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TAP1, a Yeast Gene That Activates the Expression of a tRNA Gene with a Defective Internal Promoter

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We developed a genetic selection system based on nonsense suppression in *Saccharomyces cerevisiae* to identify mutations in proteins involved in transcription initiation by RNA polymerase III. A *SUP4* tRNA^{Tyr} internal promoter mutation (*A53T61*) that was unable to suppress ochre mutations in vivo and was incapable of binding TFIIIC in vitro was used as the target for selection of *trans*-acting compensatory mutations. We identified two such mutations in the same gene, which we named *TAP1* (for transcription activation protein). The level of the *SUP4A53T61* transcript was threefold higher in the *tap1-1* mutant than in the wild type. The *tap1-1* mutant strain was also temperature sensitive for growth. The thermosensitive character cosegregated with the restorer of suppression activity, as shown by meiotic linkage analysis and coreversion of the two traits. At 1 to 2 h after a shift to the restrictive temperature, RNA synthesis was strongly inhibited in the *tap1-1* mutant, preceding any effect upon protein synthesis or growth. A marked decrease in tRNA and 5S rRNA synthesis was seen, and shortly after that, rRNA synthesis was inhibited. By complementation of the ts$^-$ growth defect, we cloned the wild-type *TAP1* gene. It is essential for yeast growth. We show in the accompanying report (T. L. Aldrich, G. Di Segni, B. L. McConaughy, N. J. Keen, S. Whelen, and B. D. Hall, Mol. Cell. Biol. 13:3434–3444, 1993) that *TAP1* is identical to *RAT1*, a yeast gene implicated in poly(A)$^+$ RNA export and that the *TAP1/RAT1* gene product has extensive sequence similarity to the protein encoded by another yeast gene (variously named *DST2*, *KEM1*, *RAR5*, *SEP1*, or *XRN1*) having exonuclease and DNA strand transfer activity (reviewed by Kearsey and Kipling [Trends Cell Biol. 1:110–112, 1991]).

The first step toward forming stable preinitiation complexes for transcription by eukaryotic RNA polymerase III (PolIII) is initiation factor binding by short sequence motifs within the transcribed region of the gene (10, 23, 35). For tRNA genes, sequences within the dhU arm (box A) and the TΨ arm (box B) are covered and protected against both nuclease action and chemical probes when the gene is combined with PolIII transcription factor TFIIIC (10, 35). Point mutations within the B box profoundly affect template activity (11, 20, 29), decrease the ability of the tRNA gene to competitively block transcription of other genes (2), and greatly lower the binding affinity of the tRNA gene for TFIIIC (6). For the mutation that changes C to G at position 56 in the B box of a yeast tRNA^{Tyr} gene, this change amounts to a 400-fold decrease in the binding affinity for TFIIIC (6).

Following TFIIIC binding, the subsequent events that lead to tRNA transcription initiation are not precisely defined. Factor TFIIIB, a complex mixture of proteins, is also required for transcription initiation (11, 32). Compared with transfer DNA (tDNA) complexes of TFIIIC alone, the TFIIIB-TFIIIC-tDNA ternary complex is more resistant to dissociation (7) and has an extended region of DNase protection that includes sequences in the 5′-flanking DNA (18). Studies done by sequential addition of transcription factors indicate that TFIIIB recognizes an existing TFIIIC-tDNA complex and binds to it through specific protein-protein, protein-DNA, or both types of contacts (18). While studies of the control sites for tRNA transcription initiation by RNA PolIII have made use of genetic selection techniques (16, 22, 33), the proteins that interact with these sites have been studied exclusively by biochemical approaches. From highly purified yeast TFIIIC, partial protein sequences have been deduced for three subunits and the corresponding genes have been cloned (24, 24a, 28, 36).

We report here the results of a genetic screen for mutations that alter proteins involved in PolIII transcription initiation. The concept we applied is that of compensatory changes. A suppressor tRNA target gene crippled in its transcription by an internal promoter mutation was transformed into yeast cells. We mutagenized the transformant yeast strain and then selected for transcription of the inactivated tRNA gene. Among the resulting mutant clones are expected to be yeast cells with heritable changes in genes for the protein factors involved in PolIII transcription initiation. The compensatory mutation can (i) activate transcription of the target gene by altering a protein bound to the defective site, or (ii) if a multiprotein interaction is involved, it can modify a coupled step in transcription initiation. By a similar approach, with a dimeric tRNA gene with a box A defect as the target gene, the *PCF1* gene was identified by Willis et al. (37, 38).

We considered as potential target genes the subset of suppressor-inactivating mutations of yeast *SUP4* tRNA^{Tyr} that greatly reduce transcription of the gene in vitro (2, 20). Several mutations in box B of this gene abolish in vivo suppression of ochre alleles, drastically lower in vitro transcription by PolIII, and severely affect the ability of TFIIIC to bind to the gene (2, 6, 22). If the reduced suppression by these *SUP4* alleles were caused only by diminished TFIIIC binding, then mutations affecting the B-box-binding domain

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of TFIIIC (25) might be able to compensate the transcription defect and restore ochre suppression. However, because of the intragenic location of the B box, the tRNA molecules transcribed from such mutant *SUP4* target genes would carry single-base substitution mutations within the TΨ arm of the molecule, a region essential for both proper tRNA conformation and function in translation. Therefore, one cannot be sure a priori that the tRNA molecules made in such a mutant strain will be biologically active.

To determine, for a given *SUP4* mutant allele, whether the control site mutation also abolished RNA function (undermining the phenotypic basis for our selection procedure), we tested a number of candidate target alleles for residual ochre suppression when on a high-copy-number plasmid. We reasoned that a mutant target gene capable of encoding active tRNA molecules might, through a very weak template activity, provide ochre suppression when present at a high gene dosage. Conversely, candidate target alleles that fail to give suppression at a high copy number would be judged unsuitable for use in selecting compensating mutations. When tested in this way, all existing strong B-block mutations (2) failed to suppress in a high copy number. Believing this to be a consequence of the effects of these mutations (*A54*, *G56*, and *C57*) in the TΨ loop upon tRNA conformation, we then designed and created a new mutant allele. The double mutation *A53T61* affects two highly conserved bases that are on the outer edges of the box B control region but retains complementary base pairing (A-U pair) at the base of the TΨ stem in $tRNA^{Tyr}$. When subjected to the high-copy-number test, the *SUP4A53T61* allele did indeed suppress. It failed to suppress when introduced into *S. cerevisiae* on a *CEN*-containing plasmid vector and showed reduced competitive ability when transcribed in vitro. With *SUP4A53T61* as the target gene, we identified two allelic *trans*-acting compensatory mutants. We describe here the isolation and characterization of one of these mutant strains and the molecular cloning of the gene in which the mutation is located.

MATERIALS AND METHODS

Media and strains. The yeast media used were those described by Sherman et al. (34), except for 5-fluoro-orotic acid plates, which were prepared as described by Boeke et al. (8). Strains R3-6 (*MATα ade2-1$_o$ lys2-1$_o$ met4-1$_o$ leu2-3,112*) and RS5 (*MATα ade2-1$_o$ lys2-1$_o$ met4-1$_o$ can1-100$_o$ trp1-1 ura3-1*) were used for transformation with plasmids pSI4 and pTC3, respectively. Strain GDS1-25A (*MATα ade2-1$_o$ lys2-1$_o$ met4-1$_o$ can1-100$_o$ leu1-12 trp1-1 ura3-1 TAP1$^+$*) was used for mutant selection. Strain PJ17-1A was as described in reference 17. Diploid strain GDS5 (*MAT***a**/*MATα leu2-1$_u$*/+ *leu1-12*/+ [*trp1-1 ura3-1 ade2-1$_o$ lys2-1$_o$ met4-1$_o$ can1-100$_o$*]) was used for gene disruption and tetrad analysis.

Plasmids. Recombinant plasmids containing the *SUP4$_o$* gene and its mutant derivatives *A54*, *G56*, and *C57* were constructed as described by Allison et al. (2). *SUP4A53T61* was produced by oligonucleotide-directed in vitro mutagenesis by the method of Kunkel (21). Plasmid vectors pTC3 and pSI4 are described in references 9 and 33, respectively. For mutant selection, we utilized the YCp50 vector (30) carrying two copies, in the same orientation, of a 266-bp *Bam*HI fragment (6) containing *SUP4A53T61*; the tandem is located inside the *MFα1* gene (in the opposite direction), into which a *Bam* linker was introduced 25 bp downstream of the AUG codon (the *MFα1*-carrying plasmid was kindly provided by Janet Kurjan). Plasmids containing *SUP4G37* and *SUP4U*(IV) are described by James and Hall (17). A plasmid containing $tRNA_3^{Leu}$ (7) was used for in vitro transcription competition.

Genetic techniques. Yeast mating, sporulation, and tetrad analysis were done as described previously (34). In vivo suppression was scored by first patching *S. cerevisiae* onto YEPD (1% yeast extract, 2% peptone, 2% dextrose) or selective medium, replica plating it after 1 day of growth to appropriate synthetic dropout media, and incubating it at 30°C for several days. Mutagenesis by UV (354 nm) was done for the time necessary to cause 50 to 75% lethality (30 to 60 s). One-step gene replacement was done as described by Rothstein (31).

Yeast extract preparation and in vitro transcription assays. Cell extracts for transcription assays were prepared and fractionated as previously described (2, 17). Transcription reactions were incubated at 25°C for 30 min in a final volume of 20 μl and included final concentrations of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–KOH (pH 7.9); 100 mM KCl; 5 mM $MgCl_2$; 5 mM dithiothreitol; 5% (vol/vol) glycerol; 0.5 mM each ATP, CTP, and GTP; 0.05 mM [α-^{32}P]UTP (5 Ci/mmol); 1 μl of a yeast cell extract; pBR322 DNA at 7 μg/ml; and other DNAs as specified in the legend to Fig. 1.

TFIIIC-binding assays. Gel retardation assays to determine TFIIIC-tDNA binding were performed essentially as previously described (5, 6). Binding experiments were done with TFIIIC fractions purified by chromatography on DEAE-Sephadex. TFIIIC-binding competition reactions contained 2 ng of a ^{32}P-labeled 260-bp *SUP4* DNA fragment, 0 to 2.5 μg of unlabeled *SUP4* or *SUP4A53T61* DNA as a competitor, 100 μg of poly(dI-dC) per ml, and 2 μl of TFIIIC from mutant 8-14 or 3 μl of TFIIIC from the wild-type strain. In some experiments, vector (pBR322 or pUC18) DNA was added to keep the total amount of unlabeled plasmid DNA at 2.5 μg for each point.

In vivo labeling of RNA and proteins. Yeast cultures were grown at 30 or 38°C in 100 ml of dropout broth containing 12 μg of uracil per ml. [^{3}H]uridine and [^{35}S]methionine were each added at a final concentration of 1 μCi/ml. One-milliliter aliquots were precipitated with 4 ml of 10% trichloroacetic acid (TCA), and cells were collected by filtration through type HA Millipore filters. The filters were washed consecutively with 5% TCA and ethanol and then dried. The dried filters were immersed in scintillation fluid for counting. In pulse-labeling of RNA, 10-ml aliquots were labeled with [^{3}H]uridine at a final concentration of 10 μCi/ml for 30-min periods. Small RNA was isolated by phenol extraction at 60°C in 10 mM Tris (pH 7.5)–10 mM EDTA–0.5% sodium dodecyl sulfate for 2 h, ethanol precipitated, and analyzed by electrophoresis on a 7 M urea–10% acrylamide gel. After staining with ethidium bromide, fluorography was performed by treating the gel with the reagent Amplify (Amersham), drying it, and exposing it to preflashed X-ray films at −80°C.

Northern (RNA) analysis. RNA from yeast cultures was isolated by extraction with phenol and chloroform as previously described (3). After precipitation with ethanol, the RNA was suspended in 8 M urea, separated by electrophoresis on a 4 M urea–10% acrylamide gel, and electrotransferred to a Zeta-Probe membrane (Bio-Rad). The membrane was prehybridized for 9 h at 42°C with 50% formamide–10× Denhardt solution–0.5 mg of salmon sperm carrier DNA per ml–3.3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridization with 30 pmol of a ^{32}P-labeled 20-mer oligonucleotide specifically complementary to *SUP4A53T61* was done overnight at 30°C in 50% formamide–5× Denhardt

TABLE 1. Suppression phenotypes of *SUP4* B-box mutants with low- and high-dosage plasmids[a]

SUP4 B-box mutation	Growth in:					
	Low copy plasmid pTC3			High-copy plasmid pSI4		
	−Ade	−Lys	−Met	−Ade	−Lys	−Met
A54	−	−	−	−	−	±
G56	−	−	−	−	−	+
C57	−	−	−	−	−	±
A53T61	−	−	−	++	++	++

[a] Plasmid pTC3 was transformed in strain RS5, and pSI4 was transformed in strain R3-6. Five transformants of each mutant were patched onto selective plates lacking either tryptophan (pTC3) or leucine (pSI4) and replicated to plates lacking adenine (−Ade), lysine (−Lys), or methionine (−Met). Growth was scored after 2 days at 30°C. ++, growth in 1 day; +, growth in 2 days; ±, poor growth in 2 days; −, no growth.

solution–0.4 mg of carrier DNA per ml–4× SSC. The membrane was washed four times at 0°C in 6× SSC for 4 min each time and then washed for 1 min at 52°C in 6× SSC. The temperature of 52°C for the stringent wash was based on the following calculation: 2°C for each AT base pair, 4°C for each GC base pair, and subtraction of 6°C from the total. The filter was exposed to X-ray films at −50°C with an intensifying screen. The oligonucleotide was labeled by [γ-^{32}P]ATP and polynucleotide kinase and purified by chromatography on Sephadex G-50.

RESULTS

High-copy-number test of defective *SUP4* alleles. To identify a suitable target *SUP4* allele, several of the existing *SUP4* alleles with defective B-box sequences (2) were tested for the ability to confer in vivo ochre suppression when present at a high copy number. These included *A54*, *G56*, and *C57*, as well as newly created *SUP4A53T61*. Each was tested at a low tRNA gene dosage (*CEN*-carrying plasmid vector pTC3) and at a very high dosage (estimated at 100 to 400 copies per yeast cell) with 2μm-carrying plasmid vector pSI4 (9). The recipient yeast strain (Table 1) contained ochre alleles *met4-1*, *lys2-1*, *ade2-1*, and *can1-100*. Of the four *SUP4* alleles tested at high and low copy numbers, only the *A53T61* mutant gene gave suppression at a high copy number (Table 1). These data permit the positive conclusion that the *A53T61* transcript, if made, is competent for subsequent function in translation. They suggest that the primary defect in *A53T61* function is the inability of this gene to make enough functional suppressor tRNA when present at a low copy number. The negative results obtained with the *A54*, *G56*, and *C57* mutations do not necessarily imply that their tRNAs are nonfunctional; however, lacking positive evidence for high-copy-number suppression, we discarded these alleles as potential target genes for the selection.

In vitro transcription competition and TFIIIC binding by the *SUP4A53T61* gene. The *SUP4A53T61* and *SUP4$_o$* genes have comparable template activities for in vitro transcription (Fig. 1, lanes 2 and 3). On the other hand, when transcription is carried out under conditions in which two genes compete for limiting components in the extract (2, 39), the *A53T61* and *SUP4$_o$* templates differ greatly in competitive ability. With the tRNA$_3^{Leu}$ gene as a reference template, an equal amount of *SUP4$_o$* DNA and double the amount of *SUP4$_o$* DNA both reduced tDNA$_3^{Leu}$ transcription markedly and proportionately. In contrast, equal amounts and double amounts of *A53T61* DNA produced little competitive effect

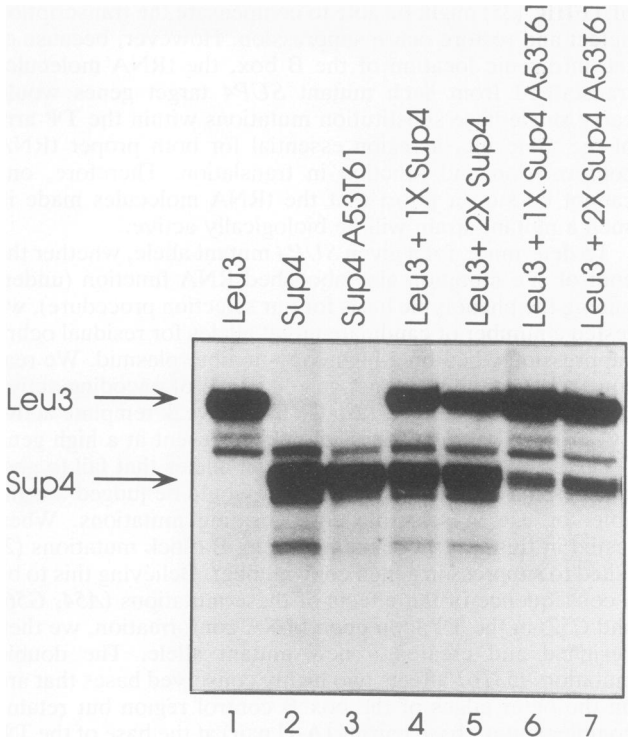

FIG. 1. In vitro transcription competition by *SUP4A53T61*. Plasmid DNAs containing the genes indicated at the top were transcribed with a crude yeast cell extract as described in Materials and Methods. RNA products were resolved by electrophoresis and visualized by autoradiography. In template activity experiments (lanes 1 to 3), DNAs were present at 3 μg/ml. In competition experiments (lanes 4 to 7), the reference gene tRNA$_3^{Leu}$ was present at 3 μg/ml and the *SUP4* and *SUP4A53T61* DNAs were present at 3 (1×) or 6 (2×) μg/ml.

upon transcription of the tRNA$_3^{Leu}$ gene (Fig. 1, lanes 6 and 7). While the latter result shows that the *SUP4A53T61* gene has a decreased affinity for some component of the PolIII transcription system, it leaves open the question of which component it is.

Because the *SUP4A53T61* mutant sites lie within the B box, a region strongly implicated in TFIIIC binding (5, 6), we compared the *SUP4A53T61* and *SUP4$_o$* alleles in this regard. As shown below (see Fig. 7), the binding affinity of *A53T61* for TFIIIC is markedly lower than that of a *SUP4* gene having the wild-type sequence in box B.

Isolation of mutants compensating the defect in the B box of the *SUP4A53T61* tRNA gene. To select for mutants able to rescue the defectiveness of the *A53T61* B-box mutation of *SUP4*, haploid strain GDS1-25A, bearing the ochre suppressible markers *ade2-1*, *lys2-1*, and *met4-1*, as well as two copies of the *SUP4A53T61* tRNA gene in centromere-based plasmid YCp50, was mutagenized with UV light and plated onto medium lacking methionine. From 5×10^7 cells plated after the mutagenesis, 3,500 colonies grew. To discriminate between *MET4*$^+$ revertants and nonsense suppressor mutants, the Met$^+$ colonies were replica plated onto plates lacking adenine or lysine: 410 mutant colonies grew on these plates, and they were therefore considered to be suppressor mutants.

Suppressor mutants might be expected to arise from a new *trans*-acting mutation that compensates *A53T61* (the desired

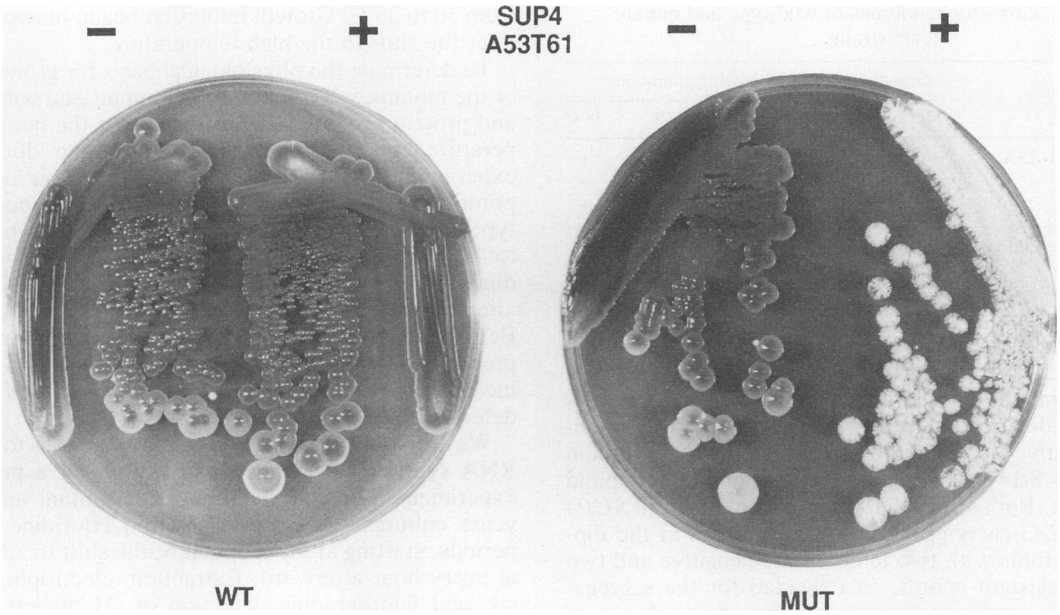

FIG. 2. Suppression ability of *SUP4A53T61* in mutant (MUT) and wild-type (WT) strains. Wild-type strain GDS1-25A and isogenic mutant strain 8-14, with (+) or without (−) the YCp50-*SUP4A53T61* plasmid, were plated on YEPD plates. Redness indicates no suppression, whereas whiteness indicates suppression ability. Red sectors in mutant white colonies derive from occasional mitotic loss of the plasmid.

class), from a mutation in one of the chromosomal tRNA genes, or from reversion or pseudoreversion of the *A53T61* mutation in the *SUP4* gene present in plasmid YCp50. Because of the relatively high number of tRNA genes in the yeast genome capable of mutating to nonsense suppressors, we utilized a quick and sensitive method to distinguish between chromosomal suppressor mutants (expected to be the majority class) and those specifically dependent on the YCp50-*SUP4A53T61* plasmid. The yeast strain we utilized bears the *ade2-1* ochre mutation, which causes the colonies to be red; *ADE2*$^+$ yeast strains and the suppressed *ade2-1* mutant are white or pink, depending on the level of suppression (26). Because the *CEN*-carrying plasmid is not completely stable, a suppressor mutant dependent upon a plasmid (for either a *cis* mutation in the plasmid itself or a *trans* mutation acting on the plasmid) forms a white (or pink) colony with red sectors, resulting from occasional mitotic loss of the plasmid, whereas a chromosomal suppressor mutation results in an unsectored white (or pink) colony. By testing putative suppressor mutant clones in this way, we found 25 (of 410 tested) that formed sectored colonies. The suppression activity of these 25 mutants was therefore considered to be plasmid dependent.

To eliminate revertants of the *A53T61* mutation or other *cis* mutations conferring suppression, we cured the mutants of the plasmid (bearing the *URA3* marker) by growing them on a plate containing 5-fluoro-orotic acid, which selects for cells that have lost the *URA3* gene (8). All of the cured mutants lost the ability to suppress. Each cured mutant clone was retransformed with a bona fide *A53T61*-carrying plasmid and then checked for ochre suppression. Only two of the mutant class, 8-14 and 10-3, regained suppression as a result of transformation by *SUP4A53T61*.

Figure 2 shows mutant 8-14 and the isogenic wild-type strain, both with and without the *A53T61*-carrying plasmid: the phenotype of the wild-type colony was red (i.e., not suppressed), even in the presence of the *A53T61*-carrying plasmid, indicating that the *SUP4A53T61* gene was not expressed, whereas mutant 8-14 was red in the absence of the plasmid but white-pink with red sectors in the presence of the A53T61 plasmid. Mutant 8-14 gained the ability to express the *SUP4A53T61* allele at a level sufficient to suppress *ade2-1*. Similar results were obtained with mutant 10-3.

To test the degree of allele specificity of the mutation in strain 8-14, we cured it of the *SUP4A53T61*-bearing plasmid and then introduced, separately, plasmids bearing *SUP4* genes with other second-site mutations that lower the level of ochre suppression. These were *SUP4G37*, carrying a mutation partially blocking intron processing (12), and *SUP4U*(IV), with a mutation that causes most transcripts to terminate prematurely (17, 20, 22). Each of these mutations was weakly compensated by the mutation in strain 8-14, but to a lesser extent relative to the *SUP4A53T61* allele. From these data, it appears that the ability of the mutation to increase *SUP4* expression is not limited to alleles in which the box B internal promoter is defective.

Mutant 8-14 was also transformed with integrating plasmid YIP-*SUP4A53T61* (see the accompanying report): in this case, too, mutant 8-14 was able to activate expression of *SUP4A53T61*.

Mutant 8-14 is temperature sensitive. To characterize the efficiency with which *A53T61* suppression is restored in mutant strains 8-14 and 10-3, we tested each of them on selective media requiring the function (separately) of the *met4-1*, *lys2-1*, and *ade2-1* ochre alleles. The level of suppression of each of these markers required for prototrophic growth is lowest for *met4-1* and highest for *ade2-1* (15). Mutant 8-14 grew well on plates lacking methionine or lysine and poorly on plates without adenine, indicating good suppression of *met4-1* and *lys2-1* and slight suppression of *ade2-1*. Mutant 10-3 grew well on plates without methionine and poorly on plates lacking lysine; it failed to grow on plates lacking adenine.

TABLE 2. Growth phenotypes of wild-type and mutant yeast strains

Strain	Growth under the following conditions[a]:				
	−Met	−Lys	−Ade	37°C	38°C
Wild-type GDS1-25A	−	−	−	+	+
Mutant 8-14	+	+	±	±	−
Mutant 10-3	+	±	−	+	±

[a] −Met, minus methionine; −Lys, minus lysine; −Ade, minus adenine.

When the ability to grow vegetatively at 38°C on complete medium was checked, mutant 8-14 was defective compared with its parent strain, while mutant 10-3 grew normally (Table 2). To determine the relationship between this temperature-sensitive (ts^-) growth phenotype and suppression ability, strain 8-14 was crossed with nonmutant haploid strain PJ17-1A. Both strains harbored plasmid YCp50-*SUP4 A53T61*, a *URA3*-carrying plasmid. Sporulation of the diploid yielded tetrads with two temperature-sensitive and two temperature-resistant spores, as expected for the segregation of a single nuclear ts^- mutation. Because ochre suppression is dependent on the presence of the plasmid, and meiotic segregation of a *CEN* plasmid is frequently imperfect, 2:2 segregation for suppression was not observed in all of the tetrads analyzed; nevertheless, all of the 43 spores (of 36 tetrads) that were suppression competent (and Ura^+) failed to grow at the high temperature; on the other hand, no segregant was found to be temperature resistant but suppression competent (Table 3). These data strongly suggest that temperature sensitivity for growth was caused by the same mutant lesion that activated suppression by the *A53T61* allele. This conclusion is supported by the nature of a temperature-resistant revertant from mutant 8-14. This revertant, in addition to becoming insensitive to high temperature, lost the ability to suppress (Fig. 3). The phenotype shown by the diploid heterozygous for the 8-14 mutant allele (Fig. 3, top center patches) illustrates that both the ts^- growth phenotype and suppressor activation are recessive traits. Complementation tests between mutants 8-14 and 10-3 showed that they are allelic to each other.

Mutant 8-14 is also cold sensitive, growing much slower than the wild-type strain at 14°C.

Physiological characterization of the ts mutant. The growth rates of mutant 8-14 at permissive and nonpermissive temperatures were determined. Figure 4A shows a growth curve for the mutant and wild-type strains after a temperature shift from 30 to 38°C. Growth inhibition began between 1 and 2 h after the shift to the high temperature.

To determine the physiological basis for growth inhibition of the mutant, we compared the mutant and wild-type RNA and protein accumulation rates both at the permissive temperature and after a shift to 38°C in a double-labeling experiment (Fig. 4). While the rate of [^{35}S]methionine incorporation into protein (Fig. 4B) increased in both the wild-type and mutant strains and was still linear in the mutant up to 4.5 h after the shift, incorporation of exogenous [^{3}H]uridine (Fig. 4C) in the mutant showed marked inhibition at 1 h after the shift and was almost totally blocked after 4.5 h. Because the cessation of isotope incorporation into RNA precedes both the effect upon growth and [^{35}S]methionine incorporation, we discount the possibility of a primary defect in protein synthesis in this mutant.

We used polyacrylamide gel electrophoresis to analyze the RNA synthesized at high temperature by a pulse-labeling experiment (Fig. 5). Aliquots of the mutant and wild-type yeast cultures were labeled with [^{3}H]uridine for 30-min periods, starting at the moment of the shift to 38°C and then at every hour afterward. Extraction, electrophoretic analysis, and fluorographic detection of ^{3}H-labeled RNA were performed as described in Materials and Methods. The amounts of total RNA present, as shown by ethidium bromide staining (Fig. 5A), showed an increase in small RNA in the wild type and a block in the accumulation of RNA in the mutant at 1 to 2 h after the shift. The fluorograph (Fig. 5B) shows that RNA synthesis during each 30-min pulse proceeded normally in the wild type following recovery from the heat shock, while in the mutant strain there was a marked decrease in the synthesis of tRNA and 5S rRNA from 2 h onward. Shortly after that, there was a very marked inhibition of the three RNA bands (5.8S, 18S, and 25S) that are processed from the PolI pre-rRNA transcript. This inhibition, like that of tRNA and 5S rRNA synthesis, was essentially complete.

Synthesis of *SUP4A53T61* tRNA is increased in the *tap1-1* mutant. The experiments described above established a relationship between the ts^- growth phenotype of mutant strain 8-14 and the in vivo synthesis of bulk tRNA, 5S rRNA, and eventually rRNA. Under temperature shift conditions, the reduction in synthesis of these RNAs preceded any large effect upon protein synthesis. In line with these findings, we will use the name *tap1-1* (transcription activation protein) henceforth to designate the mutant gene in this strain.

In view of the selection procedure used to isolate the *tap1-1* mutant allele and the temperature shift results mentioned above, it is to be expected that activation of the *SUP4A53T61* ochre suppressor allele by *tap1-1* would occur by an increase in transcription or in RNA stability rather than by a heightened efficiency of suppression by an unchanged level of *A53T61* $tRNA^{Tyr}$. To test this prediction, the levels of *A53T61* $tRNA^{Tyr}$ transcribed in vivo from plasmid-encoded tRNA genes were compared for the mutant and wild-type strains. An oligonucleotide probe exactly complementary to the *SUP4A53T61* $tRNA^{Tyr}$ (and not to the other seven yeast $tRNA^{Tyr}$ genes) was used in Northern hybridization analysis of in vivo-made RNAs (Fig. 6). Because this synthetic 20-mer is noncomplementary to wild-type tyrosine tRNA at positions 53 and 61, it should hybridize preferentially to *SUP4A53T61* $tRNA^{Tyr}$ molecules. As a negative control, we utilized cells carrying plasmid YCp50 without any *SUP4* suppressor tRNA gene in it. The RNA from these cells hybridized only weakly with the probe (Fig. 6, lanes 1 and 4). The positive control consisted of cells

TABLE 3. Suppression ability and temperature sensitivity of mutant 8-14 cosegregate[a]

Segregation of Ura^+	No. of tetrads	No. of tetrads segregating 2 Sup Ts:2 Sup^- Tr	No. of spores Sup Ts	No. of spores Sup Tr
4+:0−	8	8	16	0
3+:1−; 2+:2− 1+:3−; 0+:4−	28	NA	27	0
Total	36	8	43	0

[a] Mutant 8-14 was mated to wild-type strain PJ17-1A. Both strains harbored plasmid YCp50-*A53T61*, a *URA3 CEN*-containing plasmid. The diploid was sporulated and dissected. Sup, suppression ability; Sup^-, lack of suppression; Ts, temperature sensitivity; Tr, temperature resistance; NA, not applicable.

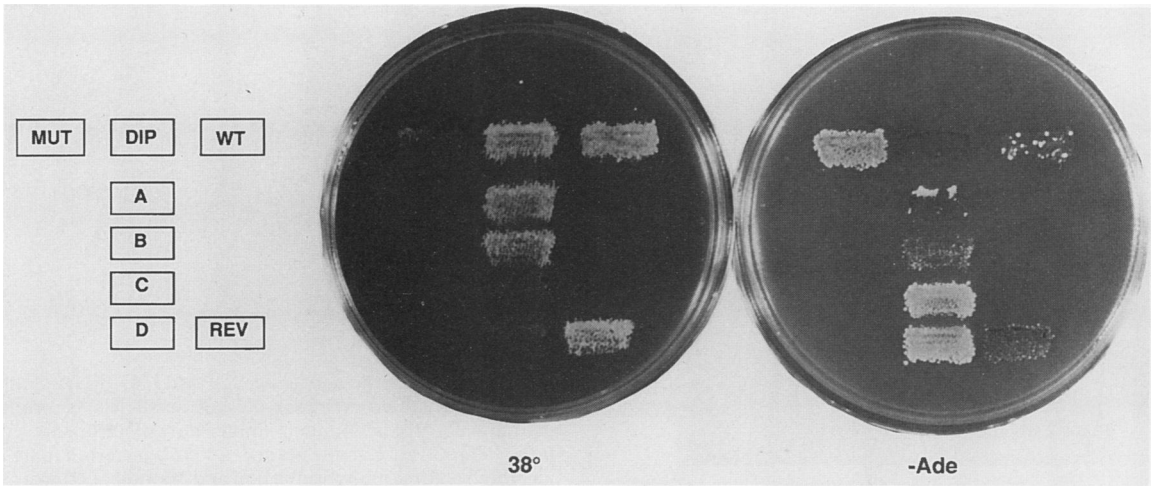

FIG. 3. The ts character is coupled to suppression ability. A plate with patches of wild-type strain PJ17-1A (WT), mutant strain 8-14 (MUT), the diploid derived from mating of the wild-type and mutant strains (DIP), the four spores of a tetrad derived from sporulation of the diploid (A, B, C, and D), and a ts^+ revertant from spore D (REV) was replica plated on a YEPD plate and on a plate lacking adenine (−Ade). The YEPD plate was then incubated at 38°C, whereas the plate without adenine was incubated at 30°C. Growth on a plate without adenine indicated suppression ability.

carrying multicopy plasmid (100 to 400 copies per cell) PSI4-*SUP4A53T61*; as we showed above, this suppressor tRNA gene is functional, even in wild-type cells, when present on a multicopy plasmid. As expected, RNA isolated from cells harboring PSI4-*SUP4A53T61* gave a strong hybridization signal both for the mutant and wild-type yeast strains (Fig. 6, lanes 3 and 6). On the other hand, RNA from cells carrying the *SUP4A53T61* gene on a *CEN* plasmid (one to two copies per cell) would be expected to give a significant increase in hybridization of the 20-mer probe in the mutant (lane 5) compared with the wild-type (lane 2) yeast strain.

Quantitation of the bands by densitometry, including both precursors and mature products, indicates that when a low-copy plasmid is utilized, the amount of *SUP4A53T61* tRNA in the mutant is increased threefold relative to that in wild-type cells carrying the same plasmid. We believe that the lack of a comparable increase in the case of wild-type and mutant cells carrying the PSI4-*A53T61* plasmid is due to phenotypic selection for a lower plasmid copy number in the mutant, because very high levels of ochre-suppressing $tRNA^{Tyr}$ are toxic (15).

The increase in the amount of *SUP4A53T61* $tRNA^{Tyr}$ brought about by the *tap1-1* mutant allele does not derive from a general increase in the transcription of all $tRNA^{Tyr}$

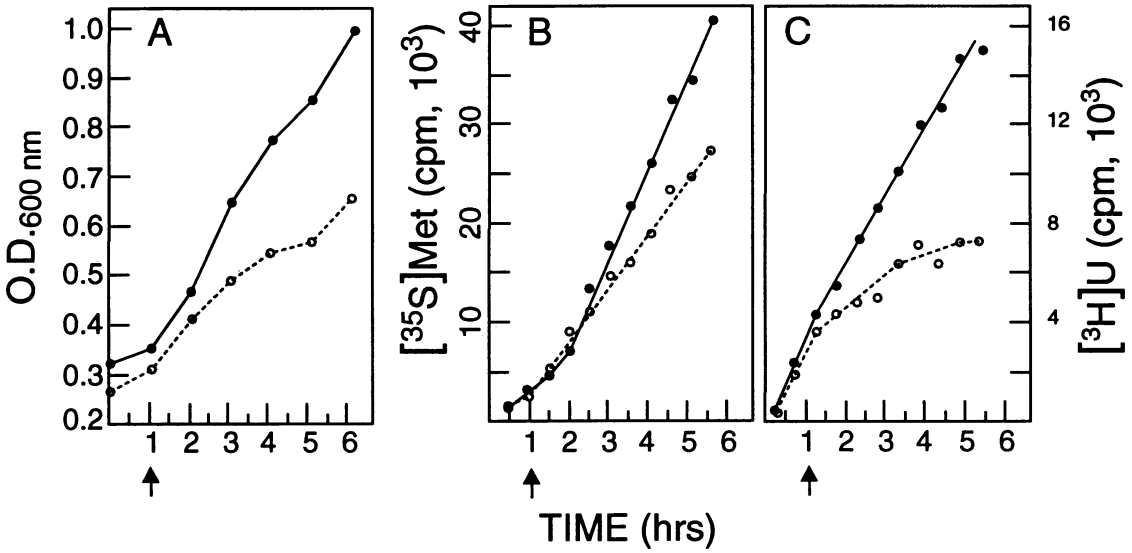

FIG. 4. Physiological characterization of the ts mutant. The wild-type (●) and mutant 8-14 (○) strains were grown for 1 h at 30°C and then shifted to 38°C (arrow). (A) Growth curve determined by measuring the optical density (O.D.) of the culture. Growth of mutant 8-14 completely ceased after 10 to 12 h. (B) Incorporation of [^{35}S]methionine into TCA-precipitable proteins. (C) Incorporation of [^{3}H]uridine into TCA-precipitable RNA. Both radioactive methionine and uridine were already present in medium broth before the shift. One-milliliter samples were taken every 30 min, precipitated with TCA, filtered, and counted with two different energy windows. The protein synthesis curve was still linear 5 h after the temperature shift, whereas RNA synthesis was clearly inhibited 1 to 2 h after the shift.

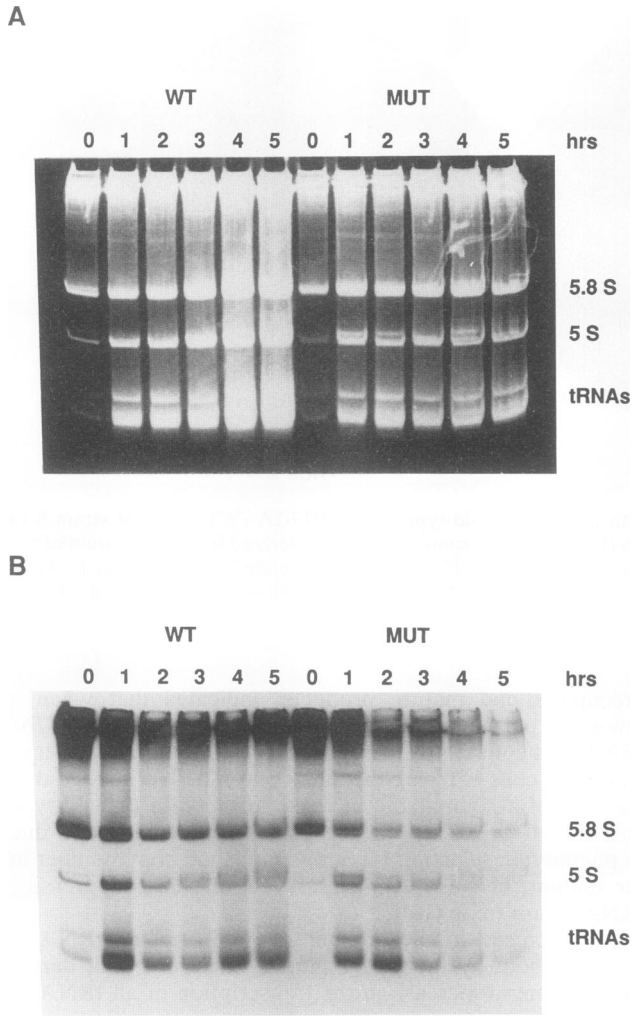

FIG. 5. Analysis of in vivo-labeled RNA at high temperature. Exponential cultures of the wild-type (WT) strain and mutant (MUT) strain 8-14 grown at 30°C were transferred at 38°C (time zero). Ten-milliliter aliquots of the cultures were labeled with [^{3}H]uridine for 30-min periods, starting at the moment of the shift to 38°C and then at every hour thereafter. Small RNA was isolated by phenol extraction and run on a 7 M urea–10% polyacrylamide gel. (A) Ethidium bromide stain of the gel. (B) Fluorograph of the gel.

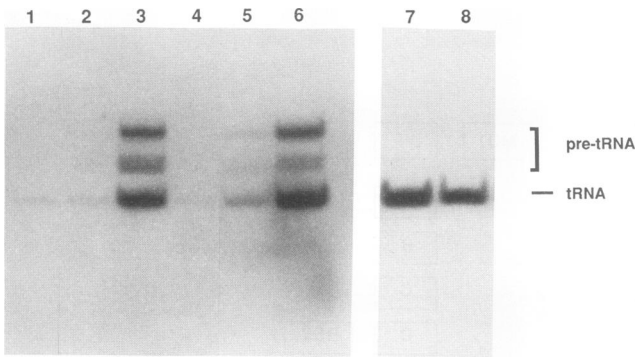

FIG. 6. Northern analysis of *SUP4A53T61* tRNA synthesized in vivo. Twenty-microgram samples of small RNAs synthesized in vivo in wild-type and mutant strains were electrophoresed in a 4 M urea–10% polyacrylamide gel and electrotransferred to a Zeta-Probe membrane. After prehybridization and hybridization to a ^{32}P-labeled 20-mer oligonucleotide specifically complementary to *SUP4A53T61* covering positions 47 to 66, the membrane was washed in stringent conditions (52°C in 6× SSC) and exposed to X-ray film overnight at −50°C with an intensifying screen. Lanes: 1 to 3, wild-type strain PJ17-1A; 4 to 6, mutant strain GDS4-19D (derived from mutant strain 8-14); 1 and 4, RNAs from strains harboring vector YCp50 only; 2 and 5, RNAs from strains with *CEN*-containing plasmid (one to two copies per cell) YCp50-*SUP4A53T61*; 3 and 6, RNAs from strains with multicopy plasmid (100 to 400 copies per cell) PSI4-*SUP4A53T61*. Quantitation of the bands by densitometry, including both precursors and mature products, indicates that the quantity of *SUP4A53T61* tRNA in lane 5 is three times higher than that in lane 2. Lane 7 contained RNA from wild-type strain GDS1-25A, and lane 8 contained RNA from mutant strain 8-14. The RNAs in lanes 7 and 8 were hybridized to wild-type ^{32}P-labeled *SUP4* tRNA.

genes. When a duplicate Northern blot was probed with the entire *SUP4* gene, the amounts of $tRNA^{Tyr}$ that hybridized in corresponding wild-type and mutant transformants were approximately the same (Fig. 6, lanes 7 and 8). While this result argues against a general increase in tRNA level in *tap1-1* cells, it does not exclude the possibility of a general increase in the level of all stable RNAs, including tRNA and rRNA species, in mutant relative to wild-type cells.

The Northern hybridization results also indicate that the pre-tRNA transcribed from *SUP4A53T61* is partially defective in processing; the ratio of precursors to mature $tRNA^{Tyr}$ molecules is higher than that for $SUP4_o$ (Fig. 6; compare lanes 1 to 6 with lanes 7 and 8). However, there was no evident difference in the processibility of pre-tRNA-*A53T61* in mutant versus wild-type yeast cells (compare lanes 3 and 6). The same result was obtained from in vitro transcription experiments of *A53T61* and $SUP4_o$ templates in crude extracts of mutant and wild-type cells at multiple temperatures and salt concentrations, indicating that the *tap1-1* mutation does not affect processing of *SUP4A53T61* pre-$tRNA^{Tyr}$ (data not shown).

Examination of the tDNA-binding properties of TFIIIC in the *tap1-1* mutant strain. To determine whether tDNA binding of PolIII factor TFIIIC was altered in affinity or specificity by the *tap1-1* mutation, we carried out parallel DNA-binding studies with TFIIIC fractions from mutant and wild-type cells. The band shift gel retardation assay was used (5), employing for all data points a constant amount of ^{32}P-labeled $SUP4_o$ DNA. The relative binding abilities of $SUP4_o$ and *SUP4A53T61* DNAs were compared by their respective abilities to prevent the tracer ^{32}P-labeled $SUP4_o$ DNA from binding to TFIIIC (5). With the same input concentration of competing DNA, the $SUP4_o$ gene was far more effective than *SUP4A53T61* DNA at competitively blocking [^{32}P]DNA binding. For example, with an input of 200×10^{-10} M competitor (nucleotide units), there was a sevenfold difference in effect between the two DNAs (Fig. 7). Because this difference was observed irrespectively of whether the TFIIIC was from mutant or wild-type cells, we conclude that the *tap1-1* mutation does not suppress the TFIIIC-binding defect of *SUP4A53T61*.

Not only does TFIIIC from *tap1-1* mutant cells discriminate between $SUP4_o$ and *SUP4A53T61* DNAs in its binding, just as TFIIIC from the wild type does, but the transcription competition behaviors of mutant and wild-type extracts are also the same. When the *SUP4A53T61* gene was used as a competitor against $tRNA_3^{Leu}$ transcription, as in Fig. 1, it competed no better in a *tap1-1* extract than in a *TAP1* extract. In each case (data not shown), the competitive effect of *SUP4A53T61* DNA was measured relative to that of

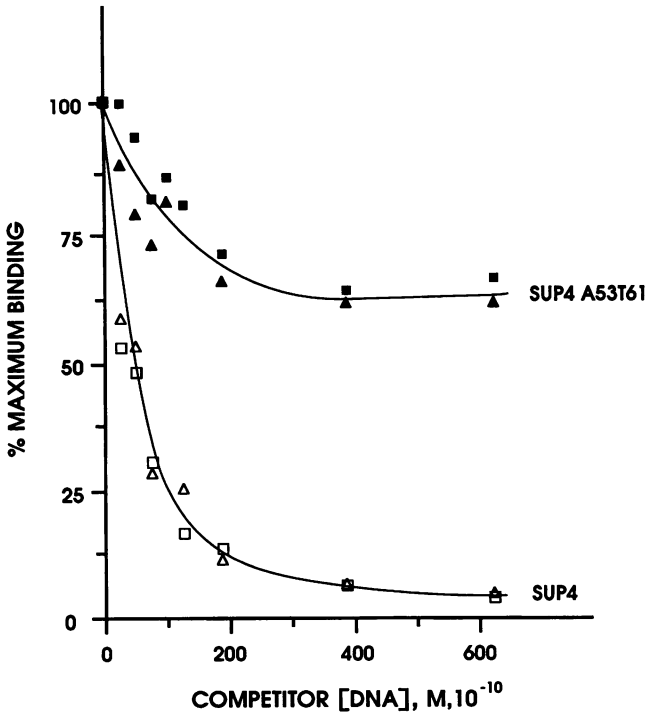

FIG. 7. TFIIIC binding by *SUP4A53T61* in mutant and wild-type strains. TFIIIC binding competition reactions contained 2 ng of a ^{32}P-labeled 260-bp *SUP4* DNA fragment, 0 to 2.5 μg of unlabeled *SUP4* or *SUP4A53T61* DNA as a competitor, 100 μg of poly(dI-dC), and 2 μl of TFIIIC from mutant 8-14 (△ or ▲) or 3 μl of TFIIIC from the wild-type strain (□ or ■) in a final volume of 20 μl.

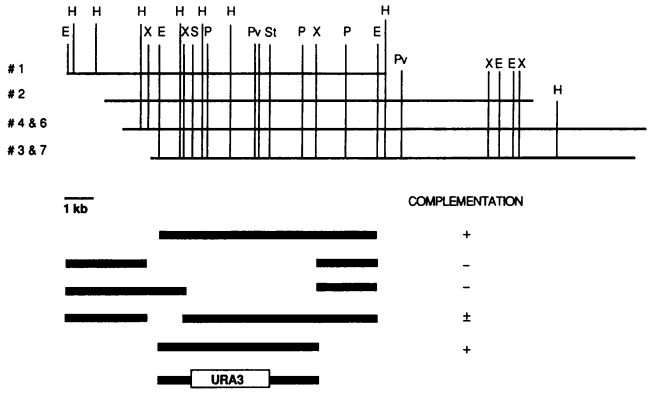

FIG. 8. Restriction map of plasmids that complement the ts lethality of strain 8-14. Mutant strain 8-14 was transformed with a library of wild-type yeast genomic fragments cloned into YCp50, a *URA3 CEN*-carrying plasmid. We screened 1,150 Ura$^+$ transformants for growth at 30 and 38°C, and only 7 were able to grow at the high temperature. Plasmid DNA was recovered from the seven transformants and examined by restriction mapping. Abbreviations: E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; S, *Sal*I; St, *Sst*I; P, *Pst*I; Pv, *Pvu*II. To localize the gene within the 8-kb *Eco*RI-*Eco*RI common fragment, deletions were made (bottom); empty spaces between black bars indicate the deleted parts. Deletion of the *Xba*I-*Xba*I 1,350-bp fragment only partially decreased the complementation of ts lethality. To determine the essentiality of *TAP1*, much of the complementing region (between *Sal*I and *Sst*I) was replaced with the *URA3* gene, as shown at the bottom; the disrupted gene was used to transform diploid yeast cells.

$SUP4_o$ DNA. Both the TFIIIC-binding and the transcription competition results point to the same anomalous feature of the *tap1-1* mutation. The selection scheme that identified it was designed to screen for alterations in the binding properties of TFIIIC and, as a consequence, increased transcription of the *SUP4A53T61* allele. The mutation we obtained strongly increased the level of *A53T61* tRNA in vivo, presumably by increasing its transcription, yet the properties of TFIIIC as regards *A53T61* DNA binding are not altered in the mutant strain. This raises the possibility that the mechanism by which the *tap1-1* mutation compensates for the defective B box of *SUP4A53T61* does not involve TFIIIC directly. To investigate that possibility, we set out to identify the *TAP1* gene product by molecular cloning (see below), DNA sequencing, and protein analysis by immunological cross-reactivity (see the accompanying report).

Molecular cloning of the *TAP1* gene. The wild-type gene allelic to *tap1-1* was cloned by complementation of the temperature-sensitive growth defect. Mutant strain 8-14 was used as the recipient for transformation by a size-selected library of yeast genomic fragments (30) in *CEN URA3*-carrying shuttle vector YCp50. Of 1,150 Ura$^+$ transformants that grew at 30°C, seven were also capable of growth at 38°C. Restriction endonuclease mapping of the plasmid DNA rescued from these seven transformants showed that the inserts fell into four classes and that all overlapped a common region approximately 8 kb long (Fig. 8). An *Eco*RI-*Eco*RI fragment that comprised most of this common region was subcloned into YCp50 and shown to be capable of complementing the *tap1-1* temperature-sensitive growth defect. Removal of the *Xba*I-*Xba*I smaller segment from this large *Eco*RI fragment caused a substantial decrease in its ability to complement *tap1-1*, whereas larger deletions completely inactivated it. The partial ability of the *Xba*I-*Xba*I truncated fragment to complement suggests that the innermost *Xba*I site is at or near one end of the *TAP1* coding region.

To determine whether the ability of the cloned fragment to complement *tap1-1* is truly indicative of allelism between the mutation and the locus of the cloned fragment, homologous integration was performed. *TAP1*-carrying strain GDS1-25A was transformed by an *Xba*I-cut YIp5 integrating plasmid bearing the 8-kb *Eco*RI-*Eco*RI fragment as well as *URA3*. One Ura$^+$ transformant was crossed to a *ura3 tap1-1* strain, and the meiotic products were analyzed. For the 24 tetrads examined, all of the Ura$^+$ spores were able to grow at high temperature, while all of the Ura$^-$ segregants were temperature sensitive, indicating allelism between the *tap1-1* mutation and the chromosomal locus at which *URA3* and the putative *TAP1* gene were integrated.

To determine whether the *TAP1* gene is essential for yeast cell viability, the gene was disrupted by the method of Rothstein (31). The *URA3* gene was inserted between the *Sal*I and *Sst*I sites internal to the *TAP1* coding region (Fig. 8), resulting in deletion of most of the *TAP1* gene. The 4.5-kb *Xba*I fragment was used to transform diploid yeast cells. The transformed diploid was sporulated, and 20 tetrads were analyzed. All of them had two viable and two dead spores. All viable spores had a Ura$^-$ phenotype, showing that the *TAP1* gene is essential for cell viability.

DISCUSSION

In this report, we describe the isolation, by direct selection, of a yeast mutation that promotes in vivo expression of

a defective suppressor tRNA gene. To provide a means for genetic selection of compensatory changes that affect PolIII transcription initiation, we tested several alleles of the *SUP4* $tRNA^{Tyr}$ gene that had in vitro defects in transcription template activity. Among these, only the *A53T61* allele, with two changes in box B of the internal promoter, showed any ability to yield active suppressor tRNA in vivo. The *SUP4A53T61* gene transformed into *S. cerevisiae* very high-copy-number (100 to 400 per cell) vector pSI4 actively suppressed the *ade2-1*, *lys2-1*, and *met4-1* ochre mutations, indicating that its tRNA transcript is capable of undergoing tRNA maturation and subsequent function in translation. On the other hand, when *SUP4A53T61* was present on a low-copy-number vector, it failed to suppress these ochre mutations. We attribute this difference to a low affinity of binding by the *A53T61* gene of some essential component of the PolIII system and to a mass action effect of a high intracellular copy number upon formation of the tDNA-protein complex. The in vitro properties of the *A53T61* template are consistent with these views. When a high concentration of *A53T61* DNA was present in the PolIII system as the sole template, it was transcribed well. However, it was poorly able to compete for one or more of the essential components of the PolIII system when challenged with another tRNA gene ($tRNA_3^{Leu}$) that is strongly recognized by the PolIII system (Fig. 1). Furthermore, in vitro binding studies indicated that TFIIIC was poorly bound by *SUP4A53T61* (Fig. 7).

Mutagenesis of a haploid yeast strain and subsequent selection for ochre suppression yielded a mutant capable of expressing the *SUP4A53T61* allele, even when present on a low-copy-number vector, at a level high enough to give prototrophy for adenine, lysine, and methionine. A single chromosomal mutation brought about this phenotypic change. An accompanying trait, temperature sensitivity for growth at 38°C, was shown by tetrad analysis to be closely linked to the suppression-altering mutation and to corevert with it. By complementation of the ts^- growth defect, we cloned the wild-type *TAP1* gene. Further experiments with cloned *TAP1* DNA have shown unequivocally that the two phenotypes, altered suppression and ts^-, result from the same single-nucleotide change (1).

Northern hybridization analysis of the in vivo tRNA population in the mutant strain gave results consistent with the suppression phenotype; the mutation was accompanied by a large increase in the level of hybridization to an *A53T61*-specific oligonucleotide probe. Thus, we conclude that this mutation, which we have named *tap1-1* for transcription activation protein, increased the level of transcript formation by the target $tRNA^{Tyr}$ gene.

TAP1 was shown by chromosomal disruption to be essential for the growth of yeast cells. An indication of the nature of the essential *TAP1* function was provided by the results of RNA labeling and electrophoretic size analysis of RNA made following a shift from the permissive to the restrictive temperature in a *tap1-1* mutant strain. From 2 h after the temperature shift, tRNA synthesis was strongly inhibited, followed shortly thereafter by decreased formation of 5S rRNA. This progression resembles that seen for ts^- mutant alleles of the core subunits of RNA PolIII (14, 27). A surprising result is the subsequent sharp decline in accumulation of all processed forms of the PolI primary transcript, namely, 5.8S, 18S, and 25S rRNAs. Our interpretation of these results is that *TAP1* plays a role directly in the formation of PolIII transcripts and either directly or indirectly in formation of the rRNAs transcribed by PolI. An indirect mechanism might involve destabilization of nascent, incompletely processed PolI transcripts due to a deficit of 5S rRNA owing to blockage of PolIII transcription.

The enhanced in vivo effectiveness of *SUP4A53T61* as a template in the mutant yeast strain was not reflected in the results of in vitro transcription studies. Neither of two tests of the interaction between *SUP4A53T61* and TFIIIC binding and transcription competition indicated that the *tap1-1* mutation had changed the properties of this transcription factor. The reactivation of in vivo transcription of *SUP4A53T61* by *tap1-1* without a concomitant reversal of the *A53T61* DNA TFIIIC-binding defect suggests the existence of a bypass mechanism whereby a tRNA gene that cannot form a stable complex with TFIIIC can nonetheless be transcribed. In the study described in the accompanying report, which characterizes the *TAP1* gene and describes recombinant fusion proteins between *TAP1* and *Escherichia coli trpE* (1), we tested for immunological cross-reactivity between TAP1 protein and yeast TFIIIC. Because none was found, we have considered the possibility that some hitherto unknown mechanism is involved in transcriptional activation of the *SUP4A53T61* $tRNA^{Tyr}$ gene by the Tap1-1 protein. One clue to the nature of this protein is provided by its extensive sequence similarity to the protein encoded by another yeast gene, variously named *KEM1*, *XRN1*, *DST2*, *SEP1*, or *RAR5* (19). The different functions attributed to this gene depend upon, or result in, alteration of the secondary structure of DNA, for example, by catalysis of DNA strand transfer like that mediated by the *E. coli recA*-encoded protein.

Equally provocative are the findings of another laboratory, where the *TAP1* gene was cloned in connection with studies of different aspects of RNA synthesis in *S. cerevisiae*. In their screen of a yeast ts^- mutant bank for cells defective in the export of $poly(A)^+$ RNA from the nucleus to the cytoplasm, Amberg et al. isolated the *rat1* mutant strain (altered in RNA trafficking). As a result of cloning and sequencing of *RAT1*, they found it to be identical to the *TAP1* gene we have characterized. However, at the restrictive temperature, *tap1-1* is reported by them not to accumulate $poly(A)^+$ RNA within the yeast nucleus (4).

While an understanding of the puzzling interrelationships between the various TAP1/RAT1 functions will come only after further research, we feel obliged to put forward a plausible explanation for the many faces shown by this gene product (1, 4). We prefer not to attribute them to independent functional domains within the protein (4) but to explain both the PolIII transcription and $poly(A)^+$ RNA export effects by a single underlying function of the protein. In the accompanying report (1), we consider in detail the implications of the low affinity of TFIIIC binding by *SUP4A53T61* and its activation by the *tap1-1* allele in relation to the interplay between TFIIIC binding and chromatin structure. This line of reasoning suggests that transcription activation by *tap1-1* and the defect in $poly(A)^+$ RNA transport in *rat1-1* yeast cells (4) are both manifestations of an effect of the TAP1/RAT1 protein upon chromatin structure. Similar pleiotropic effects have been observed for the yeast *prp20* mutation (13); *TAP1/RAT1* is, however, structurally unrelated to the *PRP20* gene.

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