MOLECULAR AND CELLULAR BIOLOGY, Apr. 1984, p. 657–665
0270-7306/84/040657-09$02.00/0

Vol. 4, No. 4

Effects of Altered 5′-Flanking Sequences on the In Vivo Expression of a *Saccharomyces cerevisiae* tRNA^{Tyr} Gene

KAREN J. SHAW† AND MAYNARD V. OLSON*

Department of Genetics, Washington University School of Medicine, St. Louis, Missouri 63110

Received 3 October 1983/Accepted 22 December 1983

Deletion mutations ending in the 5′-flanking sequences of the *Saccharomyces cerevisiae SUP4-o* gene have been analyzed for their effects on gene expression. This *ochre*-suppressing tRNA^{Tyr} gene was cloned into a *S. cerevisiae* centromeric plasmid, and its level of in vivo expression was monitored by observing the suppressor phenotype of the gene after transformation into *S. cerevisiae*. A deletion mutant that retains only four base pairs of the 5′-flanking sequence is profoundly deficient in expression; deletion mutants extending to positions −18, −17, −16, or −15 are moderately deficient; deletion mutants extending to positions −36 or −27 are slightly defective; and mutants retaining more than 60 base pairs of the original 5′-flanking DNA are expressed normally. In some cases, the cloning procedure led to the introduction of multiple *Bam*HI linkers at the *SUP4-o*-vector fusion site, and in one instance, the resulting structure dramatically affects gene function: the presence of three linkers abutting a −18 deletion completely inhibits the in vivo expression of *SUP4-o*. In contrast, three linkers that abut a −77 deletion have no effect on in vivo expression. The template properties of these plasmids in a homologous in vitro transcription system parallel the levels of in vivo expression, suggesting that the mutations predominantly affect transcription. The data demonstrate that there are significant functional constraints on the 5′-flanking sequences of this RNA polymerase III-transcribed gene. The dramatic effects of the multiple linker insertion at position −18 suggest that there may be extensive melting of the DNA in this region during normal transcription initiation.

It is now well established that sequences essential for transcription initiation are located within the coding regions of eucaryotic genes transcribed by RNA polymerase III. It has been demonstrated by deletion analysis that the essential internal sequences map between residues +50 and +83 for *Xenopus* 5S genes, and this same region has also been shown to mediate the binding of TFIIIA, a transcription factor required for the in vitro transcription of these genes (1, 8, 28, 29). Similarly, eucaryotic tRNA genes have two highly conserved sequence blocks within their coding regions that appear to provide the primary signals for transcription initiation (3, 11, 14).

Although less is known about the role of 5′-noncoding sequences in the expression of genes transcribed by RNA polymerase III, there is a variety of evidence from in vitro transcription studies that these sequences can modulate the level of RNA polymerase III transcription and may also influence the exact site of initiation. Two tRNA^{Lys} genes of *Drosophila melanogaster* are transcribed to different extents in nuclear extracts from *Xenopus* oocytes. Exchange of the 5′-flanking sequences results in higher-level expression of the poorly transcribed gene and lower-level expression of the more efficiently transcribed gene. Deletion analysis indicates that these results are due to the inhibitory effect of sequences present in the vicinity of positions −23 to −13 of the poorly transcribed gene (5, 6). Deletion of all but 14 residues flanking the mature *Bombyx mori* tRNA^{Ala} gene results in the loss of transcription, using homologous *Bombyx* extracts; however, transcription occurs with or without the normal 5′-flanking sequences, using heterologous *Xenopus* germinal vesicle extracts (31).

Several in vivo observations also imply a role for noncoding sequences in the expression of genes transcribed by RNA polymerase III. For example, although the two *Bombyx* alanine tRNAs differ only at the site of a single unmodified nucleotide at position 40, expression of the tRNA$_1^{Ala}$ species is silk gland specific, whereas tRNA$_2^{Ala}$ is constitutively expressed in several silkworm cell types (30). In the case of the adenovirus *VAI* gene, which shares a high degree of structural and functional homology with eucaryotic tRNA genes (10, 20), a viable mutant with a 2-base-pair (bp) deletion at position −22 fails to utilize one of two normal transcription initiation sites (35). In yeasts, RNA and DNA sequence data indicate that the coding regions of the eight tRNA^{Tyr} genes are identical (13, 24). However, *ochre*-suppressing alleles have been obtained at all eight loci, and some of these suppressors display distinctive phenotypes; for instance, *SUP11-o* is a weak suppressor and *SUP3-o* homozygotes show a locus-specific sporulation defect that can be eliminated by second-site, loss-of-suppression mutations (15, 27). In light of these observations, it is clear that sequences outside the coding regions can modulate the expression of genes transcribed by RNA polymerase III in vitro and in vivo.

In the present study, we explored the functional constraints on the 5′-flanking sequences of the *S. cerevisiae SUP4-o* gene by observing the suppressor phenotype of cells carrying alleles of this tyrosine-inserting *ochre* suppressor that contain deletion-fusions in the 5′-noncoding sequences. We found that the phenotypic expression of these mutant *SUP4* genes, as assayed by the extent to which they suppress seven *ochre* mutations, is impaired in all mutants that have deletion endpoints within 36 bp of the tRNA-coding region; furthermore, we found that the severity of the effect is directly correlated with the proximity of the deletion endpoints to the coding region. One exception to this rule was found for a mutant with a triple *Bam*HI linker insertion at its −18 deletion endpoint; this mutation causes a complete loss of expression. Results from a preliminary study of the

* Corresponding author.
† Present address: Unigene Laboratories, Inc., 110 Little Falls Rd., Fairfield, NJ 07006.

ability of these mutants to function as in vitro transcription templates correlate well with the in vivo phenotypes.

MATERIALS AND METHODS

Plasmid constructions. The *SUP4-o* fragment was derived from an M13mp5 clone constructed and provided by S. B. Sandmeyer. This clone contains a 262-bp *Alu*I fragment subcloned from pSU4-A (13) by blunt-end ligation into M13mp5 after the unique *Eco*RI site of the vector had been cleaved and the ends had been filled in with the Klenow fragment of DNA polymerase. Since this procedure regenerated *Eco*RI sites at both ends of the *SUP4-o* fragment, the M13mp5 clone provides a source of the *SUP4-o* gene on an artificial 266-bp *Eco*RI fragment with endpoints at positions −110 and +152 relative to the first nucleotide of the tRNA-coding region. All of the *SUP4 CEN3* plasmids used in this work were constructed by cloning derivatives of this 266-bp *Eco*RI fragment into the *Bam*HI site of the *S. cerevisiae-Escherichia coli* shuttle vector pTC3. The structure of plasmid pTC3, which was constructed and provided by K. Nasmyth, can largely be deduced from Fig. 1; pTC3 was derived from YRP7 (33) by the insertion (via blunt-end ligation) of the 2.0-kb *Bam*HI-*Hin*dIII fragment of pYE-(*CEN3*)11 (4) into the pBR322-derived *Pvu*II site of YRP7.

Two of the *SUP4 CEN3* plasmids were constructed by direct subcloning of the 266-bp *SUP4-o Eco*RI fragment. The larger of these plasmids pC66 was simply derived by filling in the *Eco*RI ends with the Klenow fragment of DNA polymerase, adding *Bam*HI linkers (see below), and cloning the resulting fragment into the *Bam*HI site of pTC3. The smaller plasmid pC689 was derived by a similar protocol, but in this case the end of the fragment upstream from the *SUP4-o* coding region was created by cleavage at the unique *Ava*II site of the fragment at position −77.

The remaining *SUP4 CEN3* plasmids were derived by a procedure that included *BAL*31 digestion of variable tracts of DNA adjacent to the −77 *Ava*II site. Starting with replicative form DNA from the original M13mp5 clone, the following series of steps was carried out: (i) *Ava*II cleavage, (ii) brief *BAL*31 digestion, (iii) *Eco*RI digestion, (iv) filling in of the *Eco*RI ends with the Klenow fragment of DNA polymerase, (v) addition of *Bam*HI linkers (CCGGATCCGG) with T4 DNA ligase, (vi) cleavage with *Bam*HI, (vii) purification of fragments in the size range of 150 to 240 bp on a preparative polyacrylamide gel, (viii) ligation of the *SUP4-o* fragments into *Bam*HI-cleaved pTC3, and (ix) transformation of *E. coli* RRI (2) to ampicillin resistance. This procedure normally preserved an *Eco*RI site immediately adjacent to the *Bam*HI site that is proximal to the 3′ end of the tRNA-coding region (Fig. 1). In the pC68 series, however, this *Eco*RI site was lost by an uncharacterized alteration that had no other detectable effect on the restriction map.

The set of *SUP4 CEN3* plasmids that was chosen for transformation into *S. cerevisiae* was enriched for cases with fusion points near the 5′ end of the *SUP4* coding-region by prescreening the original collection of *E. coli* transformants with two hybridization probes. These probes were prepared by cleaving the 266-bp *SUP4-o Eco*RI fragment (see above) at position −24 (relative to the 5′ end of the tRNA-coding region) with *Mbo*II. Colony hybridization was carried out by the method of Gergen et al. (12); hybridization to the probe containing sequences from −24 to +152, but not to the probe containing sequences from −110 to −25, was diagnostic of plasmids with fusion points near, or in, the tRNA-coding region.

***S. cerevisiae* manipulations.** Media recipes were minor modifications of those described by Rothstein et al. (27). AHA75 (**a** *ade2-o arg8-o his4-o leu2-o lys2-o trp1 tyr1-o*) was used as the host for all plasmid transformations. Transformation was carried out by the lithium acetate procedure of Ito et al. (18). Transformants were selected on plates without tryptophan after 48 to 72 h of incubation at 30°C.

To serve as controls, spontaneous *SUP4-o* and *SUP11-o* mutations were selected in the AHA75 background. Selections and allelism testing to identify *SUP4* and *SUP11* were carried out as described by Hawthorne and Mortimer (16).

Tests of the level of suppression (see Fig. 4) were carried out as follows: (i) plasmid-containing strains were grown

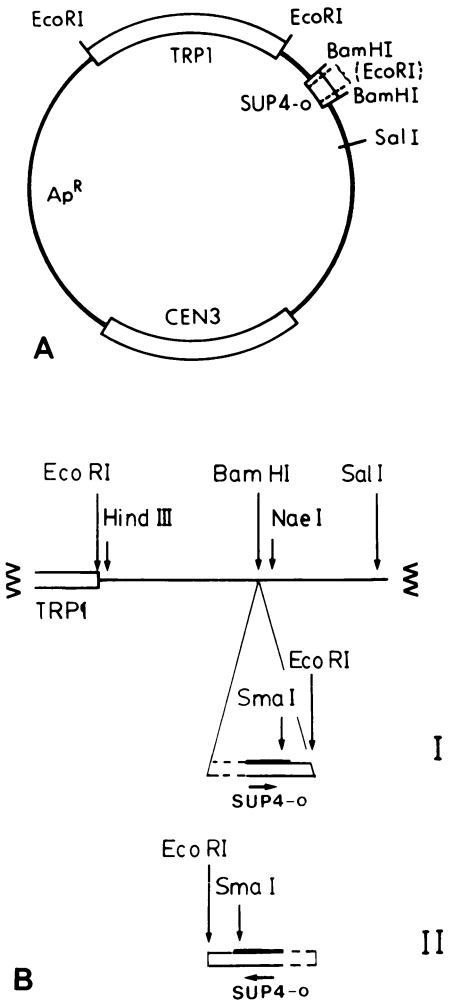

FIG. 1. Structure of *SUP4-o CEN3* plasmids. (A) Overview of the plasmid restriction maps; yeast sequences are designated by open boxes, whereas pBR322-derived sequences are designated by solid lines. *SUP4* was introduced into the plasmids on a short *Bam*HI fragment, which was cloned into the unique pBR322-derived *Bam*HI site of the *TRP1-CEN3* vector pTC3. Most of the plasmids contain an *Eco*RI site beyond the 3′ end of the tRNA-coding region; the position of this site depends on the orientation of the *SUP4* *Bam*HI fragment. For details of the construction of pTC3 and the *SUP4* inserts see the text. (B) Expanded view of the *SUP4* region. Dotted lines represent the position of sequences in the 5′-noncoding region of *SUP4* that are deleted to various extents in the different plasmids. The tRNA-coding region is marked with a heavy line, and the direction of transcription is indicated by an arrow.

TABLE 1. Suppressor phenotypes of strains containing chromosomal and plasmid-borne *ochre* suppressors

S. cerevisiae strain[a]	Deletion endpoint[b]	Action spectra of *SUP4-o* mutations on suppressible alle[c]							Orientation[b]	No. of linkers[b]
		tyr1	ura4	arg8	ade2	lys2	leu2	his4		
AHA75-4-*o*	NA	2	3	4	5	4	5	5	NA	NA
AHA75-11-*o*	NA	2	3	3	4	4	5	5	NA	NA
YC66	−100	1	3	2	5	3	5	5	II	ND
YC689	−77	2	3	4	5	4	5	5	II	3
YC44	−62	2	2	3	4	4	5	5	I	ND
YC207	−61	2	2	3	4	4	4	5	II	ND
YC225	−36	1	2	3	3	4	5	5	I	ND
YC226	−27	2	2	3	4	4	4	5	I	1
YC68	−18	0	0	0	0	0	0	0	II	3
YC68-1	−18	1	2	2	4	3	4	5	I	1
YC723	−17	1	2	2	2	3	4	5	II	1
YC235	−16	1	2	2	1	3	4	5	II	ND
YC865	−15	1	2	2	2	3	4	5	I	2
YC888-1	−4	1	2	2	1	3	3	4	I	1
YC888-2	−4	0	1	1	1	2	2	2	II	1
YC675	+9	0	0	0	0	0	0	0	II	ND

[a] Strains AHA75-4-*o* and AHA75-11-*o* contain chromosomal alleles of *SUP4-o* and *SUP11-o* selected in the AHA75 background. YC strains were derived by transforming AHA75 with the pC plasmid that has the corresponding number.

[b] NA, Not applicable; ND, not determined.

[c] Numbers (1 to 5) were derived by scoring plates such as those shown in Fig. 4. A high number reflects strong suppression of the indicated marker, and a low number reflects poor suppression. Specifically, the values indicate the number of cells that must be spotted to give significant growth on selective plates in 2 days: 5, 10^3 cells; 4, 10^4 cells; 3, 10^5 cells; 2, 10^6 cells; 1, 10^7 cells. An entry of zero implies that no significant growth was observed even when 10^7 cells were spotted.

overnight at 30°C in liquid medium without tryptophan, (ii) cells were harvested by centrifugation and resuspended in water to a concentration of 10^9 cells per ml, (iii) serial dilutions were made in water, (iv) samples containing 10 μl of the various dilutions were spotted onto appropriate single drop-out plates. Plates were scored after 48 h of incubation at 30°C; a spot was scored as positive if it contained confluent growth. Scorings for a particular strain were normally completely reproducible, although occasional variations of ±1 in the values seen in Table 1 were encountered. For strains containing stable chromosomal suppressors, the same procedure was employed except that the original overnight growth was in complete medium since these strains are Trp^-.

In vivo transcription reactions. The cell transcription extracts were generously provided by P. A. Weil. The templates were supercoiled plasmid DNA samples purified by equilibrium banding in cesium chloride-ethidium bromide gradients. The reactions were carried out by a modification (P. A. Weil, personal communication) of the method of Klekamp and Weil (19). Reaction volumes were 50 μl, and 2 μg of template DNA was employed. The ribonucleotide triphosphates ATP, CTP, and UTP were present at concentrations of 600 μM, whereas GTP was present at 25 μM. Labeling of the in vitro transcripts was achieved by including 15 μCi of [α-^{32}P]GTP in the reactions. In the competition experiments, 2 μg each of the *SUP4* and 5S templates was present. Samples were analyzed on 8% (20:1) polyacrylamide gels prepared as described by Maxam and Gilbert (25).

Characterization of plasmid structures. DNA sequencing was carried out by the method of Maxam and Gilbert (25). In most cases, the *Sma*I site at position +83 in the tRNA-coding region was 5'-end labeled and used as a sequencing origin. For sequencing the 5'-flanking region of clones in orientation I, secondary cleavage was with *Hin*dIII; for clones in orientation II, the *Sal*I site was used for secondary cleavages (Fig. 1). Because of the severe collapse of the sequencing ladder in the vicinity of the triple *Bam*HI linker insertion (see below), unequivocal determination of the structure of pC68 required sequencing of both strands. The − strand was sequenced as described above, whereas the + strand was sequenced by using the *Nae*I site as a 5'-end-labeled origin; secondary cleavage was with *Eco*RI.

Corroborative evidence on the number of linkers present in pC68 and several other plasmids (Table 1, last column) was obtained by sizing restriction fragments that span the *SUP4* vector fusion site proximal to the 5' end of the tRNA-coding region. For clones in orientation I, *Taq*I cleavage produces a convenient fragment with one end 36 bp into the pBR322 fusion sequence and the other end at position +69 in the tRNA-coding region; in the case of orientation II, a *Nae*I-*Sma*I digest was used, giving a fragment with one end (*Nae*I) 25 bp into the pBR322 fusion sequence and the other end (*Sma*I) at position +83 of the tRNA-coding region. The diagnostic fragments, which are in the 100- to 200-bp range, were sized on 6% (30:1) native polyacrylamide gels.

RESULTS

Structures of the *SUP4-o* plasmids. The method of construction placed the *SUP4-o* coding region and variable amounts of noncoding DNA on a short restriction fragment with synthetic *Bam*HI linkers on both ends. This fragment was cloned into the *Bam*HI site of the *S. cerevisiae-E. coli* shuttle vector pTC3 (Fig. 1A; K. Nasmyth, private communication). This shuttle vector contains the yeast selectable marker *TRP1* and the *TRP1*-linked replication origin *ARS1*, as well as the functional centromeric sequence *CEN3* (4, 32). The latter sequence leads to the maintenance of the plasmids in *S. cerevisiae* at a copy number of ~1 per cell (9).

All of the plasmids contain 63 bp of wild-type-*SUP4* 3'-noncoding sequences together with up to 110 bp of 5'-noncoding DNA. Depending on the orientation in which the *SUP4-o* fragment was cloned into pTC3, wild-type-*SUP4* 5'-noncoding DNA was fused to sequences that normally lie to

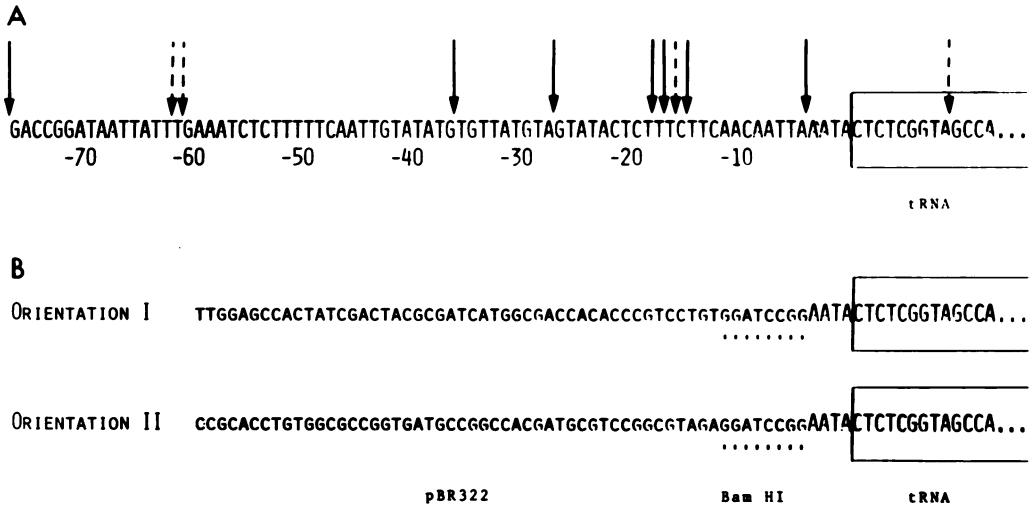

FIG. 2. Deletion endpoints and fused sequences. (A) The vector-*SUP4-o* fusion points were either determined exactly by DNA sequencing (solid arrows) or to ±1 bp by sizing an appropriate end-labeled restriction fragment on a sequencing gel (broken arrows). The 5′-noncoding sequences of *SUP4* are in agreement with but are more extensive than those reported by Goodman et al. (13). (B) The precise structures of typical deletion-fusions are illustrated by using the two −4 deletions, pC888-1 (orientation I) and pC888-2 (orientation II), as examples. *SUP4*-derived sequences are in large type. The *Bam*HI region is underlined with a dotted line; because of the particular *Bam*HI linker employed (decamer CCGGATCCGG), sequence GG was always introduced between the actual *Bam*HI site and the *SUP4*-derived sequences. The sequences of the pBR322-derived regions have been previously reported by Sutcliffe (34).

one or the other side of the unique, pBR322-derived *Bam*HI site of the vector. A detailed view of the *SUP4-o* region of the plasmids is shown in Fig. 1B. With the exception of the plasmids with deletion endponts at positions −110 and −77, which were constructed by using naturally occurring restriction sites, the variable lengths of 5′-noncoding DNA were generated by *BAL*31 digestion starting at the *Ava*II site at position −77. From an initial collection of 750 *E. coli* clones, each of which contained an independent, *BAL*31-derived deletion, 10 plasmids with deletion endpoints between positions −62 and +9 were chosen for detailed characterization. These mutants were selected on the basis of colony hybridization and restriction analysis to give the best possible coverage of the 5′-noncoding region. Figure 2 shows the precise endpoints of the deletions as well as the vector sequences to which the *SUP4* 5′-noncoding regions were fused.

Phenotypes of *S. cerevisiae* strains transformed with *SUP4-o CEN3* plasmids. Our primary assay for gene function involved transforming *SUP4-o* plasmids into *S. cerevisiae* and observing the level of in vivo expression of the *ochre* suppressor. For a host yeast strain, we constructed AHA75, which contains seven *ochre* mutations (*ade2-o*, *arg8-o*, *his4-o*, *leu2-o*, *lys2-o*, *tyr1-o*, and *ura4-o*), all of which lead to nutritional requirements when not suppressed. AHA75 also contains a nonsuppressible *trp1* mutation, which allows the presence (*trp*$^+$) or absence (*trp*$^-$) of the plasmid to be monitored independently of the expression of the suppressor.

Particularly useful in this genetic system is the *ade2-o* mutation. Unless suppressed, this mutation leads to a red colony color as well as to adenine auxotrophy (26). Suppression of *ade2-o* leads to a white, *ade*$^+$ phenotype. Because *CEN3* plasmids are not absolutely stable mitotically, *ade2-o* strains transformed with *SUP4-o CEN3* plasmids give rise to visibly sectored colonies. We find that typically 50 to 80% of the cells in a colony grown on a rich, nonselective medium contain the plasmid, a result that is consistent with the estimate by Fitzgerald-Hayes et al. (9) that *CEN3* plasmids are lost at a rate of 1 to 3% per generation.

The usefulness of this combination of phenotypes is illustrated in Fig. 3. The two lower strains contain *SUP4-o CEN3* plasmids, whereas the upper strain contains a chromosomal *SUP4-o* mutation, selected in the AHA75 genetic background. Not only does Fig. 3 illustrate the mitotic instability of the plasmids, but it also shows that the extent of suppression of the red colony color phenotype can be used as one criterion for the level of suppressor function. The lower left strain contains a *SUP4-o* gene with 5′-flanking sequences intact to position −77, whereas the lower right strain contains a gene whose deletion endpoint is at −4. In the case of the −77 deletion, the red *ade2-o* color is completely suppressed in sectors bearing the plasmid, whereas only a low level of suppression is observed in the strain containing the −4 deletion.

A more comprehensive test of the level of expression of *SUP4-o* in the different plasmids involved observing the extent to which the plasmids could mediate the suppression of all seven *ochre* mutations present in AHA75. The extent of suppression was assessed by spotting five log dilutions of cultures grown overnight in medium without tryptophan onto appropriate single drop-out plates. Growth of the spots was scored after 2 days (Fig. 4A); a wide range of phenotypes were observed when three different deletion mutants (−77, −17, −4), as well as a chromosomal *SUP4-o* mutant in the identical genetic background, were tested for *lys2-o* suppression on a plate without lysine. The control plate (Fig. 4B), in which the same dilution series was spotted onto complete medium, shows identical patterns of growth for all four strains. By using this technique, it was also possible to order the seven nonsense alleles in terms of their ease of suppression: *his4-o*, *leu2-o* (most easily suppressed) > *lys2-o*, *ade2-o*, *arg8-o* > *ura4-o* > *tyr1-o*.

Table 1 summarizes the results obtained from a similar

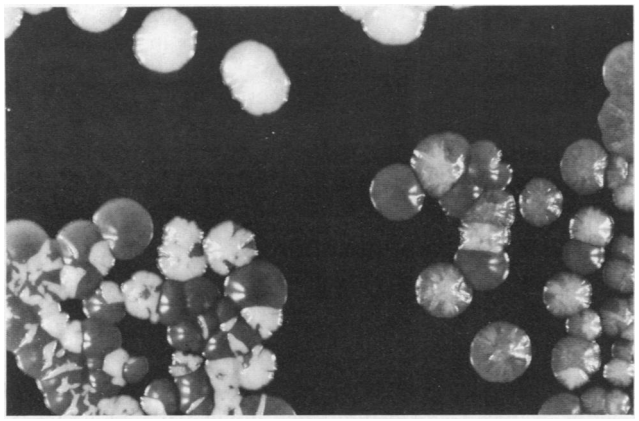

FIG. 3. Colony phenotypes of a chromosomal and two plasmid-borne *SUP4-o* alleles. The all-white colonies at the top are from a strain containing a chromosomal *SUP4-o* allele selected in the same genetic background (strain AHA75) as that employed in all of the plasmid experiments; this stable suppressor completely suppresses the red colony color phenotype of the *ade2-o* mutation. In the lower left are colonies from strain YC689 (AHA75 transformed with pC689). The *SUP4-o* allele on this −77 deletion-fusion plasmid is fully functional but mitotically unstable. All-red colonies are derived from cells that had lost the plasmid before plating. In the lower right are colonies from YC888-2 (AHA75 transformed with pC888-2); the *SUP4-o* allele on this −4 deletion-fusion plasmid is poorly expressed. In YC888-2, in addition to red sectors, occasional enhanced-white sectors are observed (e.g., at the bottom of the colony near the extreme lower-right-hand corner of the photograph); these sectors probably arise from nondisjunction events that increase the plasmid copy number.

analysis of a larger set of deletion mutants. Deletion of bases −110 through −61 has no substantial effect on the ability of the *SUP4-o* gene to suppress the seven nonsense alleles, although slight variations were observed; for example YC66 (−110) is reproducibly weaker than YC689 (−77). As has been previously observed (15, 27), the chromosomal *SUP11-o* mutant (AHA75-11-*o*) is a slightly weaker suppressor than is the *SUP4-o* mutant (AHA75-4-*o*), but this difference is small compared with the phenotypic range observed for the deletion mutants. It is notable that in this assay a fully expressed *SUP4-o* gene on the *CEN3* plasmid has a phenotype that is indistinguishable from that of a chromosomal *SUP4-o* gene in the same genetic background.

Mutants which retain only 27 or 36 bp of 5'-flanking sequences (YC226 and YC225, respectively) show a small decrease in the strength of the suppressor, as demonstrated with the more difficultly suppressible alleles *tyr1-o*, *ura4-o*, *arg8-o*, and *ade2-o*. Deletions extending to positions −18, −17, −16, and −15 (YC68-1, YC723, YC235, and YC865, respectively) show a significant loss in the ability to suppress these alleles. A deletion mutant which retains only 4 bp of the original 5'-flanking sequence (YC888-1) is severely deficient in suppressor activity, as shown by decreased suppression of all seven nonsense alleles. A mutation which deletes the first 9 bp of the coding region (YC675) is, as expected, nonfunctional. In general, these results indicate that there are sequences present in the 5'-flanking region of *SUP4-o* that play a direct role in the normal in vivo expression of the gene and that as sequences closer to the gene are deleted, more activity is lost.

Linker effects. The one exception to the good correlation between phenotype and deletion endpoint is the mutant YC68, which retains 18 bp of the normal 5'-flanking sequence but is unable to suppress any of the nonsense alleles tested (Table 1). Sequence data indicate that the coding and 5'-flanking regions are identical to the known sequence of *SUP4-o*; however, three *Bam*HI linkers are present at the vector-*SUP4-o* junction adjacent to the 5' end of the tRNA-coding region.

To determine whether the anomalous lack of *SUP4-o*

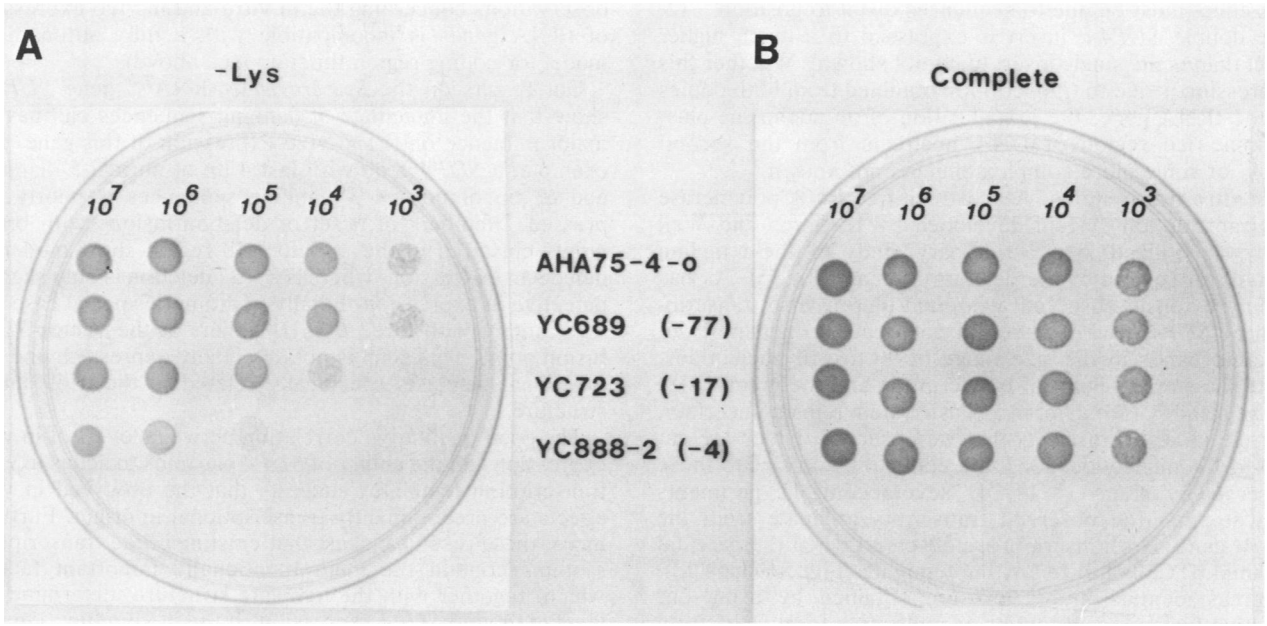

FIG. 4. Typical plate assays for the level of suppression in strains containing different *SUP4-o* alleles. The specified numbers of cells were spotted onto plates on which growth was either dependent (A) or independent (B) of *ochre* suppression. In this particular experiment, growth on plate A depended on the suppression of *lys2-o*, which is suppressed with intermediate difficulty in comparison with the full set of seven *ochre* alleles that we employed.

expression in YC68 was specifically due to the triple-linker insertion, two of the linkers were eliminated by *Bam*HI cleavage and religation. The starting point for this reconstruction was plasmid DNA from the nonexpressing *S. cerevisiae* YC68. This DNA was recloned by using it to transform *E. coli* to ampicillin resistance. DNA from this *E. coli* transformant was cleaved with *Bam*HI, religated, and again cloned in *E. coli*, producing a plasmid that contained a single linker at the *SUP4-o*-vector fusion site. Retransformation of AHA75 with this plasmid led to *S. cerevisiae* YC68-1, which showed a level of expression similar to other deletion-fusions with endpoints in the −15 to −18 region. It is apparent, therefore, that the complete lack of suppression in YC68 is directly associated with the triple-linker structure.

DNA sequence analysis revealed two other cases of multiple linkers present at the fusion point: pC689 (−77) has three linkers, and pC865 (−15) has two linkers (Table 1). When these plasmids were introduced into *S. cerevisiae* AHA75, suppressor activity was observed, and in each case the level of activity was comparable to that observed with other deletion mutants with similar endpoints.

Orientation effects. The data in Table 1 suggest that the orientation of the *SUP4-o Bam*HI fragment within pTC3 has relatively little effect on expression. Because an orientation effect seemed most likely for deletion-fusions with endpoints close to the coding region, the −4 deletion was specifically examined for this effect. The *Bam*HI insert in pC888-1 (−4) was inverted by a cleavage-religation protocol similar to that described above for pC68. *Eco*RI digests indicated that, of the transformants tested, six had *SUP4-o* in the original orientation I, three were in orientation II, and one transformant contained two tandem inserts, both in orientation I. DNA from a representative of each class was transformed into AHA75.

The phenotypes of these transformants indicate that the expression of the suppressor in orientation II (YC888-2) is somewhat lower than that in orientation I (YC888-1). Since both inserts are flanked by 8 bp of *Bam*HI linker (Fig. 2B), this effect must be due to sequences distal to position −12. The double *SUP4-o* insert is expressed to a much higher level than is the single insert (data not shown). Whether this expression is due to transcription obtained from both copies of the tRNA gene, the juxtaposition of an adenosine-plus-thymine-rich region of DNA upstream from the second copy, or some more complex effect is not known.

In vitro transcription. A yeast cell-free RNA polymerase III transcription system developed by Klekamp and Weil (19) was utilized in a preliminary study of the template activity of several of the deletion mutants (Fig. 5). As has been previously observed, we found that in vitro transcription of *SUP4-o* with crude extracts leads to a number of discrete bands in the size range of 90 to 110 nucleotides. Both the starting point of transcription and the precise size of the primary transcript in a yeast system remain uncertain, but the largest transcripts that we observed, which are ca. 104 to 106 nucleotides long, are comparable in size to those observed by others (17, 19, 21). Several control experiments indicate that the observed transcripts originate from the *SUP4* gene. No transcripts are observed when the parental plasmid pTC3 is utilized as the template (Fig. 5A, lane 12), whereas identical transcripts are obtained by using our deletion-fusion constructions or replicative form DNA from an M13mp5 clone of *SUP4-o* on its natural 262-bp *Alu*I fragment (data not shown). Finally, cleaving the *SUP4-o* centromeric plasmids with *Sal*I (which cuts 278 nucleotides away from the insert) has no effect on the size of the transcripts obtained, whereas cleavage within the *SUP4-o* gene at the unique *Sma*I site (+83) results in the complete loss of transcription (data not shown).

Plasmids are transcribed with decreasing efficiency as the deletion-fusion point approaches the beginning of the coding region (Fig. 5A, lanes 6 through 11). Furthermore, pC68 (−18), which has no suppressor activity in vivo due to the presence of three linkers at the fusion site, is also very poorly transcribed in vitro (Fig. 5A, lane 8). These data indicate a strong qualitative correlation between the amount of transcription observed in vitro and the level of expression of the suppressor in vivo.

In an effort to control for nonspecific factors that might have inhibited the in vitro transcription system when particular template preparations were employed, we cotranscribed several of the *SUP4-o* templates along with an intact *S. cerevisiae* 5S template. The results show a clear inverse correlation between the extent of transcription from *SUP4-o* and the 5S gene (Fig. 5B). Cotranscription of the weakly expressing pC888-1 (−4) DNA results in a slight inhibition of 5S transcription, whereas cotranscription of the increasingly stronger expressors pC723 (−17) and pC689 (−77) results in increasingly stronger inhibition of 5S transcription; these results suggest that in this transcription system there is competition between the *SUP4-o* and 5S genes for transcription factors that are present in limiting amounts and that the more severely impaired mutants are poorer competitors.

DISCUSSION

The demonstration of an internal promoter for RNA polymerase III has raised a question with intriguing functional and evolutionary implications: to what extent are the coding regions of eucaryotic tRNA genes self-contained functional units? On the one hand, the lack of convincing consensus sequences in the 5′-flanking regions of tRNA genes argues that the functional constraints on this domain are quite weak, whereas on the other hand, a diverse set of observations concerning the in vitro and in vivo expression of tRNA genes is incompatible with a fully autonomous model for coding region function (see above).

Our results on the *S. cerevisiae* $tRNA^{Tyr}$ gene *SUP4-o* show that the immediate 5′-flanking sequences can have a major influence on the in vivo expression of this gene. For example, a *SUP4* gene with just 4 bp of normal 5′-flanking and 63 bp of normal 3′-flanking sequences is poorly expressed. Members of a set of deletion-fusions with break points clustered in the −15 to −18 region show moderate defects in expression, whereas a −27 deletion is only slightly defective in expression. Finally, a dramatic special case is a −18 mutant with three *Bam*HI linkers at the vector-*SUP4* fusion point. This gene is not detectably expressed, and the functional defect is specifically related to the triple-linker structure.

The good qualitative correlation between levels of in vivo expression and the ability of *SUP4* plasmids to act as in vitro transcription templates suggests that the observed in vivo effects are predominantly transcriptional in origin. Furthermore, these results suggest that existing crude transcription systems contain the main functionally important factors which, together with the template structure, determine the level of in vivo *SUP4* expression. If the in vivo functionality of *SUP4* were heavily influenced by higher-order chromatin or chromosome structure, very different results might have been obtained.

Overall, our results are subject to interpretation from two

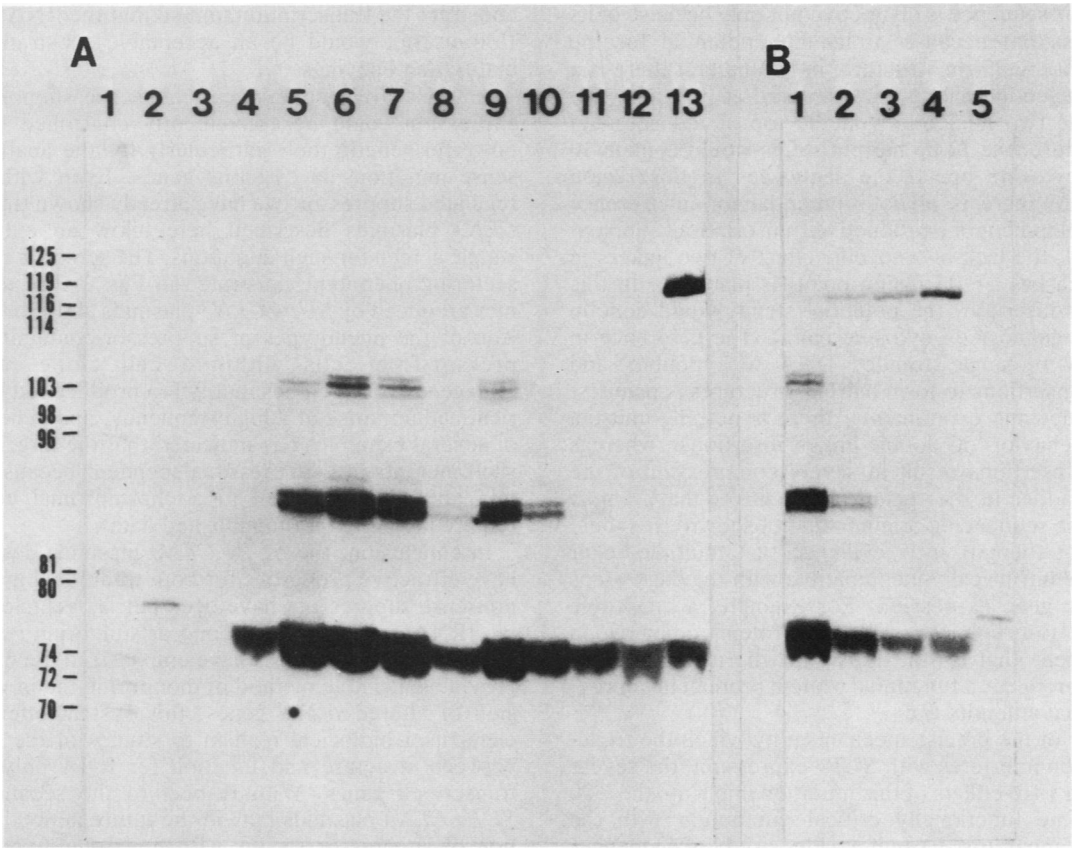

FIG. 5. Analysis of in vitro transcription products on denaturing polyacrylamide gels. (A) Comparison of *SUP4* templates with various amounts of normal 5'-flanking sequence. Lanes 1 through 5, 12, and 13 involve size markers and controls: 1, a Maxam-Gilbert sequencing ladder; 2, mature *S. cerevisiae* $tRNA^{Tyr}$, 3'-end labeled with ^{32}P-labeled pCp and RNA ligase; 3, no extract; 4, no template; 5, an alternate transcription cocktail, using the −77 deletion; 12, the vector pTC3 as template; 13, the yeast 5S gene clone pSC90 (22). Lanes 6 through 11 involve identical reactions, using six different deletion-fusions as templates: 6, pC689 (−77); 7, pC226 (−27); 8, pC68 (−18; 3 liners); 9, pC723 (−17); 10, pC865 (−15); 11, pC888-1 (−4). In all reactions containing the cell extract, a smeared band was observed in the 75-nucleotide region; this band, which is not template dependent (lane 4) presumably involves label incorporation into a component of the extract (P. A. Weil, personal communication). (B) Competition between *SUP4-o* deletion-fusion plasmids and a *S. cerevisiae* 5S gene clone (pSC90) as templates. The identities of the *SUP4-o* templates in the lanes are as follows: 1, pC689 (−77); 2, pC723 (−17); 3, pC888-1 (−4). Lane 4 contains no competing *SUP4-o* template, and lane 5 contains mature yeast $tRNA^{Tyr}$, 3'-end labeled with pCp and RNA ligase.

alternative viewpoints: a particular deletion-fusion could be regarded as causing poor expression because it either replaces a functionally critical sequence with a less acceptable one or introduces a sequence that interferes in some specific way with expression. These alternatives are genetically distinguishable only in extreme cases. If just a few of all possible sequences are functional, the former hypothesis is favored, whereas the opposite circumstance favors the latter hypothesis. On the available evidence, we favor a model for *SUP4* expression that posits few if any critical sequence-specific contacts between the transcription apparatus and the 5'-flanking sequences. This model is based on the diversity of the sequences found in this region of eucaryotic tRNA genes, the difficulty of selecting point mutations in noncoding regions of *SUP4* (23), and the observation that the only one of our in vitro mutants that completely eliminates function does so by introducing a highly peculiar sequence in the −20 region.

One unifying explanation for the observed effects is provided by presupposing extensive melting of the DNA duplex in the −20 region as part of the normal transcription initiation pathway. This hypothesis would explain the defectiveness of pBR322-*SUP4* fusions in or proximal to the −20 region on the basis of the dramatically increased guanosine-plus-cytosine (G+C) content of the DNA present in this region of the fusions. For example, whereas the first 50 bp of wild-type *SUP4*-noncoding DNA is only 22% G+C, the first 50 bp of pBR322 fusion sequence is 62% G+C in orientation I and 72% G+C in orientation II. A simple G+C rule would also explain the recovery of function in the −4 deletion in a tandem duplication of the gene that fuses the downstream gene to adenosine-plus-thymine-rich sequences normally proximal to the 3' end of the *SUP4*-coding region. Similarly, this model would resolve the apparent conflict between the phenotypes of our *SUP4* deletion-fusion series and the phenotypes of three mutants constructed by Koski et al. (21): these three fusions between *SUP4* and adenosine-plus-thymine-rich *CEN3* sequences with break points at −2, −15, and −27 appear to give higher levels of in vitro and in vivo function than do mutants with comparable fusion points in our series.

The lack of function in YC68, the mutant with three *Bam*HI liners at a −18 fusion point, is particularly suggestive of DNA melting in the −20 region during transcription

initiation. This sequence is distinctive not only because of its G+C richness but because of its high potential for the formation of a cruciform structure: in this mutant there is a perfect 26-nucleotide palindrome centered at position −33. Excluding the two nucleotides at the top of the potential hairpin structure, the 12-bp hairpin stems would contain 10 guanosine · cytosine bp. If the sequences in this region normally melt, there is obvious potential for interference with the development of a productive transcription complex. In this model, the lack of a specific effect of two linkers in YC865, which has a −15 fusion point, is plausible. In this double-linker insertion, the potential stems would contain only 7 bp (5 guanosine · cytosine pairs). The difference in the tendency of single-stranded DNA with double- and triple-linker insertions to form hairpin structures is manifest in DNA sequencing experiments: there is nothing unusual about the behavior of double-linker insertions, whereas triple-linker insertions result in severe compression of the sequencing ladder in the region of the linker that is most distal from the sequencing origin (data not shown). It should be noted that there is little evidence that multiple-linker insertions are intrinsically incompatible with the basic steps of eucaryotic gene expression. For example, a quadruple *Bam*HI linker insertion into a polyoma protein-coding region produces a gene that is still transcribed normally and, for that matter, produces a functional protein product in approximately normal amounts (7).

Regardless of the precise mechanism by which the triple-linker insertion interferes with *SUP4* expression, the severe in vivo and in vitro effects of this mutation implicate the −20 region in some functionally critical interaction with the transcription apparatus. Even if a wide variety of sequences are permissive for this interaction, the existence of a nonpermissive sequence is of both theoretical and practical interest. From a theoretical standpoint, the result has a bearing on the problem of tRNA gene regulation. Specifically, it suggests that eucaryotic tRNA genes could be regulated by conventional procaryote-like mechanisms. If a sequence alone such as the triple-linker structure can block transcription, presumably a conditionally binding repressor could also do so if an appropriate repressor-binding site were present in the −20 region. Such a model for tRNA gene regulation is appealing since the internal promoter sequences are subject to such strong functional constraints in any tRNA gene that it might be impossible for them to meet the additional constraint of serving as the binding site for a gene-specific repressor. In contrast, the −20 region sequences are only weakly constrained by the basic requirements of tRNA gene function and could presumably be readily adapted to a regulatory role.

From a more practical standpoint, the triple-linker construction, as well as several of the other plasmids described here, offers attractive opportunities to design nonsense suppressors with useful properties by genetic engineering techniques. Particularly important would be the development of a strong nonsense suppressor that could be tightly regulated by a gratuitous component of the culture medium. The regulatory hypothesis that was discussed above would suggest that a regulated nonsense suppressor could be constructed simply by inserting a suitable operator-like sequence next to *SUP4*. Alternatively, the same goal might be achievable by embedding the triple-linker insertion into a regulated RNA polymerase II transcription unit. Presumably, this structure would not be transcribed by RNA polymerase III, thereby avoiding a high basal level of unregulated expression, whereas it should be possible to construct the transcription unit so that the RNA polymerase II transcript would be an acceptable substrate for tRNA maturation enzymes.

A yeast strain containing a nonsense suppressor whose expression could be conveniently controlled would be a powerful genetic tool, particularly for the analysis of nonsense mutations in essential genes. Even without such a regulated suppressor, we have already shown that the *SUP4 CEN3* plasmids described here allow an extraordinarily simple screen for such mutations. The screen is based on the sectoring phenotype illustrated in Fig. 3. The mosaic colonies produced by *SUP4 CEN3* plasmids allow direct comparison of the phenotypes of suppressor-containing and suppressor-free cells within a cell clone. After light mutagenesis, cell clones that fail to produce red sectors on a rich medium arise at a high frequency, and genetic analysis of several examples has indicated that the large majority of such mutants are suppressor dependent because they contain an *ochre* mutation in a chromosomal gene that is required for growth (unpublished data).

In conclusion, the *SUP4 CEN3* plasmids described here have attractive properties for both traditional uses to which nonsense suppressors have been put: as vehicles for studying tRNA gene expression and as utilitarian genetic tools. Our present experiments have emphasized the first area. By providing a simple method of monitoring the in vivo expression of altered tRNA genes, this system offers a critical element of biological realism to studies of the relationship between structure and function for RNA polymerase III-transcribed genes. With respect to the second area, the *SUP4 CEN3* plasmids may in the future allow the construction of suppressor strains with novel genotypes that lend themselves to useful genetic manipulations that are beyond the reach of classical techniques.

ACKNOWLEDGMENTS

We are particularly grateful to the following individuals for material assistance in this work: P. A. Weil for transcription extracts and advice on their use, S. B. Sandmeyer for the M13 *SUP4-o* clone, and K. Nasmyth for the vector pTC3. J. C. Goodloe provided expert photographic assistance.

This work was supported by Public Health Service grant GM27839 from the National Institutes of Health. K.J.S. was supported by fellowship DRG-488-F from the Damon Runyon-Walter Winchell Cancer Fund.

LITERATURE CITED

1. **Bogenhagen, D. F., S. Sakonju, and D. D. Brown.** 1980. A control region in the center of the 5S RNA gene directs specific initiation of transcription: II. The 3′ border of the region. Cell **19**:27–35.
2. **Bolivar, F., R. L. Rodriguez, M. C. Betlach, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. Gene **2**:75–93.
3. **Ciliberto, G., G. Raugei, F. Costanzo, L. Dente, and R. Cortese.** 1983. Common and interchangeable elements in the promoters of genes transcribed by RNA polymerase III. Cell **32**:725–733.
4. **Clarke, L., and J. Carbon.** 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature (London) **287**:504–509.
5. **DeFranco, D., O. Schmidt, and D. Soll.** 1980. Two control regions for eukaryotic tRNA gene transcription. Proc. Natl. Acad. Sci. U.S.A. **77**:3365–3368.
6. **DeFranco, D., S. Sharp, and D. Soll.** 1981. Identification of regulatory sequences contained in the 5′-flanking region of *Drosophila* lysine $tRNA_2$ genes. J. Biol. Chem. **256**:12424–

12429.
7. **Dinces, N. B., and G. Milman.** 1982. Viable polyoma virus with four *Bam*HI decanucleotide linkers inserted at the 26-minute *Hin*cII site. J. Mol. Appl. Genet. **1**:395–404.
8. **Engelke, D. R., S.-Y. Ng, B. S. Shastry, and R. G. Roeder.** 1980. Specific interaction of a purified transcription factor with an internal control region of 5S RNA genes. Cell **19**:717–728.
9. **Fitzgerald-Hayes, M., L. Clarke, and J. Carbon.** 1982. Nucleotide sequence comparisons and functional analysis of yeast centromere DNA's. Cell **29**:235–244.
10. **Fowlkes, D. M., and T. Shenk.** 1980. Transcriptional control regions of the adenovirus VAI RNA gene. Cell **22**:405–413.
11. **Galli, G., H. Hofstetter, and M. L. Birnstiel.** 1981. Two conserved sequence blocks within eukaryotic tRNA genes are major promoter elements. Nature (London) **294**:626–631.
12. **Gergen, J. P., R. H. Stern, and P. C. Wensink.** 1979. Filter replicas and permanent collections of recombinant DNA plasmids. Nucleic Acids Res. **7**:2115–2136.
13. **Goodman, H. M., M. V. Olson, and B. D. Hall.** 1977. Nucleotide sequence of a mutant eukaryotic gene: the yeast tyrosine-inserting ochre suppressor SUP4-o. Proc. Natl. Acad. Sci. U.S.A. **74**:5453–5457.
14. **Hall, B. D., S. G. Clarkson, and G. Tocchini-Valentini.** 1982. Transcription initiation of eucaryotic transfer RNA genes. Cell **29**:3–5.
15. **Hawthorne, D. C., and U. Leupold.** 1974. Suppressor mutations in yeast. Curr. Top. Microbiol. Immunol. **64**:1–47.
16. **Hawthorne, D. C., and R. K. Mortimer.** 1968. Genetic mapping of nonsense suppressors in yeast. Genetics **60**:735–742.
17. **Hopper, A. K., and J. Kurjan.** 1981. tRNA synthesis: identification of in vivo precursor tRNA's from parental and mutant yeast strains. Nucleic Acids Res. **9**:1019–1029.
18. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
19. **Klekamp, M. S., and P. A. Weil.** 1982. Specific transcription of homologous class III genes in yeast-soluble cell-free extracts. J. Biol. Chem. **257**:8432–8441.
20. **Klemenz, R., D. J. Stillman, and E. P. Geiduschek.** 1982. Specific interactions of *Saccharomyces cerevisiae* proteins with a promoter region of eukaryotic tRNA genes. Proc. Natl. Acad. Sci. U.S.A. **79**:6191–6195.
21. **Koski, R. A., D. S. Allison, M. Worthington, and B. D. Hall.** 1982. An *in vitro* RNA polymerase III system from *S. cerevisiae*: effects of deletions and point mutations upon *SUP4* gene transcription. Nucleic Acids Res. **10**:8127–8143.
22. **Kramer, R. A., P. Philippsen, and R. W. Davis.** 1978. Divergent transcription in the yeast ribosomal RNA coding region as shown by hybridization to separated strands and sequence analysis of cloned DNA. J. Mol. Biol. **123**:405–416.
23. **Kurjan, J., and B. D. Hall.** 1982. Mutations at the *Saccharomyces cerevisiae SUP4* $tRNA^{Tyr}$ locus: isolation, genetic fine-structure mapping, and correlation with physical structure. Mol. Cell. Biol. **2**:1501–1513.
24. **Madison, J. T., and H.-K. Kung.** 1967. Large oligonucleotides isolated from yeast tyrosine transfer ribonucleic acid after partial digestion with ribonuclease T1. J. Biol. Chem. **242**:1324–1330.
25. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
26. **Roman, H.** 1956. Studies of gene mutation in *Saccharomyces*. Cold Spring Harbor Symp. Quant. Biol. **21**:175–185.
27. **Rothstein, R. J., R. E. Esposito, and M. S. Esposito.** 1977. The effect of *ochre* suppression on meiosis and ascospore formation in *Saccharomyces*. Genetics **85**:35–54.
28. **Sakonju, S., D. F. Bogenhagen, and D. D. Brown.** 1980. A control region in the center of the 5S RNA gene directs specific initiation of transcription. I. The 5' border of the region. Cell **19**:13–25.
29. **Sakonju, S., and D. D. Brown.** 1982. Contact points between a positive transcription factor and the *Xenopus* 5S RNA gene. Cell **31**:395–405.
30. **Sprague, K. U., O. Hagenbuchle, and M. C. Zuniga.** 1977. The nucleotide sequence of two silk gland alanine tRNAs: implications for fibroin synthesis and for initiator tRNA structure. Cell **11**:561–570.
31. **Sprague, K. U., D. Larson, and D. Morton.** 1980. 5' flanking sequence signals are required for activity of silkworm alanine tRNA genes in homologous in vitro transcription systems. Cell **22**:171–178.
32. **Stinchcomb, D. T., K. Struhl, and R. W. Davis.** 1979. Isolation and characterization of a yeast chromosomal replicator. Nature (London) **282**:39–43.
33. **Struhl, K., D. T. Stinchcomb, S. Scherer, and R. W. Davis.** 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. U.S.A. **76**:1035–1039.
34. **Sutcliffe, J. G.** 1978. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.
35. **Thimmappaya, B., N. Jones, and T. Shenk.** 1979. A mutation which alters initiation of transcription by RNA polymerase III on the Ad5 chromosome. Cell **28**:947–954.