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Enhancer and Silencerlike Sites within the Transcribed Portion of a Ty2 Transposable Element of *Saccharomyces cerevisiae*

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The Ty2-917 element is a member of the Ty2 class of retroviruslike transposable elements of *Saccharomyces cerevisiae*. We showed that regions downstream of the Ty2-917 transcription start site modulate its transcription. One region was located downstream of the transcription initiation site (position 240) and within the first 559 base pairs of the element. This region had a dramatic effect, causing an approximately 1,000-fold increase in steady-state levels of RNA. The region stimulated transcription when placed in either orientation upstream of a heterologous gene, *HIS4*, lacking its own upstream activation sequence (UAS). We termed this positively acting region an enhancer, by analogy to sites described in higher cells, to distinguish it from yeast UASs which do not function when placed within the transcribed portion of the gene. Though, like some higher eucaryotic enhancers, the Ty2-917 enhancer is located within the transcribed region, it is unlike them in that it occurs within a coding region rather than in an intron. The Ty2-917 enhancer and the Ty2-917 UAS had a synergistic effect on transcription, together stimulating transcription 15-fold over the predicted additive effect. We also identified a site which decreases RNA accumulation, located about 750 base pairs into the element. This site functioned in only one orientation when inserted upstream of the UAS-less heterologous gene. The site was similar to silencers, or negative enhancers, in that it acted to repress transcription from outside the transcribed region, but was distinct in that the function of a canonical silencer was independent of orientation.

The Ty elements of the yeast *Saccharomyces cerevisiae* are a dispersed family of approximately 30 transposons (7). They are members of a larger family of elements which replicate via an RNA intermediate, among which are the *copia*-like elements of *Drosophila* sp. (17, 35), L1Md of mice (31), and retroviruses (reviewed in reference 44). Ty elements encode two proteins termed *TYA* and *TYB*; *TYB* includes sequence homologies to the retroviral *pol* gene products, reverse transcriptase, integrase, and protease (10, 35, 45). The elements replicate by a retroviruslike mechanism (5). The replication occurs in a viruslike particle (23, 33), the proteins of which are encoded by the *TYA* gene (1). Ty elements are composed of a 5.2-kilobase (kb) internal region called ε flanked by a 0.33-kb direct repeat of a region called δ (7). Ty1 and Ty2 elements are transcribed into a 5.6-kb RNA which initiates 240 base pairs (bp) into the 5′-δ-terminal repeat and terminates 285 bp into the 3′-δ repeat at the other end, resulting in an RNA with a 45-nucleotide terminal redundancy (16). Although the 30 Ty1 elements of *S. cerevisiae* constitute only about 1% of the total genome, the Ty1 messenger constitutes about 10% of the total mRNA in haploid strains. In addition, this RNA is regulated in response to the mating type of the cell, since transcription is reduced about fivefold in *MAT***a**/*MAT*α heterozygotes (15).

Ty1 and Ty2 elements have the capacity to alter gene expression of adjacent genes by changing the activity of nearby promoters. Ty insertion mutations fall into two groups, those which inactivate adjacent genes by decreasing promoter activity (21, 39) and those which cause overproduction of adjacent genes by activating their promoter (18, 46). The insertions which cause adjacent gene activation can also impose the cell-type-specific control typical of the elements themselves, so that the RNA produced by the adjacent gene is reduced about fivefold in *MAT***a**/*MAT*α heterozygotes. In all such overproduction mutations, the Ty element inserts with its promoter δ repeat nearest the overproduced gene, which would result in divergent transcription of the Ty element and the overproduced gene. If the terminal direct repeats of a Ty element undergo homologous recombination (δ-δ recombination), the internal ε region is lost, along with one δ element, leaving a single δ inserted at the site of the original mutation (21). When Ty insertions which cause adjacent gene activation undergo δ-δ recombination, they lose the overproduction phenotype (9). This finding suggests that the ε region of the element is necessary, though perhaps not sufficient, for mating-type-dependent overproduction. Sites responsible for overexpression of adjacent genes have been identified in Ty1 (12, 19, 20, 38) and Ty2 (40) elements and have been termed enhancers. The Ty1 cell-type-specific regulator is similar to the *MAT***a**1/*MAT*α2 regulatory site of the *MAT*α gene (19). It is interesting that although a single copy of the regulator acts autonomously as a mating-type regulator, it has very little autonomous enhancer activity, instead requiring a second Ty sequence (12, 20). Reiterating the site has an additive effect on transcription, four copies stimulating transcription about eightfold (13). The region is recognized by a constitutively expressed protein factor (13). A second region of Ty1 is the major activator of adjacent gene transcription (12, 38); the region is recognized by a protein factor whose in vitro binding activity depends on an active *STE12* gene product, a gene required for expression of haploid-specific genes, including Ty1 and Ty2 elements (11). The binding site for this protein is termed a sterile responsive element (SRE) (11).

In this study, we define the sites required for transcription

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of the Ty2 element Ty2-917. We have shown that the 5'-δ-terminal repeat includes two sites required for maximal expression of the Ty2 element, an upstream activation sequence (UAS)-like site and a TATA box (30). In this work, we show that there are sites within the transcribed portion of the element which modulate the transcription of Ty2. By fusing the *Escherichia coli lacZ* gene to a collection of 3' deletions of *TYA2*, we have shown that the first 754 bp of the element contains two such sites. One of these sites, termed an enhancer, stimulates Ty2-917 transcription initiation, whether present within the Ty2-917-transcribed region or positioned in either orientation upstream of a heterologous gene. It includes a region similar to the Ty1 SRE. A second type of site decreases RNA accumulation. It can function when positioned upstream of a heterologous gene, but only in one orientation. This site is unlike a silencer (6, 29), or negative enhancer, in that the function of a canonical silencer is orientation independent.

MATERIALS AND METHODS

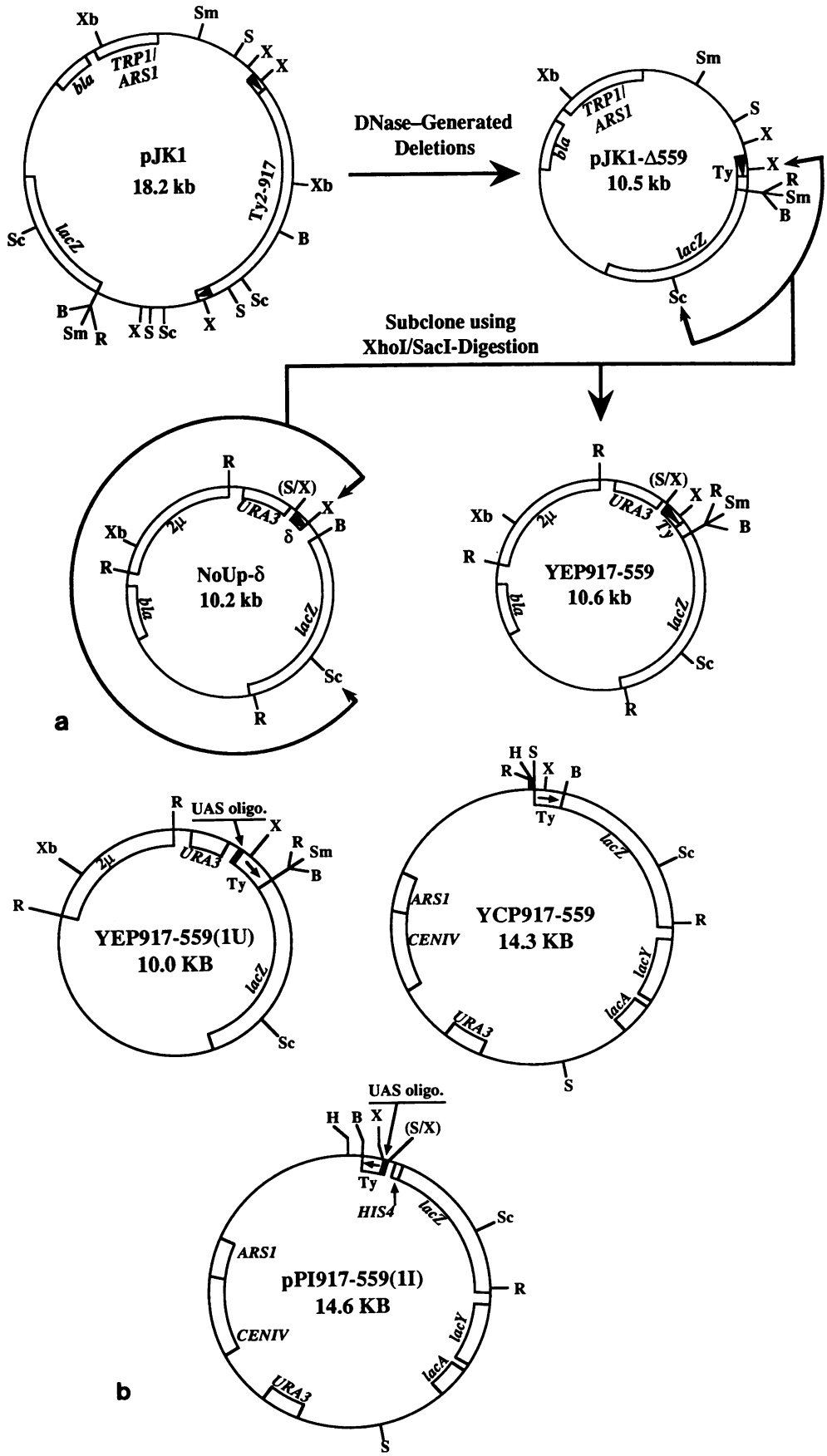
Strains and media. The *S. cerevisiae* strain used was PF387-ID (*MATα his4-38 trp1-289 ura3-52*). It was transformed with plasmids carrying the *URA3*$^+$ gene by the method of Ito et al. (28). Transformants were grown in liquid YNB minimal medium (Difco Laboratories, Detroit, Mich.) containing 2% glucose and 30 mg each of histidine and tryptophan per liter, but lacking uracil, to maintain selection for the plasmids.

Construction of Ty::*lacZ* gene fusions. Ty2-917 was isolated as an insertion into the *HIS4* promoter *his4-917*, which eliminated *HIS4* transcription (39). Plasmid pJK1 (Fig. 1a) is a vector suitable for generating Ty2-917::*lacZ* translational fusions. It was made by inserting a 9.7-kb *Pst*I/*Eco*RI fragment, which includes the entire Ty2-917 element, into the *Eco*RI site of the *TRP1*/*ARS1* shuttle vector pMC1790 (8). (The cleaved *Pst*I site was first decorated with an oligonucleotide, 5'-AATTTGCA-3', creating an *Eco*RI-compatible 5' protruding AATT sequence.) Treating the plasmid with DNase I in the presence of ethidium bromide caused double-strand breaks at random locations (4). pJK1 has a unique *Eco*RI site at the 5' end of the *lacZ* gene. Ty2-917::*lacZ* fusions were generated by attaching the random breakpoints to the unique *Eco*RI site via *Eco*RI 12-mer linkers (New England BioLabs, Inc., Beverly, Mass.). One plasmid resulting from this process, pJK1-Δ559, is diagrammed in Fig. 1a. The intrinsic instability of the pJK1-derived plasmids made them unsatisfactory for assaying Ty2-917::*lacZ* gene expression.

Each pJK1 deletion was subcloned into a 2μm DNA-based, autonomously replicating shuttle vector derived from pLGΔ312 (26), which carries a *CYC1*::*lacZ* fusion; transcription initiates within the *CYC1* promoter. We first replaced the UAS of *CYC1*, UAS_C, with a DNA fragment consisting of the region from a *Sal*I site 19 bp upstream of the Ty2-917 δ to the *Xho*I site at position 240 (the site of Ty2-917 transcription initiation); the plasmid, termed NoUp-δ, is diagrammed in Fig. 1a. The *Sal*I site had been introduced 19 bp into the flanking *HIS4* sequence upstream of the δ by attaching a *Sal*I 8-mer linker to an endpoint generated by Bal31 exonucleolytic digestion. The pJK1-derived deletions were transferred to the 2μm vector by inserting an *Xho*I/*Sac*I fragment in place of the analogous fragment of NoUp-δ; an example of the product of this construction, YEP917-559, is diagrammed. In this and all other classes to be described, the last number in the plasmid name refers to the last nucleotide of Ty2-917 retained at the Ty2-917::*lacZ* fusion junction.

Three other types of plasmids incorporating the pJK1-derived deletions were constructed. YEP917-559-1U (Fig. 1b) is an example of the first class of plasmids which have the region upstream of position 132, including the Ty2-917 UAS (30; X.-B. Liao and P. J. Farabaugh, submitted for publication), replaced by an oligonucleotide encompassing the UAS (positions 95 to 130 flanked by *Sal*I and *Xho*I sites, respectively). The oligonucleotide was first inserted into a *Sal*I site introduced by Bal31 digestion at position 132 of the element carried on YEP917-559 (30). The UAS insertion was transferred into the other YEP917 plasmids by introducing the *Xba*I/*Xho*I fragment, including a portion of the 2μm DNA, *URA3*, and the region of the UAS insertion in place of the analogous fragment of each plasmid. A second class of centromere-containing plasmids was constructed to test the effect of plasmid copy number on Ty2-917::*lacZ* gene expression. YCP917-559 (Fig. 1b) is an example of a class of *ARS1*/*CENIV*-based plasmids. A region of each pJK1-derived deletion from the *Sal*I site at position −19 to the *Sac*I site in *lacZ* was subcloned into pUC18 (47); vectors related to YEP917 which retain the *Sal*I site were the source of this fragment. A *Hin*dIII/*Sac*I fragment of the pUC18 subclone, carrying each pJK1-derived deletion, was inserted to replace the analogous *Hin*dIII/*Sac*I fragment of the YCP50-derived vector pFN8 (36), generating the YCP917 plasmids.

A third class of plasmids, termed "promoter insertions" or pPI917 plasmids, was constructed to test the ability of the Ty2-917-derived fragments to stimulate transcription when located outside a transcribed region. One such plasmid, pPI917-559(1I), is diagrammed in Fig. 1b. Starting at the unique *Hin*dIII site and proceeding clockwise, the plasmid consists of the following: the 346-bp *Hin*dIII/*Bam*HI fragment of pBR322, a fragment from the *Bam*HI site at the Ty2-917::*lacZ* fusion junction of YEP917-559 to the *Xho*I site at position 240, one copy of the UAS oligonucleotide (oriented with the *Xho*I site adjacent to position 132 to Ty2-917 upstream and the *Sal*I site downstream), and pFN8x-52 (36) from the *Xho*I site around to the *Hin*dIII site to complete the plasmid (the joining of the last two fragments by ligating *Sal*I to *Xho*I destroyed both sites [Fig. 1]). pFN8x-52 carries a *HIS4*::*lacZ* fusion in which the binding sites of all transcriptional activator proteins (*GCN4*, *BAS1*, and *BAS2*) have been deleted (2, 36). The region downstream of the *Xho*I site consists of the *HIS4* TATA region and the first 34 bp of *HIS4* fused to *lacZ*. In pPI917-559(1I), the *HIS4* TATA region is located immediately downstream of one copy of the Ty2-917 UAS, itself immediately downstream of the region from positions 240 to 559 of Ty2-917. Other versions of this plasmid were made, including either four or no copies of the UAS oligonucleotide. The constructions were made by using standard cloning techniques (32). Versions of these plasmids carrying the other pJK1-derived deletions were made by replacing the smaller *Xho*I/*Bam*HI fragment of this plasmid with the analogous fragment of the YEP917 plasmids. A version of this plasmid in which the Ty2-917 *Xho*I/*Bam*HI fragment is inserted in the opposite orientation, pPI917-559(1N), was constructed by first inserting a double-stranded oligonucleotide, 5'-GATCTCGAGCG GCCGCGGTACCGGATCCG-3' base paired with 5'-TCGA CGGATCCGGTACCGCGGCCGCTCGA-3', into *Bam*HI/*Xho*I-cleaved pPI917-559(1I). Insertion of the oligonucleotide destroys the original *Xho*I and *Bam*HI sites and creates new *Xho*I and *Bam*HI sites, in the opposite order (underlined above), flanking a unique *Not*I site. The small *Xho*I/


pJK1
18.2 kb
TRP1/ ARS1
bla
lacZ
Ty2-917
DNase-Generated Deletions
pJK1-Δ559
10.5 kb
Subclone using XhoI/SacI-Digestion
NoUp-δ
10.2 kb
URA3
2μ
δ
(S/X)
YEP917-559
10.6 kb
Ty
a
YEP917-559(1U)
10.0 KB
UAS oligo.
YCP917-559
14.3 KB
ARS1
CENIV
lacY
lacA
pPI917-559(1I)
14.6 KB
HIS4
b

*Bam*HI fragment of YEP917-559 was inserted into this plasmid that had been cleaved with *Xho*I and *Bam*HI, resulting in pPI917-559(1N).

β-Galactosidase assays. Transformants of plasmids bearing each deleted element were assayed for the level of β-galactosidase expression. Cells grown to mid-log phase (optical density at 600 nm, ≈ 1) were harvested by centrifugation, washed with distilled water, suspended in 200 μl of breaking buffer (20% glycerol, 0.1 M Tris hydrochloride, 1 mM dithiothreitol, pH 8.0) and 12.5 μl of phenylmethylsulfonyl fluoride (40 mM in ethanol; Sigma Chemical Co., St. Louis, Mo.), and permeabilized by addition of 20 μl each of chloroform and sodium dodecyl sulfate (0.1%, wt/vol). Three transformants were each assayed in triplicate as described previously (34). Units are given in micromoles of *o*-nitrophenyl-β-D-galactoside (Sigma) cleaved per minute per milligram of protein. The standard error was below 20% for all but the lowest levels of expressed β-galactosidase (<10 U).

Primer extension of RNA analysis. Total RNA was prepared as described previously (10) from logarithmically growing cultures of cells. To measure the level of transcription and to determine the site of transcription initiation of Ty2-917::*lacZ* hybrid genes, we performed primer extension with a probe specific for *lacZ*. A 40-nucleotide oligonucleotide, CCATTCGCCATTCAGGCTGCGCAACTGTTGGGA AGGGCGA, is complementary to a portion of *lacZ* from 113 to 152 bp downstream of the fusion junction of the Ty2-917::*lacZ* plasmids. The oligonucleotide was labeled with [γ-^{32}P]ATP (>5,000 Ci/mmol; Amersham Corp.), and 1.5 × 10^5 cpm were hybridized to 25 μg of total RNA. The primer was extended by using avian myoblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla.). The conditions of hybridization and extension were essentially as described before (43). Conditions of extension were adjusted so that extension would proceed to the end of the RNA molecules present. The critical parameters are the amount of reverse transcriptase and the concentration of deoxynucleotide triphosphates. A 50-μl reaction contains 100 nmol of each deoxynucleotide triphosphate and 100 U of reverse transcriptase. The extension products were separated by electrophoresis in a 6% polyacrylamide (1:20 bisacrylamide)–50% urea gel and visualized by autoradiography. The images were scanned and digitized with a model 620 video densitometer (Bio-Rad Laboratories, Richmond, Calif.). To ensure that exposure of the autoradiogram was in the linear range, autoradiograms exposed for various lengths of time were scanned. When the ratio of the most dense to least dense bands began to fall, the film was presumed to be overexposed.

Determining plasmid copy number by Southern blotting. To determine the copy number of the plasmids used in this study, DNA was extracted from transformed clones as described before (27). The DNA was restricted with *Eco*RI (YEP917 transformants) or *Eco*RI and *Sal*I (YCP917 transformants) and separated by agarose gel electrophoresis. The fragments were electrophoretically transferred to a Gene-screen-Plus membrane (Dupont, NEN Research Products, Boston, Mass.). Plasmid pR1 (P. J. Farabaugh, unpublished data), a plasmid carrying a 2.5-kb fragment of *HIS4C*, was end labeled with T4 DNA polymerase as described previously (32). This yields two equally labeled fragments, one specific for the chromosomal *HIS4* gene and the other specific for the transforming plasmid. The blotted membranes were hybridized and washed by using the manufacturer's specifications and visualized by autoradiography. The results were quantitated by scanning densitometry as given above.

RESULTS

Deletion analysis identifies multiple modulators of Ty2-917 gene expression. We constructed a series of deletions of the 3′ end of Ty2-917 to determine whether sites within the transcribed region modulate transcription of the element (the YEP917 series of plasmids). The deletions, constructed as described in Materials and Methods, fuse the *E. coli lacZ* gene to various positions within the *TYA2* gene. In each case, transcription and translation beginning within Ty2-917 resulted in expression of a *TYA2*::*lacZ* fusion protein. As an initial test of the effect of removing various amounts of the 3′ end of the element, we transformed each fusion plasmid into yeast cells and assayed the β-galactosidase activity of the fusion protein. The *TYA2*::*lacZ* fusions express levels of β-galactosidase which vary >1,000-fold. The results of assays of these fusions are shown in Table 1. Plasmid YEP917-296 retained only the first 4 bp of the *TYA2* gene and expressed nearly undetectable amounts of β-galactosidase activity. A deletion retaining the 5′ end of the element to position 388 stimulated 20- to 40-fold-higher expression. Including the region to position 458 stimulated 490-fold more activity than YEP917-296, and the region to position 559 stimulated 1,000-fold more. This increase in expression suggests that a positively acting site exists between positions 296 and 559. That the increase in expression seemed to occur in three steps, between positions 296 and 312, 388 and 458, and 501 and 559, suggests that the region may be composed of multiple distinct sites. When portions of the region past position 559 were included in the Ty2-917::*lacZ* fusion, expression was reduced, first about 2.5-fold at position 667 and then a further 4.2-fold for fusions occurring distal to position 667. Expression of all *TYA2* fusions past position 754 was the same, ca. 180 U (Table 1; data not shown). That inclusion of the region past position 559 caused a decrease in expression suggests that one or more negative sites exist in that region.

To exclude the effect of variations in plasmid copy number among the deleted plasmids, we performed two experiments. First, Southern blotting, performed as described in Materials and Methods, determined the number of copies of each plasmid construction present. Densitometry was performed on autoradiograms that had been exposed for various lengths of time to exclude the effect of overexposure. The experiment showed that all of the YEP917 plasmids were present at

FIG. 1. Plasmid maps. The maps of representative plasmids used in this study are shown. (a) Construction of plasmid YEP917-559, described in Materials and Methods. (b) Structure of representative examples of the three other classes of plasmids used in the study. The positions of relevant genes are indicated inside of the circle depicting each plasmid; the extent of each gene is represented by a boxed arc. The δ-terminal repeat of Ty2-917 is indicated by a triangle, the triangle pointing in the direction of Ty2-917 transcription. For clarity, the terminal repeat is not diagrammed in panel b, the direction of transcription being shown by an arrow. The position of the oligonucleotide corresponding to the Ty2-917 UAS (UAS oligo.) is shown as a filled rectangle. B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I.

TABLE 1. Positive and negative modulators of gene expression in the transcribed region Ty2-917

Ty 2-917::*lacZ* fusion junction	β-Galactosidase activity (U)[a]		
	YEP917		YCP917 per plasmid copy
	Total[b]	Per plasmid copy[c]	
296	2	<1	≤1
312	45	11	6
342	30	8	5
388	81	20	18
458	980	250	290
475	700	180	ND[d]
501	560	140	250
559	2,100	530	760
667	860	220	430
754	210	52	190
806	300	75	ND
934	130	33	200
1072	110	28	190

[a] Units are expressed in micromoles of *o*-nitrophenyl-β-D-galactoside cleaved per minute per milligram of protein.
[b] Units of β-galactosidase in crude protein extracts of each transformant.
[c] Units per plasmid copy (4 copies of the YEP plasmids, 1 copy of the YCP plasmids).
[d] ND, Not determined.

approximately the same copy number, estimated at 4 ± 0.6 per haploid genome (data not shown). Thus, the difference in expression seen among the transformants cannot be due to differences in copy number.

Second, we constructed a series of *ARS1/CENIV*-based plasmids carrying each of the 3′ deletions of Ty2-917. Quantitative DNA blotting demonstrated that the *CEN* plasmids were present at 1 ± 0.25 copies per haploid genome (data not shown). Transformants of each of these were assayed for β-galactosidase. The expression of the *CEN*-based plasmids (Table 1, column 4) confirmed that the differences in expression among the deletions were not due to copy number variation. Addition of the region downstream of the translation initiation site to as far as position 458 caused an increase from 1 to 290 U of β-galactosidase. Addition of the region from positions 458 to 559 caused a further increase to 760 U. This level then fell off to 430 U at position 667 and to 180(± 30) U for deletions ending past position 667. The almost 800-fold stimulation of expression when the region up to position 559 was present and the subsequent fall of 4.2-fold when the region past position 667 was included cannot derive from variations in the amount of DNA template.

A somewhat surprising result is that the expression of YCP917 and YEP917 plasmids bearing the region distal to 667 was nearly the same, despite the fact that they are present at about 1 and 4 copies per haploid genome, respectively. Expression of the YEP917 plasmids averaged 152 ± 52 U of β-galactosidase, 38 ± 13 U of β-galactosidase per plasmid copy, while the YCP917 plasmids averaged 193 ± 6 U. This contrasts with the effect of changing copy number on plasmids not retaining the region distal to position 667. In each case, expression was roughly proportional to plasmid copy number (cf. columns 3 and 4 of Table 1). Since the flanking sequences immediately upstream of the Ty2-917 insert are different in the YCP917 and YEP917 plasmids (the *bla* gene of pBR322 and the *URA3* gene, respectively), the effect could be due to the effect of these flanking sequences. This unusual behavior must be accounted for in any model describing the mechanism of action of this negative region.

Sites within the Ty2-917-transcribed region modulate steady-state RNA levels. To determine whether the differences in expression of the Ty2-917::*lacZ* fusions reflect changes in either the structure or steady-state levels of RNA, we performed primer extension analysis, as described in Materials and Methods. Total RNA expressed by each transformant (25 μg) was hybridized to an end-labeled single-stranded oligonucleotide complementary to a region of the *lacZ* gene from 113 to 152 bp downstream of the fusion junction. The primers were extended by avian myoblastosis virus reverse transcriptase, and the products were separated by electrophoresis in a urea-polyacrylamide gel. The experiment directly demonstrated both the position of all 5′ termini and the relative amount of steady-state RNA expressed by each construct. Deoxynucleotide triphosphates and reverse transcriptase were titrated to minimize pausing products (data not shown). The data presented in Fig. 2 are consistent with all of the YEP917 (lanes 2 to 9) and YCP917 (lanes 11 to 18) constructs directing initiation at the normal location, the *Xho*I site at position 240. The relative steady-state level of each transcript was determined by densitometric scanning and is proportional to the level of β-galactosidase produced in vivo, except for YEP917-296, for which no band is detectable (Table 2). Thus, the variation in expression is an effect on the transcript level, not a translational effect. The variation could result from a difference in the rate of transcriptional initiation or from some process acting during elongation or after synthesis of the RNA.

A transcribed region of Ty2-917 functions as an enhancer to stimulate a heterologous promoter. To determine whether the downstream sites act at initiation or during some subsequent step, we constructed a set of promoter insertion constructions. In these, portions of the downstream region were inserted upstream of a UAS-less *HIS4*::*lacZ* gene; the gene carries the deletion *his4Δx-52*, which removes the binding sites for both the *GCN4* product, responsible for stimulating transcription during derepression, and the *BAS1* and *BAS2* products, responsible for the basal level of transcription (2). The deletion retained only the TATA box and initiation site (I site). This gene is transcribed very poorly (36). This well-characterized DNA region can be used to test whether heterologous fragments are able to stimulate transcription dependent on the TATA box and I site. We inserted the region from the RNA initiation site (the *Xho*I site at position 240) through various portions of the transcribed region as described in Materials and Methods. Sets of plasmids include portions of the transcribed region inserted in either orientation upstream of the *HIS4* TATA box. Any transcription of these constructions is dependent on activation by sites within the inserted Ty2-917 DNA. Including as little as the region from 240 to 559, in either orientation, allowed 50- to 100-fold stimulation of transcription (Table 3, columns 2 and 5). The amount of expression was similar to that promoted by the same region when present in its normal context without the UAS being present, 33 U per copy (30). We have previously shown that the UAS and the positive site stimulate transcription synergistically (30). The effect of the sites in combination was 10-fold higher than the predicted additive effect. To test whether the UAS and positive site synergistically stimulate transcription of the heterologous promoter, the UAS was introduced between the various portions of the Ty2-917-transcribed region and the *HIS4* TATA box. Table 3, columns 3 and 6, shows that the sites did synergistically stimulate the heterologous promoter, stimulating fourfold more than the predicted additive effect in one orientation (480 versus 127) and 17-fold more in the

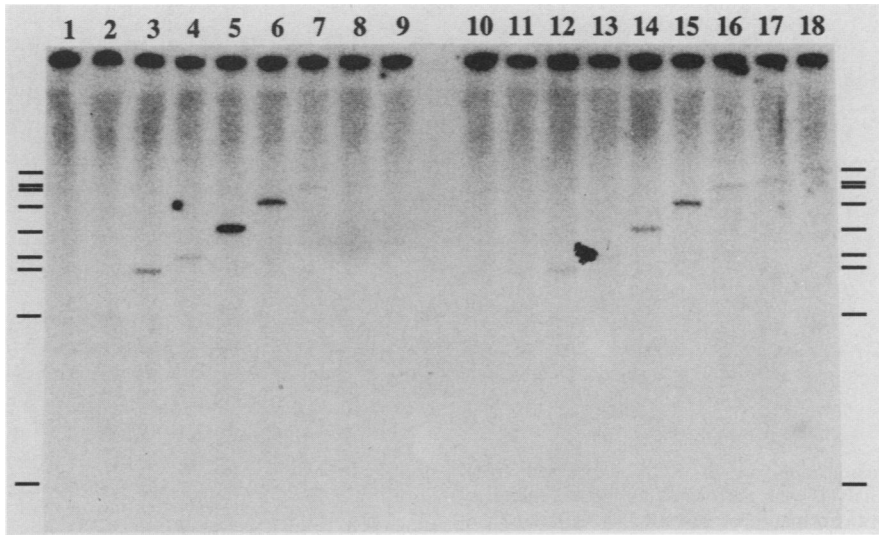


FIG. 2. Primer extension analysis of RNA expressed from YEP917 and YCP917 plasmids. The amount of Ty2-917::*lacZ*-specific RNA from 25 μg of RNA isolated from cells transformed with YEP917 and YCP917 plasmids was assayed by primer extension as described in Materials and Methods. Lanes 1 to 9 correspond to preparations from YEP917 transformants, and lanes 10 to 18 correspond to preparations from YCP917 transformants. The lanes correspond to fusions to *lacZ* at the following positions within the element: 296 (lanes 1 and 10), 388 (lanes 2 and 11), 458 (lanes 3 and 12), 501 (lanes 4 and 13), 559 (lanes 5 and 14), 667 (lanes 6 and 15), 754 (lanes 7 and 16), 806 (lanes 8 and 17), and 934 (lanes 9 and 18). The positions expected for products corresponding to initiation at the normal site (position 240) are indicated by bars. A portion of the loaded material nonspecifically bound to the wells can be seen as a dark band at the top.

other (1,100 versus 66). Figure 3 shows that the enzyme activity expressed by the constructs with and without the UAS correlates with the amount of steady-state transcript.

Since the region stimulated transcription when inserted outside the transcribed region, in either orientation we conclude that it functions as a transcriptional activator. Yeast UASs have been shown not to function when positioned within the transcribed portion of a gene (25, 42). To distinguish the Ty2-917-positive region from a UAS, we term it an enhancer since it shares features of a metazoan enhancer, functioning downstream or inverted (41).

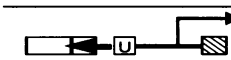
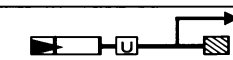
A downstream silencerlike site represses heterologous gene transcription in an orientation-specific manner. To determine whether the transcribed region negative site can function to regulate a heterologous promoter from outside the transcribed region, we constructed promoter insertion plasmids as above, including the region downstream of position 559. Insertions in either orientation upstream of the *his4Δx-52* TATA box include the enhancer region plus increasing amounts of the distal region. The region downstream of position 667 repressed transcription when inserted outside of the *his4::lacZ* gene, when inserted in one orientation but not the other (Table 3, cf. columns 2 and 3 with columns 5 and 6). In one orientation, addition of this region caused about a 30-fold decrease in expression of constructs having the enhancer and either no or one UAS. In the other orientation, addition of the region downstream of position 667 had no effect on expression. The negative site resembled a silencer, or negative enhancer (6, 29), in that it repressed transcription of a heterologous promoter from outside the transcribed region. Silencers are known to act, like enhancers, in an orientation-independent fashion. We were unable to demonstrate orientation independence for the negative site. This was because either the site is intrinsically orientation sensitive or some feature of one of the constructions interferes

TABLE 2. Evidence that differences in expression of 3′ deletions occur at the level of transcript accumulation

Ty2-917::*lacZ* fusion junction	Ratio to value for Ty2-917Δ559	
	β-Galactosidase[a]	Primer extension[b]
296	0.00	0.00
388	0.06	0.04
458	0.4	0.5
501	0.2	0.3
559	1.0	1.0
667	0.6	0.4
754	0.09	0.1
806	0.09	0.1
934	0.09	0.1

[a] Ratio of values from Table 1, column 2.
[b] Ratio of values obtained from densitometric scanning of Fig. 2, lanes 1 to 9.

TABLE 3. Stimulation of a heterologous gene by promoter insertion

Portion of the transcribed region	β-Galactosidase activity (U per plasmid copy)[a]					
						
	No UAS	1 UAS	4 UAS	No UAS	1 UAS	4 UAS
None	1	7	830	1	10	840
240–501	10	52	1,200	24	120	1,100
240–559	120	480	1,600	56	1,100	1,600
240–667	130	440	1,300	130	1,000	1,500
240–1033	100	410	1,400	2	46	1,300
240–1072	140	450	1,300	1	31	1,000

[a] Units are expressed as in Table 1, footnote *a*. Plasmids are present in 1 copy per genome. The diagram shows the orientation of the Ty2-917 insertion upstream of the *his4Δx-52* promoter. The leftmost box represents the portion of the transcribed region inserted; the triangle denotes the δ long terminal repeat region, pointing toward the transcribed region. The boxed U denotes the site at which the UAS, when present, is inserted, and the hatched box denotes the *his4::lacZ* fusion gene. The arrow shows the start site and direction of transcription.

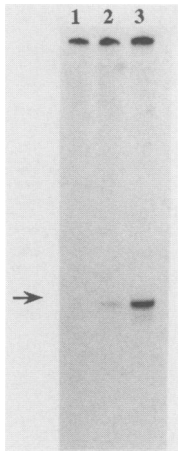


FIG. 3. Primer extension analysis of RNA expressed from pPI917-559 plasmids. Analysis was performed as in the legend to Fig. 2 on RNA from transformants of pPI917-559(NU) (lane 1), pPI917-559(1U) (lane 2), and pPI917-559(4U) (lane 3). The arrow indicates the expected position of the primer extension product. Nonspecific label bound to the well appears at the top.

with its activity. The interference could be caused by flanking sequences or by the increased distance between the negative site and the RNA initiation site in the inactive construction. That the site can repress transcription from a position outside of the transcribed region argues against its functioning during or after elongation.

The region between 559 and 667 reduced steady-state RNA accumulation approximately twofold when present within the transcribed region. This region did not have this effect when inserted in either orientation upstream of the heterologous *HIS4* promoter. These results suggest that the site functions only when present within the transcribed region; they are compatible with the region having an effect during transcriptional elongation or after transcription termination (e.g., affecting RNA stability).

Effect of duplicating the Ty2-917 UAS on level of transcription of the 3′ deletions. That plasmid YEP917-296 expresses very low steady-state levels of transcript, levels undetectable in any experiment we have performed (Fig. 2; data not shown), suggested that the region was not sufficient to promote detectable transcription. An alternative explanation might be that the transcript expressed by YEP917-296 is so unstable as to be undetectable. We have shown recently that an oligonucleotide corresponding to positions 95 to 130 of the element can substitute for the entire region upstream of the TATA region in stimulating transcription dependent on the downstream site (Liao and Farabaugh, submitted). Significantly, when tandemly reiterated, this oligonucleotide is sufficient to promote nearly normal levels of the Ty2-917::*lacZ* transcript from YEP917-296 (Fig. 4, lane 5). The transcript made by this construction initiates at the normal site. Thus, the lack of expression by YEP917-296 results from inefficient transcription initiation, which can be overcome by duplicating the UAS.

By combining the single or reiterated UAS with the 3′ deletions retaining all or part of the downstream enhancer, we investigated whether the UAS and enhancer have a synergistic effect on transcription. We have done this in constructions in which the enhancer is either in its normal context (downstream within the Ty2-917 element) or located upstream of the UAS-less *HIS4* promoter. When the sites were located in their normal context, they were highly

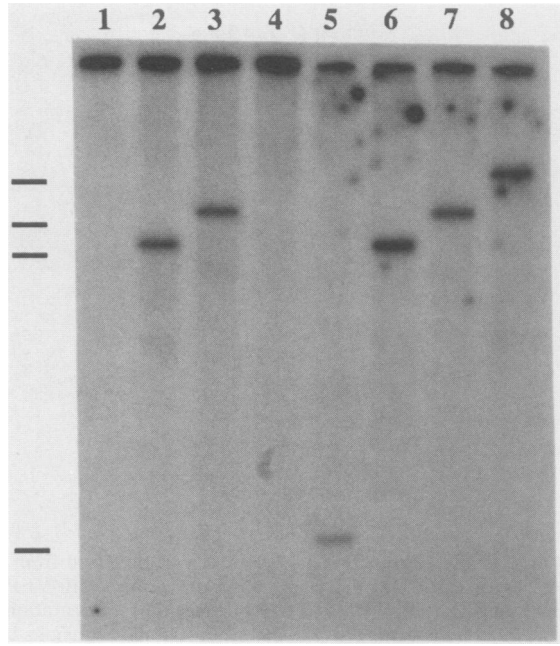


FIG. 4. Primer extension analysis of RNA expressed from YEP917(1U) and YEP917(4U) plasmids. Products derived from primer extension of 25 μg of RNA isolated from YEP917(1U) (lanes 1 to 4) and YEP917(4U) (lanes 5 to 8) transformants. The lanes correspond to fusions to *lacZ* at the following positions within the element: 296 (lanes 1 and 5), 559 (lanes 2 and 6), 667 (lanes 3 and 7), and 934 (lanes 4 and 8). The positions of expected product are indicated by bars, as in Fig. 2. Nonspecific label bound to the well appears at the top.

synergistic (Table 4). The enhancer stimulated 130 U of β-galactosidase (30), or 33 U per copy, while the UAS stimulated <1 U per copy; together they stimulated 500 U per copy, an excess of 15-fold over the predicted additive effect (34 U). Four copies of the UAS stimulated 180 U per copy in a similar YEP construct; combining the reiterated UAS with the enhancer stimulated 3,000 U per copy, an excess of more than 14-fold over an additive effect (213 U).

Combining the UAS with the enhancer present upstream of the truncated *his4* promoter resulted in less synergy

TABLE 4. Effect on Ty2-917 gene expression of reiterating the Ty2 UAS

Ty2-917::*lacZ* fusion junction	β-Galactosidase activity (U)[a]			
	YEP917(1U)		YEP917(4U)	
	Total	Per plasmid copy	Total	Per plasmid copy
296	2	<1	720	180
312	27	7	3,000	750
342	14	4	3,500	880
388	32	8	ND	ND
458	620	160	16,000	4,000
501	370	93	9,600	2,400
559	2,000	500	12,000	3,000
667	990	250	5,200	1,300
754	190	48	8,800	2,200
934	250	63	5,300	1,300
1072	170	43	ND	ND

[a] Total units and values corrected for copy number are expressed as in Table 1. ND, Not determined.

(Table 3). The enhancer combined with a single UAS has a synergistic effect of over three times the predicted additive effect. However, combining the enhancer with the reiterated UAS resulted in an only slightly greater than additive effect, about twofold. The reiterated UAS and enhancer individually stimulated more expression in these constructs (840 and 56 to 120 U, respectively) as they did in their normal context (180 and 33 U), while together they stimulated less (1,600 versus 3,000 U). It is not clear why changing the geometry of the sites should reduce the degree of synergy.

Reiterating the Ty2-917 UAS eliminated the repressive effect of the Ty2-917-negative site, whether present in its normal location or upstream of the UAS-less *HIS4* promoter. Introducing the region between positions 667 and 754 had no effect on YEP917 expression (Table 4, column 4); the effect occurred at the level of RNA accumulation [Fig. 4, cf. lanes 4, YEP917-934(1U), and 8, YEP917-934(4U)]. Likewise, adding the region distal to position 667 had no effect on pPI917 expression (Table 3, column 7). It is significant that for the promoter insertion constructions, the level of expression seen when the reiterated UAS and enhancer were combined with the negative site was identical to that obtained when the negative site was absent. The negative site did not eliminate the synergistic effect evidenced by combining the two positive elements. By contrast, the negative site completely eliminated the activation afforded by the enhancer alone (Table 3, column 5) and eliminated the synergistic effect of the enhancer acting with a single UAS. These facts taken together suggest that the negative site may interfere with the synergistic interaction between a single UAS and the enhancer.

DISCUSSION

The transcribed portion of the element Ty2-917 includes sites which modulate steady-state levels of mRNA transcribed from the element. The region between the transcription initiation site (position 240) and position 559 stimulates transcription by the upstream Ty2-917 promoter. The site has no effect on the copy number of the plasmid vector, eliminating an indirect effect on transcription by varying the amount of template. Since the site is located downstream of the transcription start site, it could exert its effect at a step after transcription initiation, for example, during elongation. A site which modulates an event after transcription initiation could only have an effect if it were located within the transcribed region. When the region is inserted upstream of the TATA box of a heterologous gene, the *HIS4* gene, it stimulates amounts of transcription per plasmid copy similar to that expressed when it is within the transcript. The site stimulated transcription when inserted in either orientation, an attribute typical of metazoan enhancers and of yeast UASs (reviewed in reference 41). This site is therefore clearly a transcriptional activator, regulating the rate of transcriptional initiation. Since for the two cases tested a yeast UAS is unable to stimulate transcription when positioned downstream of the RNA initiation site (25, 42), we have termed the positive site an enhancer.

A closer look at our data suggests that the enhancer is probably a complex site. A fusion between *TYA2* and *lacZ* at position 559 expresses the largest amount of β-galactosidase and Ty2-917::*lacZ* mRNA. This level falls to approximately 35% of this maximal value for fusions to positions between 501 and 458. The next fusions, to positions 312, 342, and 388, express 0.7 to 2% of the maximal amount. Finally, a fusion to position 296 expresses only 0.1% of the maximal level. There are at least two, and perhaps three, distinct stages to the decline in expression, with decreases in transcription occurring when the regions between positions 559 and 501, 458 and 388, and, possibly, 312 and 296 are deleted. This indicates that the enhancer may be composed of multiple distinct sites, perhaps corresponding to binding sites of multiple DNA-binding proteins. More detailed genetic analysis of the structure of the enhancer is in progress.

We previously identified two types of sites in the 5′-nontranscribed region of Ty2-917 which are required for maximal transcription initiation, TATA boxes and a UAS; this work has been described elsewhere (30). The TATA boxes are absolutely required for transcription initiating at position 240. Deleting them causes a 10,000-fold decrease in Ty2-917 expression; we detect no transcripts initiating within the first 934 bp of the element. Deletion of the UAS has a less dramatic effect on transcription, causing a 7- to 50-fold decrease in steady-state levels of RNA. The UAS does not autonomously promote transcription, since a construction lacking the region past position 296 expresses no detectable RNA. Reiterating the UAS enables it to stimulate transcription 500- to 1,000-fold (Liao and Farabaugh, submitted). This demonstrates unambiguously that the UAS is, in fact, a transcriptional activation site.

Combining the two positively acting sites, UAS and enhancer, has a synergistic effect on transcription. The reiterated UASs are highly synergistic since one UAS stimulates about 500-fold less transcription than do two tandemly repeated UASs. Combining a single UAS with the enhancer in its normal context within the transcript synergistically stimulates transcription about 15-fold above the expected additive effect. Combining the reiterated UAS with the enhancer has a further synergistic effect, >4-fold above additive. The degree of synergy obtained when the sites are combined in the promoter insertion plasmids is much less; combining multiple UASs with the enhancer shows little synergy. The synergy seen among UASs or between UAS and enhancer could result from cooperative effects on DNA binding between the proteins which recognize them. In particular, the highly synergistic effect of reiterating the UAS could be explained if the transcription activation proteins only unstably recognize a single UAS but bind cooperatively to reiterated sites. Alternatively, as proposed by Ptashne (37), the synergism might reside not in cooperative interaction with the DNA but in cooperative association with other protein factors or the transcriptional machinery.

A second site with a 3′ boundary between 667 and 754 into the element causes a decrease in the steady-state level of mRNA expressed when it is present within the transcript. The site can repress the expression of a heterologous gene from outside the transcription unit. This is similar to the effect of a silencer or negative enhancer (6, 29). The function of a silencer is orientation independent. Unlike the enhancer, we have been unable to show orientation independence of the function of the Ty2-917-negative site. The negative site also differs from the enhancer in that in constructions including the site, the steady-state level of RNA expressed is insensitive to plasmid copy number; that is, plasmids present in 1 or 4 copies express indistinguishable amounts of β-galactosidase and of the Ty2-917::*lacZ* transcript. This means that availability of the template is not the rate-limiting factor in transcription of constructions retaining the site. This is an unusual phenotype for a transcriptional control site. The effect of the negative site is eliminated in constructions carrying multiple copies of the Ty2-917 UAS. The simplest interpretation of these results is that the

negative site represses transcriptional initiation. The site is either an unusual silencer, whose function is not orientation independent, or an example of a different type of site. The simplest model for the function of the negative site is that it is recognized by a negatively acting transcription factor which interferes with synergistic transcriptional activation by the UAS and enhancer. We are pursuing experiments to distinguish between this and several other models for the mechanism of action of the negative site.

Ty1 and Ty2 are related families of elements which appear to derive from a common progenitor. The primary sequence of canonical Ty1 and Ty2 elements are nearly identical for approximately the first 700 bp and then diverge; the elements are highly divergent over the next 4.2 kb, showing as little as 50% homology (Farabaugh, unpublished observations). Because the families have diverged, results of studies of genetic control from one family may not be applicable to the other. Several groups have attempted to map the sites within Ty1 elements which are responsible for activation of Ty or adjacent gene expression (12, 14, 19, 20, 22, 38). The clear conclusion of these experiments is that multiple sites within the transcribed portion of the element modulate Ty1 gene expression. Company and Errede (12) have identified the first 211 bp of the transcribed region as an activator of adjacent gene transcription (the analogous region of Ty2-917 being from positions 266 to 475). Rathjen et al. (38) have identified an activator of a different Ty1 element corresponding to the region from 142 to 236 bp into the transcribed region (corresponding to positions 382 to 475 of Ty2-917). This activation region functions outside its normal context when inserted upstream of a heterologous promoter. Both groups have also identified a more distal site as having an activation function. Errede's laboratory (12, 20) identified an activator region of 112 bp (corresponding to positions 804 to 915 of Ty2-917) containing a 28-bp block of similarity to the simian virus 40 enhancer and the *MATa1*/*MATα2* control site of *MATα*. The region, or just the 28-bp homologous region, acts as a cell-type-specific activator when present in combination with the upstream activator (20) or as tandemly reiterated copies (11). Rathjen et al. (38) identified a nearly identical fragment as an activator, though they ascribe cell-type-dependent regulation to the upstream activator. Rathjen et al. (38) found that each of the two activators is active only in one orientation relative to the site of transcription initiation, whereas Errede's laboratory found that both are able to function in either orientation (11–13, 20). Company et al. (11) have demonstrated that the upstream activator is specifically recognized by a protein factor dependent for its activity on an active *STE12* gene, a gene known to be required for expression of haploid-specific genes including Ty1 and Ty2 elements; they term the region bound by the protein an SRE. Company and Errede (13) have identified a constitutively expressed protein factor which recognizes the downstream activation site.

Data presented here for the Ty2-917 element demonstrate the importance of the region immediately downstream of the transcription initiation site as the major activator of Ty2 transcription. Adding the region immediately upstream of position 435 causes the greatest increment in Ty2-917 expression, about 12-fold in YEP917-435. This region includes the region homologous to the SRE (which corresponds to nucleotides 388 to 425 of Ty2-917). Ty2-917 transcription is also stimulated by the sequences downstream of position 425, including sequences to position 559, as determined by our deletion analysis. It is interesting that when the region encompassing the SRE was inserted into our promoter insertion tester plasmid (pPI917-501), it stimulated little transcription (Table 3, line 2), but adding the region to position 559 allowed maximum transcription. In constructing the pPI917 class of plasmids, we were careful to use as our "activator trap" a promoter which had been well characterized as lacking binding sites for any activator proteins (2, 36). Expression of this plasmid is undetectable (36), so any expression of a pPI917 plasmid is solely dependent on factors bound to the inserted DNA acting autonomously. These results suggest that the activity of the SRE may require the region between positions 501 and 559 for maximum autonomous activity but that it can stimulate when inserted near other proteins in another context, e.g., the partially deleted *CYC7* (12, 20) or *PGK* (38) promoter.

Coney and Roeder (14) suggest that two distinct regions of Ty2-917 are involved in adjacent gene activation. A mutation at position 612 of the element increases adjacent gene expression 8- to 19-fold but has a modest effect on Ty2-917 transcription. Adjacent gene expression is increased an additional threefold when the mutation at position 612 is combined with a mutation inactivating the Ty2-917 promoter. These results support the existence of an adjacent gene activator whose effect on an adjacent gene is attenuated by an intervening active Ty2-917 promoter. Goel and Pearlman (24) have identified a protein which binds to the region surrounding position 612 of Ty2-917. The protein binds to the mutant but not the wild-type form of the site. Binding of this *trans*-acting factor may be required for activation of the adjacent gene. It is not clear what role this site, in its wild-type form, plays in transcription of Ty2-917. The data presented here suggest that the region does not have a major role in activating transcription of wild-type Ty2-917 since it can be deleted without effect. This result is consistent with the results of Coney and Roeder (14) showing that the mutation at position 612 has little effect on Ty2-917 transcription. The data of Coney and Roeder (14) do not rule out the possibility that the site at position 612 acts to modulate the effect of the upstream SRE-containing activator.

Company and Errede (12) have reported evidence of negatively acting transcriptional regulators within the transcribed portion of a Ty1 element. Two fragments of this element, corresponding to nucleotides 475 to 1204 and 1205 to 1689 of Ty2-917, reduce transcription of a *CYC7* promoter lacking its UAS, indicating that they had a negative effect on transcription. They had this effect regardless of their orientation. Interestingly, individually the fragments in their normal context had a positive effect on transcription, though deletion of both fragments at the same time had no effect on expression. Company and Errede (12) proposed that the two regions might include both positive and negative transcriptional control sites, the negative site operating in either orientation. It is useful to note that the sequence elements responsible for the low level of expression directed by the UAS-less *CYC7* promoter are not known. A promoter lacking any activator proteins should show no detectable expression (2, 3), suggesting that expression of the deleted *CYC7* gene might depend on one or more weakly acting positive elements. The effect of inserting large segments of DNA into such a promoter would be unpredictable, as noted by Company and Errede (12). In performing similar experiments, we have used a UAS-less promoter which has been rigorously demonstrated to lack any positive-acting transcription sites and to promote little or no transcription (2, 3). We find that the region immediately upstream of position 754 acts in an orientation-specific fashion to repress enhancer-dependent transcription of this promoter. The differences between

these results and those of Company and Errede (12) make it unlikely that the same element is responsible for both effects. The region immediately downstream of position 667 shows no more than 75% identity with the canonical Ty1 sequence and includes a 12-bp deletion (Farabaugh, unpublished data), so it is not necessary that the site found in Ty2-917 be present in a Ty1 element.

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