An *in vitro* **RNA polymerase III system from *S. cerevisiae*: effects of deletions and point mutations upon *SUP4* gene transcription**

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ABSTRACT

A soluble cell-free extract containing RNA polymerase III and factors essential for selective transcription of the yeast SUP4-o tRNA$^{Tyr}$ gene was prepared from Saccharomyces cerevisiae cells. An intragenic promoter for yeast RNA polymerase III was identified within the yeast tRNA$^{Tyr}$ coding sequence by testing several sup4 genes with 5'- and 3'-terminal deletions in the homologous transcription system.

Thirty-four different sup4 genes with spontaneous mutations were also tested in the in vitro system. Two point mutations drastically reduced transcription initiation and two other mutations caused premature termination. These mutations have nearly identical effects on SUP4 gene transcription by Xenopus RNA polymerase III (1), which demonstrates that the essential features of RNA polymerase III transcription initiation and termination signals have been conserved throughout the course of eukaryotic evolution.

INTRODUCTION

The mechanics of transcription initiation by eukaryotic RNA polymerase III are unique amongst DNA reading processes in that the major signals specifying initiation lie downstream from the transcription starting point. The transcription phenotypes of mutationally altered 5S rRNA genes (2, 3), tRNA genes (1, 4-8) and adenovirus VA-RNA genes (9, 10) are in each case most severely affected by DNA changes within the transcribed region.

A partial insight into the molecular mechanisms governing PolIII transcription initiation has come from studies of soluble RNA polymerase III transcription factors (11) and of their interaction with DNA (12). A more detailed analysis of the molecular interactions involved in RNA polymerase III transcription initiation would be aided by using, in these biochemical studies, PolIII transcription components mutationally altered in their function.

In the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe, genetic studies of nonsense suppressor genes have made possible very detailed mutational analyses of a number of tRNA genes (13-15). Even

though a given tRNA gene may belong to a repeated gene family, the occurrence of a single base-pair change within its anticodon region can impart to the individual gene a distinctive dominant phenotype (16).

The appearance, from a nonsense-suppressing parental strain, of progeny cell clones which lack suppressor activity signals the occurrence of mutations which, in one way or another, block the synthesis of functional suppressor tRNA molecules. Techniques for screening and selecting loss-of-suppression mutants not only can identify second-site mutations in the suppressor tRNA gene itself (14); they also should be capable of identifying mutations in genes encoding proteins required for the transcription of tRNA molecules. Our interest in eventually studying these proteins by mutation and in vitro complementation has motivated us to develop a cell-free extract system from yeast for specific transcription of tRNA genes by RNA polymerase III. The existing in vitro eukaryotic PolIII transcription systems all are derived from organisms (mammals, amphibians, insects) that do not lend themselves readily to in vivo tRNA genetics via suppressor mutations.

In this paper, we report initial experiments which have led to an in vitro yeast transcription system which accurately transcribes tRNA genes. The response of this system to 34 different mutational changes in the SUP4 tRNA$^{Tyr}$ gene has been tested and found identical in each case to that observed previously with the same mutant genes in a <u>Xenopus laevis</u> S100 system (1). These results illustrate the great similarities which exist between the PolIII transcription components of yeast and of vertebrates. Consequently, results obtained in the genetically more tractable fungal systems should elucidate general rules for tRNA gene transcription which will apply to the vertebrate systems as well.

METHODS
Mutant sup4 genes
    Thirty-three mutant <u>sup</u>4 genes used in this study were previously described by Kurjan <u>et al</u>. (14) and Koski <u>et al</u>. (1). One additional mutant <u>sup</u>4 gene was cloned and sequenced from the J15-13C M146 strain (obtained from Janet Kurjan) using methods previously described (14). The <u>sup</u>4 genes with spontaneous mutations were added to transcription reactions as recombinant plasmid DNA containing a 4.5 kb <u>EcoRI-HindIII</u> yeast DNA fragment inserted into pBR322.
    All of the <u>sup</u>4 genes with deletions constructed in vitro were inserted

in vector pYZ between the yeast CEN3 sequence on the 5' side and the yeast cycl gene on the 3' side.  In each plasmid, described below, the fragments:

|  | CEN3 |  |  | SUP4 |  |  | CYC1 |  |  |
|---|---|---|---|---|---|---|---|---|---|
| BglII | ———————— | BamHI | 5' | ———— | 3' | XhoI | ———————— | HindIII |

are located between the BamHI and HindIII sites of the yeast plasmid vector YRp7 (17).  The CEN3 sequence is contained in a 2.2 kb BamHI-BglII fragment from pYe(CEN3)41 (18).  Cycl is present on a 0.6 kb XhoI-HindIII fragment originating from deletion ΔX-9 (19).

The 5' deletion mutants were generated from plasmid pXSU4, which is identical to psu4-A (20) except that in pXSU4 the unique HindIII site 0.8 kb 3' to SUP4 is replaced by an XhoI site.  An AvaII site 75 bp 5' to SUP4 was a convenient starting point for making deletions.  pXSU4 was cleaved with AvaII and treated with BAL-31 nuclease.  BamHI linkers were attached and the material digested with BamHI and XhoI.  This treatment produced a series of fragments with BamHI ends at variable positions 5' to and within SUP4, and XhoI ends at a constant distance (0.8 kb) 3' to SUP4.  These fragments were inserted between CEN3 and cycl in pYZ as diagrammed above to create the pCS4 series of plasmids (Figure 3A).

In the construction of all the sup4 plasmids described below, a BamHI site located 2 kb 5' to SUP4 was utilized.  To make pBSU4, the fragment extending from this BamHI site to the XhoI site on the 3' side of sup4 in pXSU4 was ligated into the corresponding site in pYZ.  pBSU4 thus contains a sup4 gene with normal 5' and 3' flanking sequences (Figure 3A).  Both of the 3' deletion mutants (pAS4 and pDS4) are missing normal sequences 3' to the SmaI site at position 83 in SUP4.  In pAS4, the 2.1 kb BamHI-SmaI fragment from pXSU4 was ligated into the BamHI-XhoI site of pYZ (the XhoI 5' extension in the vector pYZ was first converted to a flush end with Klenow DNA polymerase).  pDS4 resulted from insertion of the 2.1 kb BamHI-SmaI fragment, with a BamHI linker attached to the SmaI end, into the unique BamHI site of the sup4 plasmid pCS4-2 (see above and figure 3).

The structure of the various plasmids was confirmed by restriction analysis and, in some cases, DNA sequencing.  The deletion endpoints of pCS4-2, pCS4-21 and pCS4-23 were determined by DNA sequencing.  The endpoints of pCS4-9, pCS4-1 and pCS4-42 were estimated by sizing on polyacrylamide gels and are within ±1 nucleotide.

Preparation of yeast cell-free extract

The Saccharomyces cerevisiae RNase⁻3 strain (21; obtained from B. Littlewood, University of Wisconsin) was grown at 30° with aeration to 2.5

x $10^7$ cells/ml in two 2 liter cultures containing YEPD medium (1% yeast extract; 2% peptone; 2% dextrose; 40 µg/ml adenine; 40 µg/ml uracil). Cell harvesting and fractionation procedures were performed at 0-4°C. The cells were collected by centrifugation at 5,000 rpm in a Sorvall GS-3 rotor for 10 min. The cell pellets were resuspended in a total of 120 ml ice-cold $H_2O$ and centrifuged for 10 min at 5,000 rpm in a Sorvall HB-4 rotor. The washing procedure was then repeated with 60 ml $H_2O$. The packed cell volume was 8.6 ml. Two cell pellet volumes of extraction buffer containing 100 mM Tris-HCl, pH 7.9 (22°C), 2 mM EDTA, 1 mM phenylmethyl sulfonylflouride (PMSF), 1 mM dithiothreitol (DTT), 40% (v/v) glycerol and 20% (w/v) sucrose were added to the cells. Then two cell pellet volumes of 0.45-0.5 mm glass beads (VWR), which had been washed with concentrated HCl and $H_2O$, and one cell pellet volume $H_2O$ were added to the cells. The cells were then resuspended and disrupted by agitation in a Braun homogenizer (model MSK 2876, Brownwill Scientific, Inc.) for a total of 2 minutes with intermittant cooling. Cell lysis was 90% complete as determined by phase contrast microscopy. The volume of the homogenate was measured and 1/10 volume of saturated ammonium sulfate (pH 7.6) was added. After gently mixing at 0°C for 20 minutes, the homogenate was centrifuged at 50,000 rpm for 4 hours in a Spinco Type 65 rotor at 4°C. The clear supernatant was removed and ammonium sulfate (0.33 g/ml) was gradually added to the supernatant with rapid stirring. When all of the ammonium sulfate had dissolved, 10 µl of 1 N NaOH was added per gram of ammonium sulfate. After mixing for 30 minutes, the precipitate was collected by centrifugation at 30,000 rpm for 30 minutes in a Spinco Ti60 rotor. The precipitate was resuspended in 2 ml buffer A containing 50 mM Tris-HCl, pH 7.9, 50 mM ammonium sulfate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.01 mM PMSF and 25% (v/v) glycerol, and loaded onto a 1.5 x 34 cm Sephadex G25 (fine) column that had been previously equilibrated with buffer A. The extract was eluted from the column with buffer A. The $A_{280}$ peak fractions (8.4 ml) were pooled and dialyzed for a total of eight hours against 3 one liter changes of Buffer B containing 40 mM HEPES-KOH, pH 7.9 (22°C), 140 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM DTT, 0.01 mM PMSF and 50% (v/v) glycerol. The G25 peak fraction retained transcription activity for several months when stored in small aliquots at -70°C and thawed once immediately before use. The protein concentration in the G25 fraction (12 mg/ml) was determined by the coomassie blue-dye binding assay (22).

## In vitro transcription reactions

Unless otherwise noted, each transcription reaction contained a final volume of 20 µl, was incubated 60 minutes at 25°C, and included the following components: 20 mM HEPES-KOH, pH 7.9 (22°C), 70 mM KCl, 5 mM MgCl$_2$, 0.1 mM DTT, 0.1 mM EDTA, 0.005 mM PMSF, 25% (v/v) glycerol, 1 µl G25 fraction, 1-2 µg/ml DNA, 0.5 mM ATP, CTP and GTP and 0.05 mM α-$^{32}$P-UTP (10 Ci/mmol; Amersham). Reactions were terminated by adding 1 µl 10% sodium dodecyl sulfate and 1 µl 2 mg/ml proteinase K. After an additional 15 minutes at 25°C, the reaction mixtures were diluted with 190 µl 0.2 M sodium acetate, pH 5.3, and 10 µl 1 mg/ml yeast tRNA, and then extracted with 200 µl H$_2$O saturated phenol-CHCl$_3$ (1:1, v/v). The aqueous phase was removed and the organic phase was extracted with 200 µl 0.2 M sodium acetate. RNA was recovered from the pooled aqueous phases by ethanol precipitation. The RNA pellets were resuspended in 200 µl 0.2% sodium dodecyl sulfate, and the RNA was precipitated again after the addition of 200 µl 2 M ammonium acetate, pH 5.3 and 800 µl ethanol. This high salt precipitation removes most of the unincorporated label (23). The RNA pellets were then resuspended in 400 µl 0.2 M sodium acetate, pH 5.2, and precipitated with 800 µl ethanol. Finally 1 ml ethanol was added to rinse the RNA pellets. The ethanol was removed after centrifugation, and the RNA was dried in vacuo. All precipitations were performed by cooling the solutions in a dry ice-ethanol bath for at least 5 minutes, and then centrifuging for 5 minutes in an Eppendorf centrifuge at room temperature.

Phosphatase activity in the transcription reactions was monitored by spotting 1 µl of the first ethanol supernatant, together with 1 µl 20 mM UTP, on polyethyleneimine-cellulose thin layers, followed by stepwise chromatography with LiCl solvents (1, 24). The extent of α-$^{32}$P-UTP degradation was determined by autoradiography.

## Polyacrylamide gel electrophoresis

RNA samples were dissolved in 5 µl 10 mM Tris-Borate, pH 8.3, 2 mM EDTA, 7 M urea, 0.05% xylene cyanole FF. They were heated to 60°C for 2 minutes, chilled in an ice bath, and loaded onto 0.04 x 14 x 24 cm 10% polyacrylamide gels (1:29, bisacrylamide:acrylamide) containing 7 M urea and 83 mM Tris-Borate, pH 8.3, 1 mM EDTA. Electrophoresis was at 5 watts for 4-4.5 hours at room temperature. Labeled RNA bands were detected by autoradiography with Kodak XR-2 film. Following autoradiography, the gels were stained with 0.2% (w/v) methylene blue in 0.8 M sodium acetate, pH 5.4, and destained with H$_2$O to determine the positions of unlabeled 5.8S

rRNA, 5S rRNA and tRNA, and to estimate the recovery of RNA from the transcription reactions.

## RESULTS

### Preparation of a yeast RNA polymerase III in vitro transcription system

The initial steps of RNA polymerase purification procedures (25, 26) were used to concentrate RNA polymerase III transcription activity from a whole cell yeast extract, and to partially remove interfering proteases, nucleases and phosphatases. A similar procedure for preparing yeast extracts containing selective polymerase III transcription activity was simultaneously developed by Klekamp and Weil (27). We disrupted log phase yeast cells with glass beads in a Braun homogenizer, and then made the homogenate approximately 0.4 M in ammonium sulfate to lyse nuclei and release proteins from chromatin. The homogenate was centrifuged at 159,000 x g, and the components required for selective SUP4 gene transcription were concentrated from the supernatant by ammonium sulfate precipitation. We used the Saccharomyces cerevisiae RNase⁻3 strain isolated by Littlewood, Shaffer and Davies (21) to reduce ribonuclease levels. Glycerol and phenylmethyl sulfonyl flouride were added to all buffers to inhibit protease activity. Phosphatase activity in the final extract was reduced by precipitating the polymerase and essential transcription factors in the absence of magnesium (J. Thorner, personal communication). After passing the extract through a Sephadex G25 column, which partially removed an unidentified inhibitor, the peak protein fractions were concentrated by dialysis against a buffer containing 50% glycerol and stored at -70°C. Additional purification of the extract on a DEAE-cellulose column separated the RNA polymerase III transcription activity into two essential components, but only 10% of the activity loaded onto the column was recovered in reconstituted fractions (data not shown).

### Transcription of the SUP4-o tRNA$^{Tyr}$ gene

When the concentrated yeast cell-free extract was incubated with ribonucleoside triphosphates and a cloned yeast SUP4-o tRNA$^{Tyr}$ (20), a major transcription product between 105 and 110 nucleotides in size accumulated with the kinetics of a primary transcription product (Figure 1, lanes a-d). The ~110 nucleotide SUP4 gene transcript is the major transcription product after a 60 minute reaction (Figure 1, lane d). A RNA molecule approximately 3 nucleotides shorter also accumulates, perhaps due to removal of precursor-specific 3-'terminal nucleotides. Very little other nucleolytic
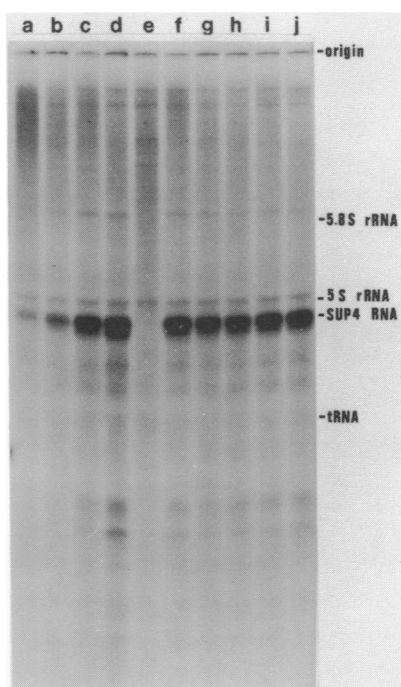
Figure 1. Transcription time course and α-amanitin inhibition experiments. pBR322 DNA containing a 4.5 kb fragment of S. cerevisiae DNA with the SUP4-o tRNA^Tyr gene (lanes a-d, f-j), or pBR322 DNA alone (lane e), was incubated in transcription reactions with yeast G25 fraction cell-free extract, ATP, CTP, GTP and α-^32 P-UTP for 10 minutes (lane a), 20 minutes (lane b), 30 minutes (lanes c, f-j), or 60 minutes (lanes d and e). α-amanitin was added to reactions g (10 µg/ml), h (100 µg/ml), i (1,000 µg/ml) and j (2,000 µg/ml). RNA synthesized in each reaction was extracted and analyzed in a 7 M urea, 10% polyacrylamide gel. The positions of unlabeled 5.8S rRNA, 5S rRNA and tRNA in the gel are indicated.

processing or degradation of the RNA transcripts occurs in the reactions.

The major transcripts generated from the SUP4-containing plasmid were shown to originate from the tRNA^Tyr gene by the effects of deletion and premature termination mutations upon in vitro transcription (see below). Although RNA sequence analysis is required to precisely define the SUP4 transcript structure, the size of the in vitro transcript, as judged by electrophoretic mobility relative to 5.8S rRNA, 5S rRNA and tRNA, is consistent with the estimated sizes of yeast tRNA^Tyr gene primary tran-scripts found in vivo (28) and made upon injection of the SUP4 gene into Xenopus oocytes (29). In contrast, Xenopus RNA polymerase III SUP4 gene transcripts produced in vitro (1) are approximately 10 nucleotides smaller.
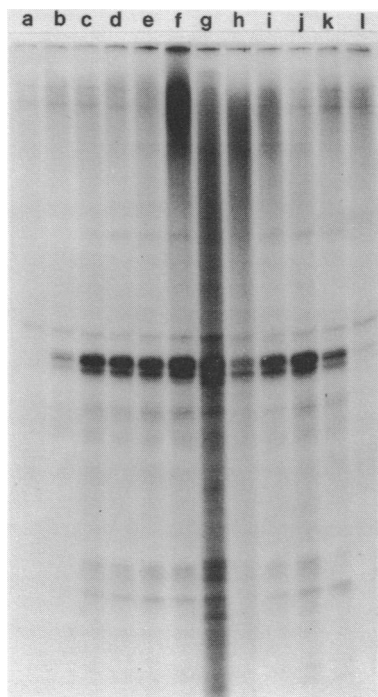
Figure 2. Optimal DNA and extract concentrations. Transcription reactions were incubated for 60 min at 25°C and contained no DNA (lane a), or SUP4-o DNA at 0.2 μg/ml (lane b), 0.5 μg/ml (lane c), 1 μg/ml (lane d), 2 μg/ml (lanes e, h-1), 5 μg/ml (lane f); 10 μg/ml (lane g); and G25 fraction extract: 1 μl (lanes a-g, j), 0.2 μl (lane h), 0.5 μl (lane i), 2 μl (lane k), 5 μl (lane 1).

The transcription reaction is not inhibited by α-amanitin (Figure 1, lanes f-j), which demonstrates that yeast RNA polymerase III (30) in the cell-free extract transcribes the tRNA$^{Tyr}$ gene, and that very little background transcription by RNA polymerase I or II occurs in the reactions. The optimal DNA concentration for SUP4 gene transcription is 1-5 μg/ml (Figure 2), and the optimal extract concentration is approximately 0.6 mg protein/ml (1 μl extract/20 μl reaction; Figure 2). Nonspecific transcription increased relative to SUP4 gene transcription at higher DNA concentrations and excess cell-free extract resulted in degraded RNA transcripts and ribonucleoside triphosphates.

Deletion mapping of SUP4 tRNA$^{Tyr}$ gene promoter sequences

We constructed recombinant SUP4-o genes lacking their normal 5'-flanking sequences by cutting at an AvaII restriction site 75 bp upstream from the tRNA$^{Tyr}$ coding sequences and trimming the AvaII restriction fragment with nuclease BAL-31 (Figure 3). SUP4-o genes with 72 bp, 27 bp, 15 bp or 2 bp of 5'-flanking sequences were ligated into a yeast vector adjacent to yeast CEN3 centromere sequences (18). Each recombinant DNA is active in the in vitro transcription reactions (Figure 4, lanes b-e). The sizes of the sup4
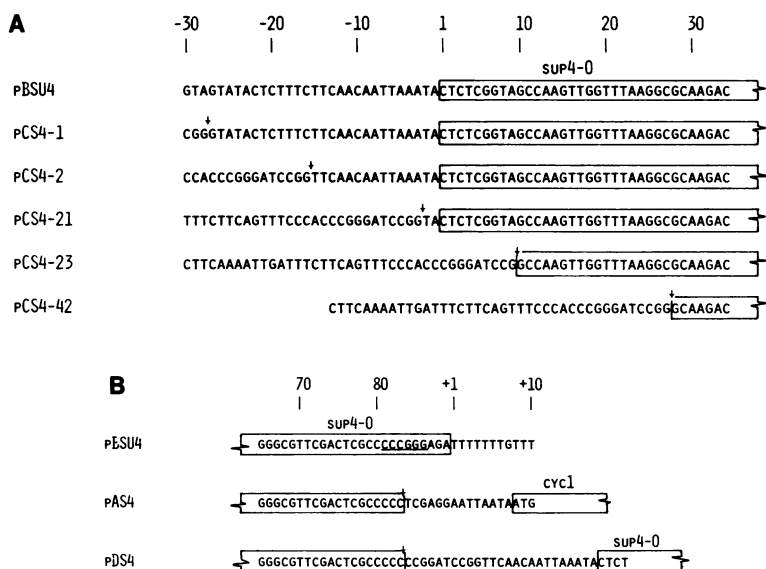
**A**

| | -30 | -20 | -10 | 1 | 10 | 20 | 30 |
|---|---|---|---|---|---|---|---|

sup4-0

pBSU4  GTAGTATACTCTTTCTTCAACAATTAAATA CTCTCGGTAGCCAAGTTGGTTTAAGGCGCAAGAC

pCS4-1  CGGGTATACTCTTTCTTCAACAATTAAATA CTCTCGGTAGCCAAGTTGGTTTAAGGCGCAAGAC

pCS4-2  CCACCCGGGATCCGGTTCAACAATTAAATA CTCTCGGTAGCCAAGTTGGTTTAAGGCGCAAGAC

pCS4-21  TTTCTTCAGTTTCCCACCCGGGATCCGGTA CTCTCGGTAGCCAAGTTGGTTTAAGGCGCAAGAC

pCS4-23  CTTCAAAATTGATTTCTTCAGTTTCCCACCCGGGATCCGGCCAAGTTGGTTTAAGGCGCAAGAC

pCS4-42  CTTCAAAATTGATTTCTTCAGTTTCCCACCCGGGATCCGGCAAGAC

**B**

| | 70 | 80 | +1 | +10 |
|---|---|---|---|---|

sup4-0

pBSU4  GGGCGTTCGACTCGCCCCCGGGAGATTTTTTTGTTT

cyc1

pAS4  GGGCGTTCGACTCGCCCCCTCGAGGAATTAATAATG

sup4-0

pDS4  GGGCGTTCGACTCGCCCCCCCGGATCCGGTTCAACAATTAAATACTCT

Figure 3.  Structure of sup4 genes with experimentally constructed deletions. (A) 5' deletion mutants.  The boxed region indicates sup4-o coding sequence. Arrows show the extent of the various deletions.  The plasmids were constructed by resection with nuclease BAL-31 from an upstream AvaII site. BamHI linkers were added and the sup4 gene fragments were ligated into a yeast vector adjacent to yeast CEN3 centromere sequences.  (B) 3' deletion mutants.  Boxed regions indicate either sup4-o or cyc1 coding sequence. Arrows show deletion endpoints.  The SmaI site is underlined.  The recombinant plasmids were created by cutting the SUP4 gene with SmaI, and ligating the 5'-terminal SUP4 gene fragment into a yeast vector adjacent to either iso-1-cytochrome c (cyc1) coding sequences or to a second SUP4 gene via XhoI or BamHI sites, respectively.

gene transcripts are unaltered by the deletions, even when the putative transcription initiation site is deleted.  SUP4-o genes with CEN3 sequences ligated to the -27 or -15 positions have reduced transcription activity in vitro (lanes c and d) and are less active as ochre suppressor genes in vivo (D.A., unpublished data) than SUP4-o genes with unaltered flanking sequences. Mutant sup4 genes with 5'-terminal deletions extending up to +10 or +28 bp in the tRNA coding region had little or no transcription activity in vitro (lanes f and g).  These data demonstrate that the SUP4 tRNA$^{Tyr}$ gene 5'-flanking sequences are not part of an essential transcription control region.  Furthermore, unless the CEN3-BamHI linker sequences are unusually inhibitory when ligated to the +10 position, the results also locate the 5' border of an essential SUP4 gene transcription control region in the -2 to +10 region.
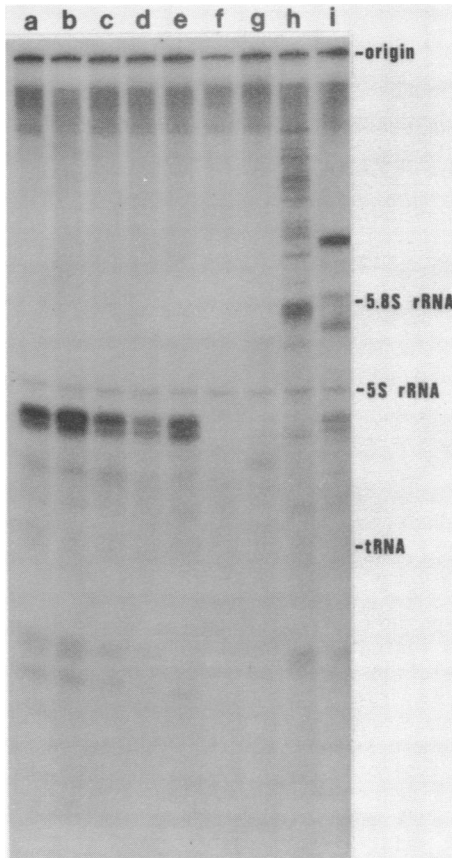
Figure 4. Transcription of sup4 genes with experimentally constructed deletions. Plasmids containing deletions of SUP4 gene sequences (Figure 3) were incubated in transcription reactions under standard conditions. a) pBSU4; b) pCS4-9 (a 5' terminal deletion to position -72); c) pCS4-1; d) pCS4-2; e) pCS4-21; f) pCS4-23; g) pCS4-42; h) pAS4; i) pDS4.

3'-terminal deletions of the SUP4 gene were created by cutting within the coding sequences at a SmaI site located 83 bp downstream from the first nucleotide of the tRNA coding sequence. The truncated sup4 genes were then ligated either to another SUP4 gene cut at the -15 position or to a yeast iso-1-cytochrome c (cyc1) gene (31) cut 8 bp upstream from the beginning of the protein coding sequence (Figure 3). The fused sup4-SUP4 gene was active in vitro (Figure 4, lane i) and in vivo (D. A., unpublished data). Both in vivo and in vitro, a new transcript was produced that corresponded in size (~210 nucleotides) to initiation occurring upstream from the truncated sup4 gene and terminating at the $T_7$ cluster following the down-stream SUP4 gene. The fused sup4-cyc1 gene produced a major in vitro transcript with approximately 160 nucleotides and also a series of longer transcripts (lane h). The size of this prominent RNA indicates that transcription initiates upstream from the truncated sup4 gene and ineffi-

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        m²      m        m²₂     i⁶                              m⁵      m¹
    pCUCUCGGUAGCCAAGDDGGDDDAAGGCGCAAGACUUᴪAA         AᴪCUUGAGADCGGGCGTᴪCGACUCGCCCCCGGGAGACCA    suppressor
                                                                                                tRNAᵗʸʳ
                                   -A36A37                             U56
              A      G          TT   AA               T   T                    A
              T      C          AA  △TT               A   A                    T
              ↑      ↑          ↑↑   ↑↑                ↑   ↑                    ↑
    AATTAAATACTCTCGGTAGCCAAGTTGGTTTAAGGCGCAAGACTTTAATTTATCACTACGAAATCTTGAGATCGGCGTTCGACTCGCCCCCGGGAGA┌─┐TTTTTTTGTTTTTT
    TTAATTTATGAGAGCCATCGGTTCAACCAAATTCCGCGTTCTGAAATTAAATAGTGATGCTTTAGAACTCTAGCCCGCAAGCTGAGCGGGGGCCCTCT└─┘AAAAAAACAAAAAA
              /\ ↑  ↑  ↑ /\ ↑   ↑  ↑ /\↑↑ ↑          ↑  ↑↑  ↑↑   ↑  ↑ ↑↑↑ ↑ ↑ ↑↑                    SUP4 gene
              TG T  ↑  GT    T   G GATCG T            A  AG  AA   G  G C△CTC TT
              AC A  A  CA    A   C CTAGC A            T  TC  TT   C  C G G AG A A
                   U15    U21              U(IV)         G46      G56
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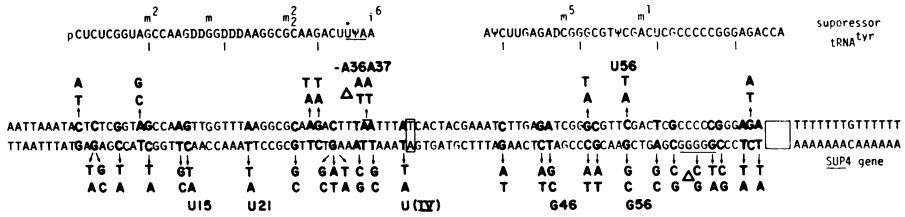
Figure 5. Sequence changes in SUP4 genes with spontaneous loss-of-suppression mutations. Positions at which loss-of-suppression mutations have been isolated are indicated with boldface type with mutational alterations indicated above and below the SUP4-o gene sequence. Each mutation is named according to the position in the tRNA and the nucleotide alteration as described by Celis (33).

ciently terminates at a $T_4$ cluster in the noncoding strand of the cycl coding sequence. The transcription activity of the truncated sup4 genes demonstrates that the last six coding nucleotides of the SUP4 tRNA gene and the 3'-flanking sequences are not required to promote RNA polymerase III transcription.

## Transcription of sup4 genes with spontaneous loss-of-suppression mutations

Selection for loss of in vivo SUP4-o ochre suppressor activity and genetic fine structure mapping identified 98 independent mutations at the SUP4 locus (32). Molecular cloning and DNA sequence analysis of the tRNA genes provided 32 cloned sup4 genes with different mutational alterations (14). Almost all of the known sequence changes within the mutant genes are point mutations in the tRNA coding sequence (Figure 5). Here we report the isolation of one additional mutant sup4 gene, which contains an A → T transversion at position 71 of the tRNA gene (mutation U71, Figure 5).

Most of the mutations we have studied lie between the 5' and 3' boundaries of the promoter region as defined by the deletion mutations. However, only four of the mutations (U56, G56, U(IV) and -A36A37) significantly altered tRNA$^{Tyr}$ gene transcription in vitro. The G56 and U56 mutations alter the same base pair in the 3' half of the SUP4 gene and both reduce transcription approximately five fold (Figure 6). Both mutations alter a base pair located approximately 85 bp downstream from the transcription initiation start site. This base pair encodes the invariant C of the highly conserved GTᴪCGA tRNA sequence. The weak nature of the G56 and U56 gene promoters permits increased expression of minor transcripts from the 4.5 kb yeast DNA fragment (Figure 6), presumably because the two mutations make the SUP4 gene less effective in competing for limiting transcription
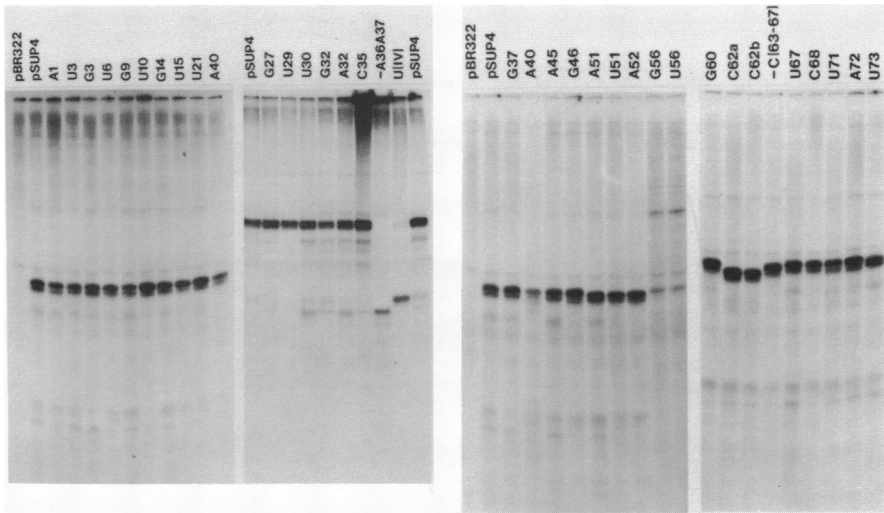
Figure 6. Transcription of sup4 genes with spontaneous mutations. pBR322 DNA, or recombinant DNAs with the SUP4-o gene or one of its mutant alleles, were incubated in transcription reactions under standard conditions. C62a and C62b are recombinant DNAs from two independently isolated mutants.

factors in the in vitro reactions. The G56 and U56 mutations might also make the tRNA$^{Tyr}$ precursor unusually susceptible to degradative nucleases and thereby reduce the yield of SUP4 gene transcripts. However, we consider this possibility unlikely since other mutations that more severely disrupt the tRNA precursor structure do not result in degradation of the mutant transcripts.

The -A36A37 deletion and the U(IV) intervening sequence mutation perturb SUP4 gene transcription by causing premature transcription termination. Both mutations create new T clusters within the tRNA gene that resemble the T tract which immediately follows the tRNA$^{Tyr}$ coding sequence (Figure 5). The -A36A37 gene generates a major in vitro transcript with approximately 50 nucleotides and the U(IV) gene transcript has approximately 55 nucleotides (Figure 6). These RNAs accumulate with time according to the kinetics expected for primary transcripts and are not degradation products of full-length SUP4 gene transcripts (data not shown). The transcript sizes are consistent with transcription initiating approximately 15 bp upstream from the tRNA$^{Tyr}$ coding sequence and terminating within the new T clusters created by the mutations.

Several other mutations result in slightly shortened SUP4 transcripts as judged by relative mobilities of the transcripts in 7 M urea gels

(Figure 6). The -C(63-67) gene contains a 1 bp deletion and produces a shortened transcript. The U73 mutation causes the T cluster following the tRNA$^{Tyr}$ coding sequence to begin one nucleotide earlier and induces slightly premature transcription termination. All four mutations that alter base pairs encoding the tRNA TΨ stem (A51, U51, A52, and C62) also result in faster migrating transcripts; which could be due to an altered conformation.

We previously reported that the U15 and U21 mutant genes yield aberrant transcripts in Xenopus RNA polymerase III in vitro transcription reactions (1). This result has since been found to be due to a cloning artifact. Because of partial HindIII digestion during cloning, the plasmids bearing the U15 and U21 mutant genes contained an extra DNA fragment adjoining the sup4 locus in the yeast genome. This fragment was subsequently found to contain a tRNA$^{Arg}$ gene (P. Philippsen, personal communication) and RNA sequence studies indicate that the above mentioned "aberrant" transcripts are derived from this tRNA$^{Arg}$ gene. The U15 and U21 alleles, when tested alone, were transcribed normally as compared to the wild type SUP4 gene.

DISCUSSION

We developed a yeast RNA polymerase III in vitro transcription system capable of accurate and selective tRNA gene transcription. RNA polymerase III and essential transcription factors were concentrated from whole-cell yeast extracts with a procedure that partially removes interfering enzyme activities. The yeast in vitro transcription system was used to identify DNA sequences that control yeast RNA polymerase III transcription of the yeast SUP4 tRNA$^{Tyr}$ gene. Forty-two cloned sup4 genes with different sequence alterations were tested in the homologous transcription system. Eight mutant sup4 genes were constructed in vitro by deleting sequences from the 5' and 3' ends of the gene and substituting unrelated yeast DNA. The other 34 sup4 genes contained spontaneous mutations that inactivated the gene in vivo.

In vitro transcription experiments with the sup4 genes containing 5'- or 3'-terminal sequence substitutions established that the sequences flanking the tRNA coding sequences are not essential for selective transcription of the tRNA gene in vitro. The mutant sup4 gene that lacks all but 2 bp of 5'-flanking sequence still contains promoter sequences that direct yeast RNA polymerase III transcription initiation approximately 15 bp upstream. A more extensive deletion that also removes the first nine tRNA coding nucleotides inactivates transcription in vitro. In contrast,

neither the last six 3'-terminal coding nucleotides nor the 3'-flanking sequences are required for transcription initiation. These deletion experiments demonstrate that DNA sequences in the -2 to +83 region of the SUP4 gene promote RNA polymerase III transcription. The intragenic location of the SUP4 promoter is consistent with our inability to isolate spontaneous loss-of-suppression mutations in the DNA sequences flanking the SUP4 tRNA$^{Tyr}$ coding sequences and it also correlates with the absence of strong sequence homologies upstream from the eight different yeast tRNA$^{Tyr}$ genes (20, S. Goh, personal communication).

Many different sequence variations are tolerated within the SUP4 transcription control region by the yeast RNA polymerase III transcription initiation complex. Thirty-four different sup4 genes with spontaneous mutations were tested in vitro, and only two mutations (G56 and U56) significantly affected transcription initiation under our assay conditions. The G56 and U56 mutations implicate the highly conserved TΨ region of tRNA genes in RNA polymerase III transcription initiation. Both mutations alter the invariant C of the conserved GTΨCGA tRNA sequence. This C is invariant in prokaryotic as well as eukaryotic tRNAs (34), which suggests that the yeast RNA polymerase III transcription initiation complex recognizes at least one of the tRNA gene base pairs that are conserved for tRNA biosynthesis and function, and utilizes it as a transcription initiation signal.

The SUP4 gene promoter resembles RNA polymerase III transcription control regions defined for Xenopus tRNA$^{Met}$ and tRNA$^{Leu}$ genes, a Drosophila tRNA$^{Arg}$ gene, a Caenorhabditis elegans tRNA$^{Pro}$ gene, and for the adenovirus VA RNA I gene (4-10). The RNA polymerase III promoter in each case is located within coding sequences approximately 10 to 80 bp downstream from the transcription start site. The essential control regions within the tRNA genes include the TΨ region of each gene. DNA sequences homologous to tRNA gene TΨ regions are found in other eukaryotic genes transcribed by RNA polymerase III, including the adenovirus VA RNA I and VA RNA II genes, the rat and mouse 4.5 S RNA genes, and the Epstein-Barr virus EBER 1 and EBER 2 genes (10, 35). The Xenopus borealis somatic 5S rRNA gene also contains an intragenic promoter that directs RNA polymerase III transcription (2, 3). However, 5S rRNA gene transcription has different transcription factor requirements than tRNA gene transcription (11). Each of these genes also contains numerous palindromic regions within the coding sequences, which could act in concert with the intragenic nucleotide sequences in directing RNA polymerase III transcription initiation (36).

The SUP4 5'-flanking sequences are apparently neutral with respect to modulating yeast RNA polymerase III transcription efficiency. When the 5'-flanking sequences are deleted and substituted with other DNA sequences, SUP4 gene transcription is neither consistently reduced nor enhanced as observed for other rearranged tRNA genes (37-39). Yeast CEN3 sequences (18) exert a negative influence on SUP4 gene expression in vitro and in vivo when ligated 15 or 27 bp upstream from the tRNA coding sequences, but have little or no effect on transcription when ligated to the -2 or -72 positions. The reduced expression of the SUP4 gene could be due to place-ment of GC-rich CEN3-BamHI linker sequences at or near the transcription initiation site. Alternatively, the centromere sequence inserted upstream from the SUP4 gene may exert a position effect upon tRNA$^{Tyr}$ transcription.

The mutant sup4 genes also establish that yeast RNA polymerase III, like Xenopus RNA polymerase III (40), recognizes clusters of four or more consecutive Ts in the noncoding strand as transcription termination signals. The U(IV) and -A36A37 genes create $T_5$ and $T_6$ clusters within the SUP4 gene and cause premature transcription termination. Although a new $T_4$ cluster generated by mutation U21 does not terminate transcription, a similar $T_4$ tract in the fused sup4-cyc1 gene apparently functions as a par-tially efficient terminator.

In summary, the yeast RNA polymerase III transcription initiation and termination signals that we have identified to date closely resemble DNA sequences that direct HeLa cell and Xenopus RNA polymerase III function. DNA sequences within the SUP4 gene tRNA$^{Tyr}$ coding region contain a promoter that directs upstream transcription initiation by yeast RNA polymerase III. The nearly identical responses of yeast and Xenopus RNA polymerase III to the sup4 mutations (1) demonstrate that essential features of RNA polymerase III promoter and terminator signals have been conserved throughout the course of eukaryotic evolution.

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