcription by polymerase II transcriptional readthrough from the sigma promoter could result from low occupancy of tRNA genes by stable transcription complexes. Although there are no quantitative data regarding the percentage of tRNA genes which are occupied by stable transcription complexes, Huibregtse and Engelke (38) demonstrated that a single member of the $tRNA^{Leu}$ gene family present on a high-copy plasmid shows an *in vivo* footprint comparable to the *in vitro* pattern of protection produced by the stable complex. These results suggest that a high percentage of tRNA genes are occupied and would argue against model 2. In the present work, a single point mutation which made the *sup2+b* gene transcriptionally inactive also resulted in increased levels of the 530 nt readthrough transcript from the sigma promoter. This result is most consistent with model 3, in which the polymerase III stable complex would not only be unaffected by induction of sigma transcription, but would be refractory to readthrough by polymerase II.

Our data demonstrate a modest increase in tRNA expression from the *sup2+b* gene associated with sigma and Ty3 elements, compared to the isolated tRNA gene. This is consistent with results from other laboratories showing 5′-flanking sequences can affect tRNA gene expression. It should be noted, however, that the relative magnitude of the increase in expression from the tRNA gene which accompanies association with Ty3 or sigma, is likely to be as much a function of the original activity of the particular gene as of the Ty3 substitution. Therefore, insertions of Ty3 sequences upstream of other tRNA genes are predicted to permit tRNA gene expression, but to possibly differ in the magnitude of the effect on target tRNA gene expression. Our results also showed that the downstream polymerase III promoter in *sup2+b* is refractory to transcription from an upstream polymerase II promoter. This suggests an etiological explanation of the association between Ty3 and tRNA genes, namely that Ty3 is targeted to regions of the yeast genome in which it is unlikely to have a deleterious impact. The stability of the TFIIIB-DNA interaction (39), coupled with the absence of nucleotide sequence constraints on the DNA to which it binds, makes tRNA gene 5′-flanking regions a stable genomic niche for the maintenance of foreign sequences which are transcriptionally active. Furthermore, proliferation of Ty3 elements could act as a mechanism for incremental increases in expression of tRNA genes.

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