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Termination-Altering Mutations in the Second-Largest Subunit of Yeast RNA Polymerase III

SALAM A. SHAABAN, BRENT M. KRUPP, AND BENJAMIN D. HALL*

Department of Genetics, University of Washington, Seattle, Washington 98195

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In order to identify catalytically important amino acid changes within the second-largest subunit of yeast RNA polymerase III, we mutagenized selected regions of its gene (*RET1*) and devised in vivo assays for both increased and decreased transcription termination by this enzyme. Using as the reporter gene a mutant *SUP4-o* tRNA gene that in one case terminates prematurely and in the other case fails to terminate, we screened mutagenized *RET1* libraries for reduced and increased transcription termination, respectively. The gain in suppression phenotype was in both cases scored as a reduction in the accumulation of red pigment in yeast strains harboring the *ade2-1* ochre mutation. Termination-altering mutations were obtained in regions of the *RET1* gene encoding amino acids 300 to 325, 455 to 486, 487 to 521, and 1061 to 1082 of the protein. In degree of amino acid sequence conservation, these range from highly variable in the first to highly conserved in the last two regions. Residues 300 to 325 yielded mainly reduced-termination mutants, while in region 1061 to 1082, increased-termination mutants were obtained exclusively. All mutants recovered, while causing gain of suppression with one *SUP4* allele, brought about a reduction in suppression with the other allele, thus confirming that the phenotype is due to altered termination rather than an elevated level of transcription initiation. In vitro transcription reactions performed with extracts from several strong mutants demonstrated that the mutant polymerases respond to RNA terminator sequences in a manner that matches their in vivo termination phenotypes.

In eukaryotes, RNA polymerase III transcribes the genes for tRNA, 5S RNA, and several other nuclear genes encoding small RNAs. Studies of the termination signal for RNA polymerase III from *Saccharomyces cerevisiae* and from metazoans have defined a run of consecutive T residues in the nontemplate strand of DNA as the necessary signal for termination (2, 6). The efficiency of termination correlates with the length of the T stretch. The RNA polymerase III enzyme from vertebrate organisms can terminate at a sequence containing four or more T's (6), while yeast RNA polymerase III requires six or seven T's for efficient termination (2). Sequences surrounding the T stretch can affect the efficiency of termination (6, 33); however, the rules governing the dependence of termination upon the context of the T cluster are not yet clear.

In comparative terms, transcription termination by eukaryotic RNA polymerase III resembles more closely rho-independent termination by *Escherichia coli* RNA polymerase than termination by eukaryotic RNA polymerases I and II. In both the mouse (26, 47) and yeast (22, 28) rDNA transcription systems, termination by RNA polymerase I requires a specific DNA-binding protein that recognizes a conserved DNA element downstream of the termination site. RNA polymerase II termination appears to be dependent upon cleavage and polyadenylation of mRNA 3′ ends (10, 12, 30), as well as polymerase pausing at sites downstream of the poly(A) site (12, 13). For RNA polymerase III termination and for rho-independent termination by *E. coli* RNA polymerase, the core enzyme recognizes and responds directly to termination signals encoded in the template (11, 52, 53). The canonical rho-independent termination signal for *E. coli* RNA polymerase is an RNA hairpin structure followed by a stretch of U's at which RNA release occurs (53). While eukaryotic RNA polymerase III also terminates at a U stretch, in contrast to *E. coli* RNA polymerase it has no requirement for secondary structure in the RNA upstream of the termination site (33).

A generally accepted view is that transcription termination occurs by a two-step process involving first the pausing of RNA polymerase at the termination site followed by release of nascent RNA and subsequent polymerase dissociation. Direct evidence for this two-step model exists only for rho-dependent termination in *E. coli* (29, 34, 41). In the absence of rho factor, the terminator sequences cause pausing without actual RNA release. For RNA polymerase III from *Xenopus laevis*, Campbell and Setzer (9) obtained evidence that supports a two-step model for termination. After transcription initiation from the single-stranded junction of a poly(dC)-tailed template, the polymerase was frequently unable to displace nascent RNA from the template DNA strand. When no RNA displacement occurred, a downstream termination signal (T_4) acted as a strong pause site but did not cause termination. In interpreting their experiments, these authors concluded that the pausing required for transcription termination was uncoupled from the RNA and RNA polymerase release steps because of the lack of strand displacement. This implies that there is a positive role in transcription termination for the continuous displacement of product RNA strand that normally accompanies promoter-mediated transcription by RNA polymerase III.

Two models have been proposed for the mechanism by which nascent RNA is released from the ternary complex at rho-independent termination sites. Arndt and Chamberlin (3) attribute the great stability of transcribing ternary complexes to nonelectrostatic (salt-resistant) interactions of an RNA polymerase product-binding site with the nascent RNA. At a termination site, the polymerase isomerizes to a termination-proficient state in which the RNA is only loosely bound through electrostatic interactions. Yager and von Hippel (54)

* Corresponding author. Mailing address: Department of Genetics, SK-50, University of Washington, Seattle, WA 98195. Phone: (206) 543-1100. Fax: (206) 543-0754.

have proposed a contrasting model in which the occurrence of termination is governed by the thermodynamic stability of an RNA-DNA heteroduplex within the transcription bubble. Termination at a rho-independent terminator is brought about by the combined effects of two structures that destabilize this heteroduplex. One of them, formation of a hairpin stem-loop in the nascent RNA, may act to shorten the hybrid region. While this is occurring, the nascent hybrid contains an adjacent ribo(U)-deoxy(A) tract, which is inherently unstable. So, while the first model suggests an all-important role for the polymerase in catalyzing termination, the second model accords no active role to the enzyme.

Genetic studies of *E. coli* RNA polymerase and of yeast RNA polymerase III show that the structure of RNA polymerase directly affects transcription termination. Many mutations that alter the efficiency of termination were isolated in four different domains of the *E. coli* RNA polymerase β subunit (27) (see Fig. 4). A reduced-termination mutation was isolated in the *RET1* gene of yeast RNA polymerase III (17). These results show that the second-largest subunits of both prokaryotic and eukaryotic RNA polymerases play a role in the elongation-termination process. The second-largest subunits of different RNA polymerases from a variety of organisms show considerable similarity in their amino acid sequences (see Fig. 4), implying evolutionary conservation of enzyme structural organization and functional domains or both. In this study, our aim was to find out whether protein sequences involved in termination are similarly located and whether their sequences have been conserved between the corresponding prokaryotic and eukaryotic RNA polymerase subunits. This question has been addressed for regions of *RET1* with high sequence similarity to *E. coli rpoB* and for evolutionarily divergent sequences corresponding in their position to regions of *rpoB* that are involved in termination.

Using intensive regional mutagenesis, we isolated a large number of termination-altering mutations in four regions of *RET1*. These were between the codons for amino acids 300 to 325, 455 to 486, 487 to 521, and 1061 to 1082 (see Fig. 4). Region 300 to 325 spans the original *ret1-1* mutation (18); regions 455 to 486 and 487 to 521 correspond to regions of the *E. coli* β subunit in which termination-altering mutations, some of which also confer rifampin resistance, have been isolated (19, 21); region 1061 to 1082 is part of a conserved protein domain which is close to the initiating nucleotide-binding site (14, 15, 40). The latter two regions show considerable sequence conservation (see Fig. 4), whereas the protein sequence of the *RET1* gene product between amino acids 300 to 325 and 455 to 486 has little similarity to the regions of *E. coli* β subunit that occupy corresponding positions. Nonetheless, these similarly placed but structurally dissimilar regions of *RET1* and *rpoB* give rise in each case to mutant alleles with altered transcription termination.

MATERIALS AND METHODS

Media and strains. Yeast media were those listed by Sherman et al. (45). Yeast strains are described in Table 1. Strain RT was used in the screen for reduced-efficiency-of-termination mutants, and strain IT was used to screen for increased-termination mutants. Strains SA22-11 and SA26 were used to confirm that the mutants isolated in strains RT and IT, respectively, were bona fide termination rather than initiation mutants. In all strains, the *SUP4* alleles were integrated at the chromosomal *sup4* locus by using the YIp5-U(IV) and YIp5-Δ94 plasmids (17).

Mutagenesis oligonucleotides. DNA sequences encoding *RET1* amino acids 300 to 325, 455 to 489, 489 to 521, and 1061 to 1082 were mutagenized with 84-mer, 112-mer, 117-mer, and 65-mer oligonucleotides (Table 2). The oligonucleotides were spiked at all bases coding for the affected amino acids with a contamination mix which contained all four nucleotides. The levels of contamination with the three wrong nucleotides at each position were 2, 1.5, 1.5, and 2.5%, respectively, for the four oligonucleotides. This should result, on average, in 1.5 base changes per oligonucleotide.

Plasmids. pSA39 is the phagemid used for the mutagenesis of the DNA sequence corresponding to amino acid regions 455 to 489 and 489 to 521. It is a Bluescript KS(+)-based vector with a 312-bp fragment from the *RET1* gene (between bases 1287 and 1599) cloned in as an *Spe*I-*Sna*BI piece.

pSA70 is the phagemid used for mutagenesis of the DNA sequence corresponding to amino acids 300 through 325. It was constructed by ligating the *Bam*HI-*Pst*I fragment of *RET1* into the phagemid pUC118 (50). An *Mlu*I site was created at position 1056 in the gene, and the *Bst*BI site at position 1161 was destroyed by in vitro mutagenesis. The mutant pool could then be shuffled into the yeast plasmid pSA71 as a 187-bp *Bst*BI-*Mlu*I fragment.

pBK2 is the phagemid used for mutagenesis of the C-terminal region covering amino acids 1061 to 1082. It has a 560-bp *Sst*I-*Sal*I *RET1* fragment, isolated from plasmid pRS316-RET1 (18), cloned in pUC119 (50).

pSA23 is pRS315 (46) cut with *Xba*I and *Sal*I and ligated to an *Spe*I-*Sal*I fragment from pRS316-RET1 containing the complete *RET1* gene. Then, a ~2-kb *Sph*I-*Pst*I fragment containing the *CYH2*^{s} gene, which codes for the wild-type ribosomal protein L29, was cloned into the *Xho*I polylinker site after it had been blunt ended with T4 DNA polymerase and fitted with *Sal*I linkers.

pSA34 is pRS314 (46) cut with *Pvu*II and then ligated to a T4 DNA polymerase-treated *Bst*XI-*Pvu*II fragment from pRS316-RET1 containing the complete *RET1* gene. Other manipulations were done to destroy the *Sna*BI site in the 5′ end of the *TRP1* gene and the *Kpn*I site in the polylinker and create *Spe*I and *Sna*BI sites at positions 1287 and 1599, respectively, in the *RET1* gene.

pSA71 is similar to pSA34 except that two of the *Bst*BI sites in the *RET1* gene (one at position 1161 and the other at 3533, 87 bp downstream of the *RET1* translation stop signal) were destroyed and an *Mlu*I site at position 1056 was created. The *Sna*BI site of the *TRP1* gene is still present.

pSA16 is pRS314 with the *RET1* gene cloned in as an *Spe*I-*Sal*I fragment from pRS316-RET1 between the *Xba*I and *Sal*I sites of the polylinker.

pSA11 is pRS316-RET1 cut with *Hin*dIII, which cuts at positions 898 and 2677 in the *RET1* gene. It was then ligated to a *Bam*HI-*Xho*I 1.3-kb fragment, containing the *HIS3* gene, isolated from plasmid CMP171 (31) after both were treated with T4 DNA polymerase. An *Eco*RI-*Sst*I fragment that contains the *ret1*::*HIS3* disruption was used to replace the chromosomal *RET1* copy.

In vitro mutagenesis. Single-stranded DNA was prepared according to the method described by Vieira and Messing (50). The bacterial strain used was TG1, and the helper phage was M13K07. Mutagenesis was carried out with reagents from the Amersham in vitro mutagenesis kit (36) except that T7 exonuclease was substituted for exonuclease III. The efficiency of mutagenesis was checked by sequencing plasmids isolated from a random sample of bacterial transformants.

From 10,000 to 20,000 bacterial transformants were pooled for each mutagenesis run, and DNA was prepared from an overnight culture. The mutagenized DNAs were then used to replace the corresponding wild-type fragments in one of the yeast vectors pSA71, pSA34, and pSA16. For region 300 to 325, the DNA was subcloned as a *Bst*BI-*Mlu*I 187-bp fragment into pSA71. For region 455 to 489, the DNA was subcloned as an *Spe*I-*Pst*I 208-bp fragment into pSA34. For region 489 to 521, the DNA was subcloned as an *Spe*I-*Sna*BI 312-bp fragment into pSA34. For region 1061 to 1082, the DNA was subcloned into pSA16 as an *Sst*I-*Sal*I 560-bp fragment. This ligation step was followed by a bacterial transformation. From 10,000 to 20,000 transformants were pooled and grown overnight, and plasmid DNA was prepared.

Screening for termination mutants. DNA from the mutant pools was transformed into yeast strains RT and IT. The transformants were selected on plates without Trp. Afterwards, they were patched onto YEPD-cycloheximide plates to select for the cells that have lost the original *RET1*-containing plasmid pSA23. Cells that still carry the pSA23 plasmid fail to grow on cycloheximide-containing media because of the dominant *CYH2*$^+$ allele on the plasmid. The Trp$^+$ transformants that fail to give cells growing on YEPD-cycloheximide presumably have either a plasmid that contains a recessive lethal *ret1* allele or a rearranged plasmid (rearranged plasmids were often observed after the ligation-transformation step). The survivors were patched again onto YEPD-cycloheximide and grown for a few days, after which they were scored for color phenotype. RT transformants were scored for color phenotype at 30°C, while IT transformants were checked after growth at 23°C. That is because the increased-termination phenotype is greatly masked at 30°C and the mutants are almost indistinguishable in color from the wild type. Transformants that showed lighter coloration than the wild-type control were picked because it was assumed that the lighter coloration was the result of gain of suppression, which in turn resulted from either reduced termination in strain RT or increased termination in strain IT. The transformants from the mutant pools were also tested for conditional phenotypes by making replicas of the YEPD-cycloheximide plates and incubating these replicas at 14 and 37°C.

Putative mutants were grown and total DNA was prepared from them by the method of Braus et al. (8). The plasmid containing a mutant *RET1* gene was cloned and amplified in *E. coli* and then transformed again into a yeast recipient strain to verify that the original phenotype was conferred by the cloned mutant plasmid. The *RET1* fragments in the mutant plasmids were then sequenced, and the amino acid changes were determined.

TABLE 1. Yeast strains used in this study

Strain	Relevant genotype
SA11-2B	*MATα RET1 trp1 ura3-1 met4-1o lys2-1o ade2-1o leu2 his3-Δ200 can1-100o cyh2*^{r} 2×(*SUP4-U(IV)*:*URA3*)
SA15-2B	*MATα RET1 trp1 ura3-1 met4-1o lys2-1o ade2-1o leu2 his3-Δ200 can1-100o cyh2*^{r} *SUP4-Δ94*:*URA3*
RT	SA11-2B *ret1*::*HIS3*/pSA23 (*LEU2 CYH2*^{s} *RET1*)
IT	SA15-2B *ret1*::*HIS3*/pSA23 (*LEU2 CYH2*^{s} *RET1*)
SA26	Similar to RT except that it has multiple copies (≥3) of *SUP4-U(IV)*:*URA3*
SA27-2	Similar to RT except that it has 2 copies of *SUP4-Δ94*:*URA3* instead of *SUP4-U(IV)*:*URA3*

Double-mutant constructions. Several mutations between amino acids 501 and 521 were subcloned, as *Pst*I-*Sst*I fragments, into plasmids carrying *RET1* genes with the following mutations: the T-to-K mutation at position 311 (T311K), T455I, T471R, T471S, E478D, E478K, Q490L, Q493H, and G495V. Several mutations from both regions 455 to 489 and 489 to 521 were subcloned, as *Age*I-*Cla*I fragments, into a plasmid with the K310T mutation.

In vitro extract preparation. In vitro transcription extracts were prepared as described by Allison et al. (1) with the following modifications. Yeast cells were resuspended in a solubilization buffer that lacked $MgCl_2$. Buffers also included a cocktail of protease inhibitors: phenylmethylsulfonyl fluoride (1 mM), leupeptin (10 μg/ml), pepstatin (10 μg/ml), benzamidine (1 mM), aprotinin (10 μg/ml), and bacitracin (10 μg/ml). Dialysis of the extract was replaced by a desalting step on a Sephadex G-25 column with buffer C (with no KCl added) as the elution buffer. Protein content was monitored by the Bradford assay (Bio-Rad), and peak fractions were pooled.

The pooled fractions from the desalting step were adjusted to 100 mM KCl and adsorbed to a phosphocellulose column equilibrated with buffer C plus 100 mM KCl. The column was washed with three column volumes of buffer C plus 100 mM KCl. The proteins were then step eluted with buffer C plus 600 mM KCl. Protein content was assayed, peak fractions were pooled, and conductivity was measured to determine the final KCl concentration of the pooled fractions.

In vitro transcription reactions. Transcription was limited to a single round by initiating with a subset of the ribonucleotides (rNTPs) (ATP, CTP, and UTP) to form an arrested ternary complex containing a 17-nucleotide-long RNA product. Subsequently, synthesis was continued by addition of a chasing mix containing all four rNTPs. The initiation reaction was carried out in a 20-μl final volume with 7.5 μl of transcription extract (phosphocellulose P-11 fraction), 100 ng of the YIp5-U(IV) DNA template (17), 140 mM KCl, 0.5 mM each ATP and CTP, 0.5 μM cold UTP, and 5 μCi of [α-^{32}P]UTP (10 mCi/ml; 3,000 Ci/mmol). The 10× buffer used provided final concentrations of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.9), 7 mM $MgCl_2$, and 3 mM dithiothreitol. The reaction was allowed to proceed for 30 min before 30 μl of a GTP-containing chase mix was added. The final concentrations of the reaction components were 0.5 mM each of the four rNTPs, 140 mM KCl, and 1 mg of heparin per ml. Heparin was added in order to prevent reinitiation and to inhibit posttranscriptional processing. The chase reaction was allowed to proceed for 5 min before being stopped by adding 50 μl of a stop mix that contained sodium dodecyl sulfate (SDS), EDTA, proteinase K, and yeast total RNA to final concentrations of 1%, 5 mM, 0.4 mg/ml, and 0.2 mg/ml, respectively. Recovery and electrophoresis of transcription products were carried out as previously described (4).

RESULTS AND DISCUSSION

Mutant isolation strategy. We identified mutations in the *RET1* gene by their in vivo effects on polymerase III transcription termination. Mutant polymerases were identified by a change in colony color that results from increased suppression of the *ade2-1* ochre mutant allele. Unsuppressed cells are red, whereas *ade2-1o* cells that efficiently express a tyrosine-inserting ochre suppressor gene (*SUP4* tRNA^{Tyr}) give white colonies. In order to make colony color respond to transcription termination by RNA polymerase III, we constructed yeast strains bearing partially defective *SUP4* alleles and employed these strains in the genetic screen.

SUP4-U(IV) (Fig. 1) is a mutant allele that contains a tract of five T's in the intron (17). The five consecutive T's act as a moderate-strength terminator for yeast RNA polymerase III. Because the exon of *SUP4-U(IV)* is identical in sequence to that of *SUP4-o*, the full-length RNAs transcribed from it are capable of maturation to normal, active, suppressor tRNA molecules. *SUP4-Δ94* (Fig. 1) is a mutant allele of *SUP4* in which the natural *SUP4* terminator T_7GT_6 is shortened to five T's (2). Considerable read-through occurs with this template, producing a long tRNA species that terminates at a back-up terminator, T_7GT_6, placed about 200 bp downstream of the five T's. This long RNA product is not processed in the yeast nucleus to yield a functional suppressor tRNA (2).

Yeast strain RT (Table 1) has two integrated copies of *SUP4-U(IV)* and is red. Mutant RNA polymerases with a reduced-efficiency-of-termination phenotype, like the original *ret1-1* mutant (17), are expected to bring about an increase in suppressor tRNA level with a concomitant increase in the *ADE2* enzyme activity, changing the colony color from red toward pink or white (Fig. 2). This occurs because the intensity of color of the yeast cells is inversely related to the level of the ADE2 enzyme, which increases as more *SUP4-o* tRNA is made.

Yeast strain IT has one integrated copy of *SUP4-Δ94* and is red. Mutant RNA polymerases with an increased-efficiency-of-termination phenotype will increase the level of suppressor tRNA, shifting the colony color from red toward pink or white (Fig. 2). To facilitate screening for mutant *ret1* alleles by the plasmid shuffle procedure (31), both the RT and IT strains were chromosomally disrupted for *RET1*. The disruption was covered by a resident *RET1 LEU2 CYH2*^{s} *CEN* plasmid (Fig. 3).

Isolation of mutations in the *RET1* gene. Pools of mutations in distinct regions of *RET1* were created by regional random mutagenesis using "spiked" oligonucleotides (16). The level of contamination with the incorrect nucleotides was intended to give, on average, 1.5 base changes per oligonucleotide. By this

TABLE 2. Sequences of the four mutagenesis oligonucleotides used in the in vitro reactions

Size (bp)	Sequence[a]	% Contamination	Amino acids mutagenized	Strand
84	5′ GGC CTC TAT ACC CTC TTG CAG GAT AGT CAG TTT TTG CCT TCT CAT TGT CTT TAC TTT TGC ACC AAT ATA CTC TAA AGC TTG CTG 3′	2	300–325	Antisense
112	5′ GGT GTT ACA CAT GTT CTT TCC AGA CTA TCT TAT ATT TCC GCA TTG GGT ATG ATG ACA AGA ATT TCT TCT CAG TTT GAA AAA TCA AGA AAA GTT TCC GGT CCA AGA GCA TTA C 3′	1.5	455–487	Sense
117	5′ CCA AGA GCA TTA CAA CCT TCT CAA TTT GGT ATG CTT TGT ACT GCA GAT ACT CCA GAA GGT GAG GCG TGT GGT CTA GTG AAA AAT TTA GCA TTA ATG ACT CAT ATT ACT ACA GAT GAC 3′	1.5	489–521	Sense
65	5′ GG TTG GGT GAG ATG GAG AGG GAT TGT GTA ATC GCG TAT GGT GCT TCA CAA TTG CTG TTA GAA AGG 3′	3	1063–1080	Sense

[a] The nonunderlined bases at the 5′ and 3′ ends were not contaminated.

means, every amino acid in the target region can be changed to any of five or six other amino acids.

The pools of mutant *RET1* genes on a *TRP1-CEN* shuttle plasmid were amplified in *E. coli* and then transformed into the RT and IT yeast strains. After a plasmid shuffle step (Fig. 3), in which the resident *RET1* gene on a plasmid was removed from the strain, surviving transformants were patched onto YEPD plates and scored for color after 3 to 7 days of growth. Conditional mutants were scored by incubating replicas of the plates at 14 and 37°C.

The screen for reduced-termination mutants was done at 30°C, and that for increased-termination mutants was done at 23°C. Increased-termination mutants were almost indistinguishable in color phenotype from the wild type in strain IT at 30°C. Wild-type cells carrying *SUP4-U(IV)* developed a darker red color at 23°C than at 30°C, while those carrying *SUP4-Δ94* developed a darker red color at 30°C. These results are consistent with the efficiency of termination being higher at the lower temperature. The lack of phenotypic difference between the wild-type and increased-termination *ret1* alleles at 30°C could therefore be attributed either to the inability of the human eye to clearly distinguish degrees of accumulation of red pigment in the dark red to red range or to a stronger termination phenotype of the increased-termination mutants at 23°C.

Table 3 summarizes the numerical data from the in vivo screening of the mutant pools. The difference between the total number of transformants screened and the number surviving the plasmid shuffle does not reflect solely the number of lethal mutants in those regions, since all the mutant pools had, to various degrees, plasmids which sustained deletions during the cloning and amplification in *E. coli*. We have observed that when the *RET1* gene is present in a high-copy-number bacterial plasmid (as with pRS314, used in this study), it is somewhat toxic to *E. coli*, leading to favorable conditions for the propagation of plasmids with deletions and rearrangements. The *RET1* plasmids that survive the plasmid shuffle but show no termination phenotype are ones that carry either a wild-type *RET1* gene or a neutral *ret1* mutation.

***RET1* regions studied.** The regions of *RET1* encoding amino acids 300 to 325, 455 to 486, 487 to 521, and 1061 to 1082 were selected for mutagenesis. These four regions (Fig. 4) correspond in position to all but one of those that yielded numerous termination-altering mutant changes in the β subunit of *E. coli* RNA polymerase (27). The most N terminal of these (300 to 325) surrounds residue T311, at which the *ret1-1* mutation (T311K) occurred (18). Flanking this region are the sequences most variable in length and amino acid sequence among the second-largest subunits of different RNA polymerases. In the case of the β subunit of *E. coli* RNA polymerase, this region can tolerate large insertions and deletions (23), suggesting that it does not have an essential role in transcription and that it is probably part of a flexible region on the surface of the protein.

Amino acids 455 to 486 of the RET1 protein occupy a region with evolutionary conservation of an unusual type. For these sequences, the second-largest subunits of different RNA polymerases fall into two distinct categories: the archaebacterial and eukaryotic nuclear enzymes on the one hand and eubacterial together with chloroplast RNA polymerases on the other. Within each group, sequences align with few gaps (Fig. 4), but between groups, they do not. Nonetheless, there are distinct structural and functional similarities spanning this region for the two groups, as may be seen from simple sequence motifs and from the phenotypes of mutations observed for *RET1* and *E. coli rpoB*.

Region 487 to 521 shows considerable phylogenetic conservation when sequences from all the known second-largest subunits of DNA-dependent RNA polymerases from prokaryotes and eukaryotes are compared. Residues R-487, C-498, T-502, G-509, and L-510 are absolutely conserved in all organisms. F-494, E-504, and V-510 exhibit only conservative substitutions between different enzymes (Fig. 4).

Region 1061 to 1082 is among the most conserved phylogenetically. Residues G-1063, E-1064, M-1065, E-1066, and G-1074 are invariant, while L-1079 and E-1081 undergo only conservative substitutions. This region is part of the longest conserved domain among the second-largest subunits of bacterial and eukaryotic RNA polymerases (49). Several residues in that domain have been shown to be in close proximity to the gamma phosphate of the initiating nucleoside triphosphate by affinity labeling studies (14, 15, 35, 40). Moreover, for yeast RNA polymerase II, that part of the second-largest subunit was implicated in an interaction with homology domain H of *RPB1* (32). A conditional mutation, *rpb2-2*, which is defective in function rather than enzyme assembly, also maps in that domain (25).

Region 300 to 325. Mutagenesis of region 300 to 325 and subsequent screening of the pool of mutations in yeast strains RT and IT yielded many mutant yeast clones with altered transcription termination by RNA polymerase III (Fig. 5). A much higher yield of reduced- than increased-termination mutants was obtained (Table 3); moreover, only two of the increased-termination mutants, K310T and K310T I324K, show a strong phenotype. These two mutants are indistinguishable in phenotype, suggesting that the I324K change is neutral.

In a preliminary characterization of the efficiency of mutagenesis by the mixture of contaminated oligonucleotides, 12 plasmids from the mutant pool were isolated from *E. coli* transformants and sequenced. The plasmids were then transformed individually into yeast strains RT and IT, and their growth and termination phenotypes were examined. Eleven of the plasmids had mutations, and all gave viable yeast transformants after the plasmid shuffle step. Five of the mutant *ret1* alleles showed termination phenotypes, and one of these was a deletion-insertion change (deletion of 17 amino acids and insertion of 7 new amino acids), with the strongest reduced-termination phenotype of any mutation in this region (Fig. 5).

The relatively high number of mutations that cause a reduced-termination phenotype and the observation that many amino acid residues in this region give reduced-termination phenotypes when substituted in any one of several possible ways suggest that this region participates actively in the process of termination, either by binding a cellular termination factor or through involvement in a protein-nucleic acid interaction that facilitates termination. The reduced-termination phenotype would then result from a loss or impairment of function, while the increased-termination mutants result from some gain in function. The corresponding region in *E. coli* RNA polymerase has been implicated in binding of the phage T4 Alc protein (44). Alc binds to elongating ternary complexes, enhancing premature termination in *E. coli* transcription units, but does not affect transcription elongation on DNA, such as T4 DNA, that contains hydroxymethylcytosine rather than cytosine (24).

Region 455 to 486. The in vivo screening of a pool of *RET1* genes mutagenized in region 455 to 486 produced many termination-altering mutations, with both increased and reduced efficiency of termination (Table 3). There were approximately equal numbers of conservative and nonconservative substitutions; however, there was no simple correlation between the nature of the amino acid substitutions and the nature of the observed termination phenotype. The mutations that caused

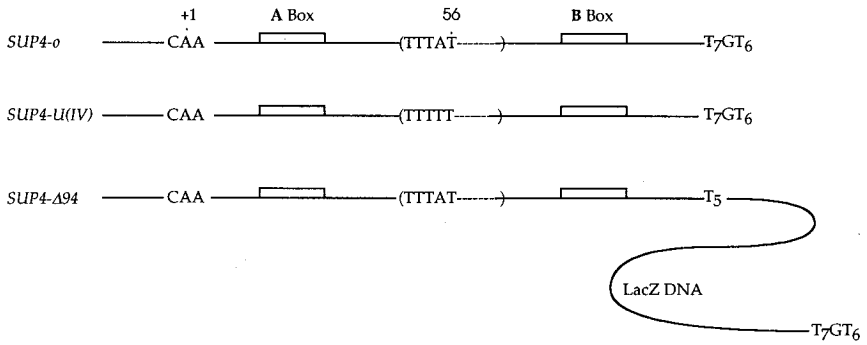

FIG. 1. *SUP4-o* and the two derived alleles *SUP4-U(IV)* and *SUP4-Δ94*. The A box and B box are the internal promoter elements. The "+1" designates the initiation site of transcription. The sequence between the parentheses is the *SUP4* intron.

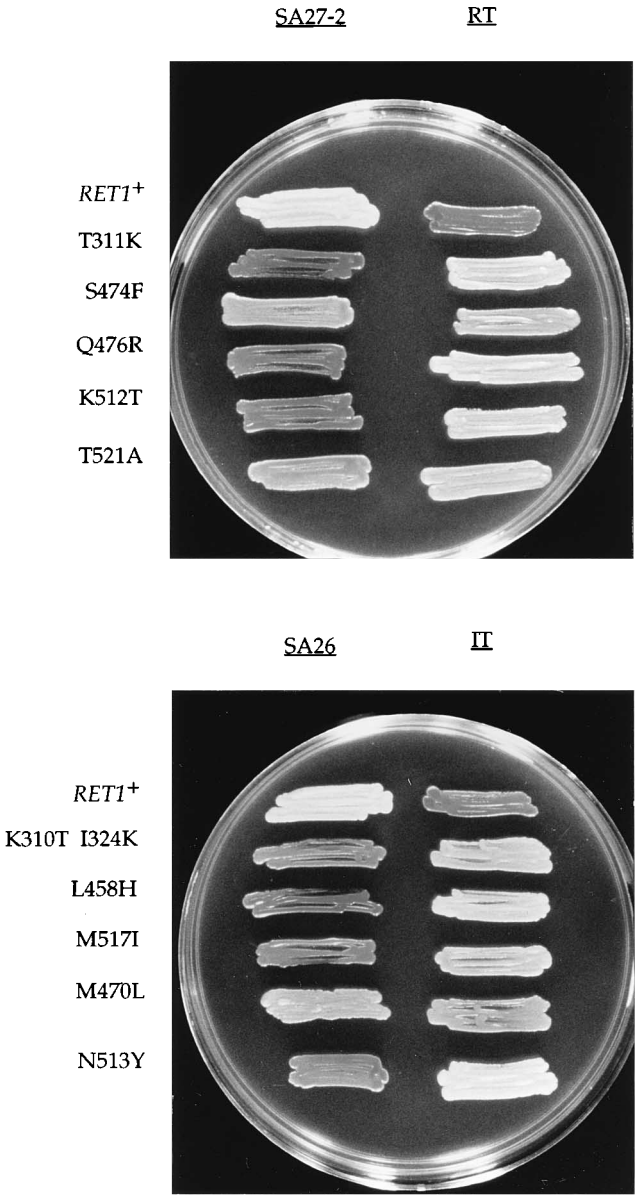

FIG. 2. In vivo color phenotypes of *ret1* mutants. The top plate shows yeast strains RT and SA27-2 with either *RET1*$^+$ or *ret1* reduced-termination mutant alleles. The bottom plate shows yeast strains IT and SA26 with either *RET1*$^+$ or *ret1* increased-termination alleles. Colors range from dark red (T311K [left] and L458H [left]) to white (*RET1*$^+$ [left]).

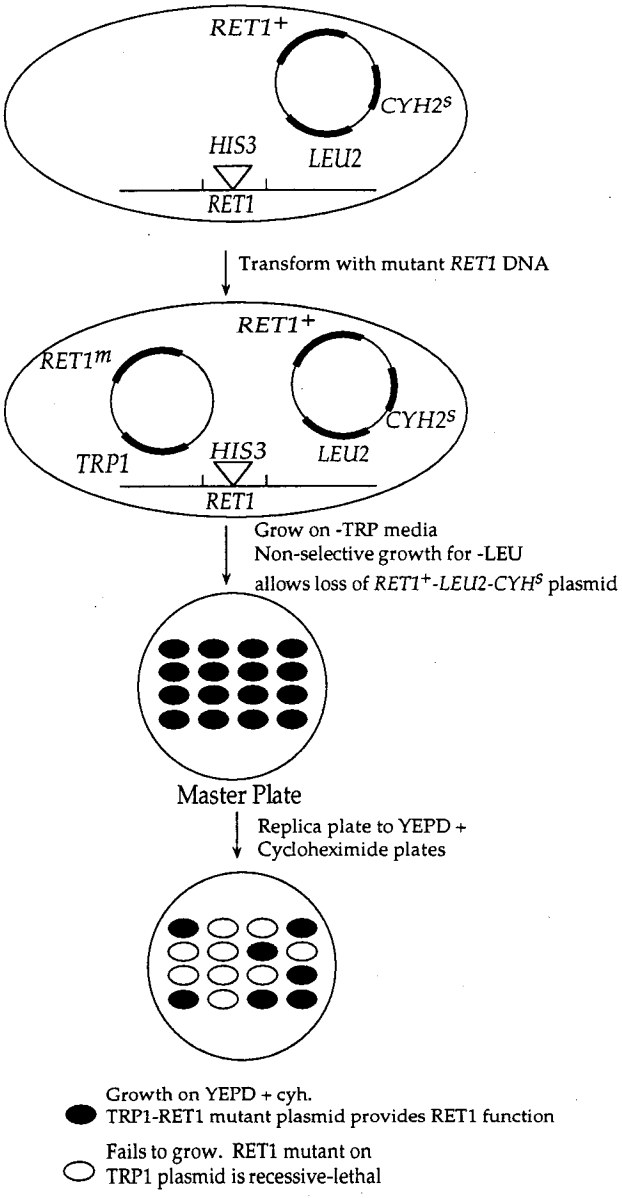

FIG. 3. Plasmid shuffle procedure. The mutant *RET1* pool is introduced on a *TRP1* centromeric plasmid. The resident *RET1* gene on the *CYH2*^{s}*-LEU2* centromeric plasmid is shuffled out by plating on YEPD-cycloheximide (cyh). Survivors are then checked for color and conditional growth phenotypes.

TABLE 3. Genetic screening data[a]

Region	Strain	No. of transformants screened	No. surviving plasmid shuffle	No. of putative mutants	No. of plasmids recovered and sequenced	No. of plasmids that reproduce phenotype	No. of single mutants[b]	No. of multiple mutants[b]	% Termination mutants[c]
300–325	RT	1,000	300	26	16	16	13 (9)	3	9
	IT	1,000	300	23	23	9	7 (4)	2	3
455–489	RT	832	331	43	36	35	15 (13)	20 (15)	13
	IT	832	385	91	31	26	15 (10)	11 (10)	20
489–521	RT	2,100	700	86	34	31	22 (12)	9 (6)	11
	IT	2,040	700	74	29	29	23 (13)	6 (5)	11
1061–1082	RT	2,735	465	6	6	0	0	0	0
	IT	660	101	39	39	32	23 (14)	9 (8)	32

[a] Numerical data for the in vivo screening of the mutant pools and the ensuing steps of plasmid recovery, retransformation, and sequencing.
[b] Numbers in parentheses refer to the number of unique mutants.
[c] The percentage of termination-altering mutants among the survivors of the plasmid shuffle was calculated by the following formula: (plasmids that reproduce phenotype/plasmids recovered and sequenced) × (putative mutants/survivors of plasmid shuffle).

an increased-termination phenotype mapped throughout the region, while the ones that gave a reduced-termination phenotype clustered in the C-terminal half of the region, with the sole exception of the T455I mutation (Fig. 6). The increased-termination mutations in the N-terminal half of the region affect hydrophobic amino acid residues that occur at every third amino acid position (Fig. 6).

Comparison of the RET1 protein sequence from amino acids 455 to 477 with the corresponding regions of other eukaryotic and archaebacterial second-largest subunits reveals the

FIG. 4. Complete sequence alignment of two eukaryotic and two eubacterial second-largest RNA polymerase subunits. Protein sequences are those predicted from their respective gene sequences: III, yeast *RET1* (18); II, yeast *RPB2* (49); B.s., the gene encoding *Bacillus subtilis* β subunit; E.c., *E. coli rpoB*. The regions overlined above "III" are those mutagenized in this study (Fig. 5 to 8); the underlined regions of *E. coli* are those mutagenized in a screen for altered-termination mutations in *E. coli* (27). The sequence encoding the *B. subtilis* β subunit was obtained from C. Price, University of California, Davis. The boldface letters mark regions that are conserved among all known members of the *rpoB* gene family, including those from vaccinia virus, four archaebacteria, four eubacteria, three higher plant chloroplasts, and, for nuclear RNA polymerases, three polymerase I's, six polymerase II's, and two polymerase III's (24a).

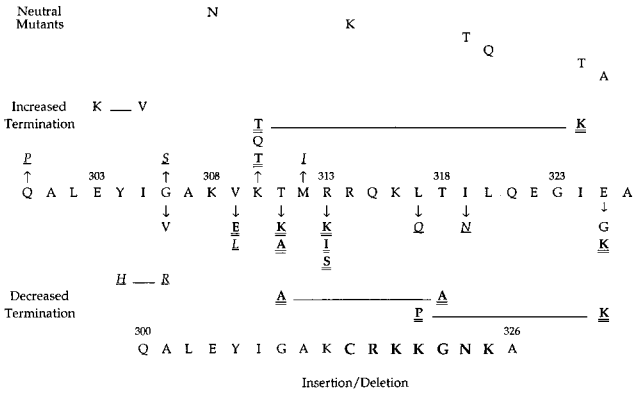

FIG. 5. Region 300 to 325 of *RET1* and sequence changes of the termination mutations in the region. Sequence changes of increased-termination mutants are shown above the wild-type *RET1* sequence, while those of reduced-termination mutants are shown under it. Both single and multiple changes are presented. The last line has the sequence of the deletion-insertion mutant. Mutants are classified into three groups: strong, symbolized by double underlining and boldface text; medium strength, presented in single underlining and italics; and weak, written in plain (lightface roman) text. The classification is based on the color phenotypes that a mutant imparts on the yeast strain RT or IT (for reduced- or increased-termination mutants, respectively). Mutations producing white or light pink colonies are classified as strong, those that change the color to medium-pink or pink are classified as medium strength, and those that only slightly alter the color of the cells from dark red to light red or dark pink are classified as weak. Mutants with multiple amino acid substitutions are shown with horizontal lines connecting the substitutions.

existence of a repeated tripeptide motif, S/T-X-Hb (Hb, hydrophobic residue) (Fig. 4). Mutant changes were recovered at positions T-455, T-471, and S-475, while none were obtained in S-459, S-462, or S-465. Mutations in every one of the hydrophobic residues of the tripeptides were recovered. Some were changes to other hydrophobic residues, while others were changes to polar residues.

In the region spanning the amino acids between Q-476 and P-486, there were many mutations with altered termination phenotypes. Q-476 and G-485 seem to be important for efficient termination because substitution at these positions by any of several other amino acids gives a strong reduced-termination phenotype. The conservative change Q476H has a weak phenotype, while nonconservative changes Q476L and Q476R have strong phenotypes. The region in *E. coli* RNA polymerase corresponding to E478 to P486 of *RET1* is not part of the Rif cluster I (Fig. 4) and is separated from it by the sequence GPGG, a hinge sequence (5) missing in eukaryotic and archaebacterial polymerases.

Region 487 to 521. Mutations that increase and mutations that reduce the efficiency of transcription termination were recovered in region 487 to 521 (Fig. 7). No termination-altering mutations that change the conserved residues C-498, T-502, G-509, and L-510 were recovered by the plasmid shuffle procedure. A mutation changing T-502 (T502P) was obtained in one of six plasmids that were recovered from the mutant pool and sequenced in order to assess the efficiency of mutagenesis by the spiked oligonucleotide. The T502P allele gave a recessive lethal phenotype when introduced into the yeast strains. The same amino acid substitution in *E. coli* RNA polymerase is known to confer rifampin resistance and a reduced-termination phenotype (19, 21). The total absence of changes at these highly conserved positions among mutants recovered by plasmid shuffling suggests that mutations at positions 498, 509, and 510 as well as 502 may cause lethality. Such lethal effects could be a consequence either of a very strong termination phenotype or of gross structural changes in the enzyme. Four other mutations were observed among the six plasmids sequenced, three of which were recessive lethal (C508S, S492A N513S, and P491H K512N) and one of which was neutral (Q490H).

An interesting feature of the primary sequence of this region is the presence of highly conserved proline and glycine resi-

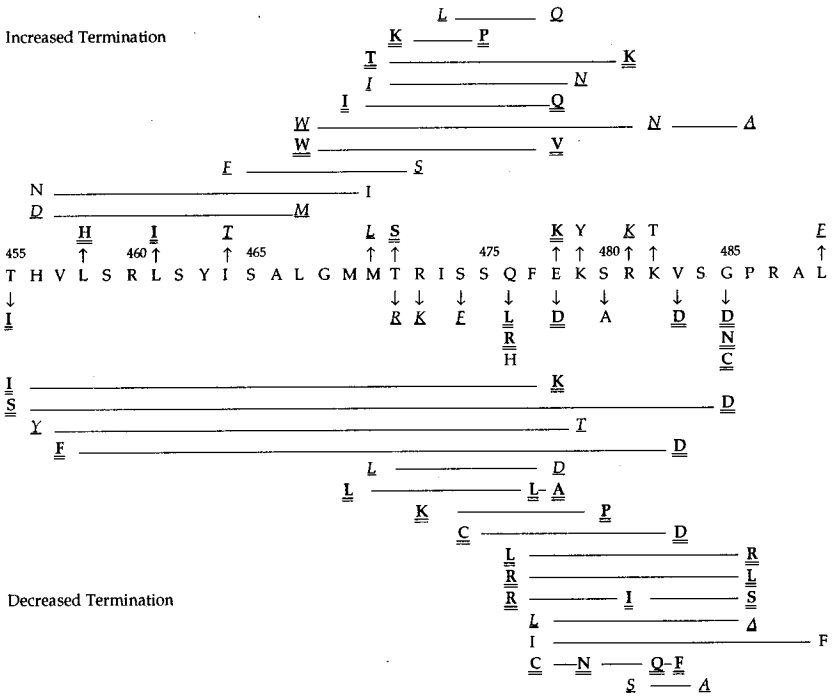

FIG. 6. Region 455 to 489 of *RET1* and sequence changes of the termination mutants there. Refer to the legend to Fig. 5 for the details of mutant classification.

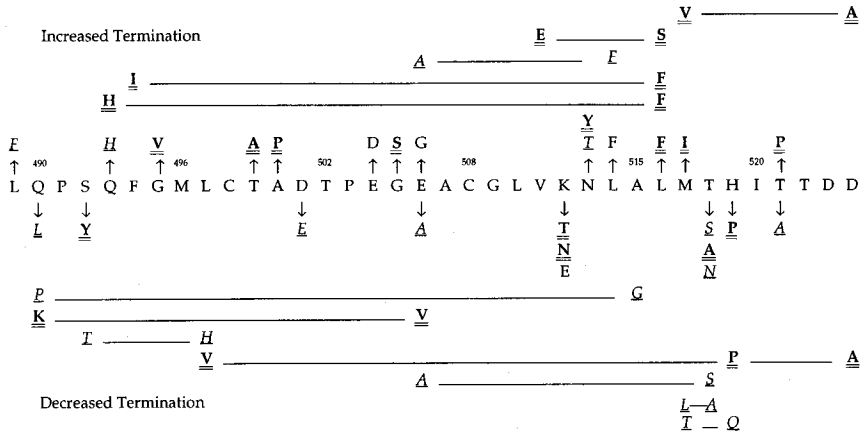

FIG. 7. Region 489 to 521 of *RET1* and sequence changes of the termination mutants there. Refer to the legend to Fig. 5 for the details of mutant classification.

dues (two prolines and three glycines) interspersed within the first 24 amino acids, suggesting the presence of conserved beta-turn structures of some type (48). Mutations in two of the glycines were recovered as strong increased-termination mutants (G495V and G505S).

The large number of residues in this region (487 to 521) and the preceding one (455 to 486) that can mutate so as to alter termination suggests that both regions are involved in the response of polymerase III to termination signals. This possibility is attractive because mutational analysis has implicated the corresponding regions in the β subunit of *E. coli* RNA polymerase in transcription termination and shown their involvement in binding rifampin (19, 21). Rifampin is thought to inhibit transcription by occupying the product binding site (43). Rifampin does not bind to yeast RNA polymerase III, but primary amino acid conservation and apparent functional conservation indicated by the appearance of termination-altering mutations in both polymerases support a model in which region 455 to 521 is involved in binding the nascent transcript.

Region 1061 to 1082. Region 1061 to 1082 is unique in that only increased-termination mutants were obtained. Moreover, some of those mutants showed conditional growth phenotypes (cold sensitive, temperature sensitive, or both). While all of the conditional mutants had an increased-termination phenotype, the converse was not found to be true. In fact, no correlation was observed between the conditional growth phenotype and the strength of the termination phenotype at the permissive temperature. The methionine at position 1065 was the only invariant residue in which a termination-altering mutation (M1065L) was recovered.

The fact that only increased-termination mutations were recovered in this region suggests that it is not directly involved in the recognition and response to termination signals. Instead, we propose that the region plays an important role in transcription elongation. To the extent that termination and continued elongation are directly competitive processes (20, 51), mutations that slow down the rate of polymerization would be expected to show an increased-termination phenotype. Indeed, it has been shown (43a) that the strong increased-termination mutant R1061K E1081D has a diminished rate of transcript elongation. The slowing of elongation by mutant substitutions in this region might act by reducing the affinity for nucleotides or the rate of catalysis. The proximity of this conserved region to the initiating nucleoside triphosphate binding site (40) at 1029 to 1034 (Fig. 4) suggests a role for the former in substrate binding or catalysis. E-1064 and E-1066 are two of seven invariant acidic amino acids in the RET1 protein, making them good potential chelators of the metal ions likely to be involved either in catalysis or in binding of the DNA template and/or nucleotide substrates. Such roles for invariant acidic amino acids have been seen in DNA polymerases and in bacteriophage RNA polymerases (7, 37–39, 42).

To explain the absence from this region of mutations that speed elongation and decrease termination, we posit that the catalytic role of this region is normally not rate limiting for elongation by the polymerase. An analogous situation exists for

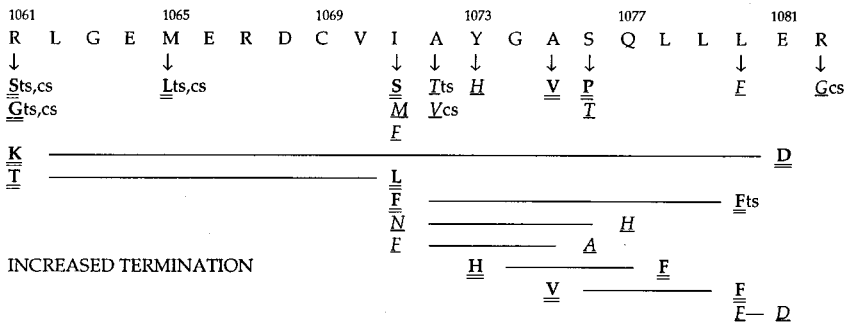

FIG. 8. Region 1061 to 1082 of *RET1* and sequence changes of the increased-termination mutations isolated there. No reduced-termination mutants were identified in the screen. Mutants with a conditional growth phenotype are shown with ts, cs, or ts,cs written as a subscript to the change to indicate temperature sensitive, cold sensitive, or both.

TABLE 4. Phenotypes of double mutants[a]

First mutation (phenotype, suppression index)	Phenotype and suppression index with second mutation (phenotype, suppression index):													
	L461I (I-5, +++)	I464T (I-3, +++)	M470L (I-4, +++)	T471S (I-5, +++)	Q493H (I-3, +++)	G505S (I-6, +++)	E506G (I-1, +++)	N513Y (I-6, +++)	N513T (I-3, +++)	L514F (I-1, +++)	L516F (I-5, +++)	V511E, L516S (I-6, +++)	M517I (I-5, +++)	T521P (I-5, +++)
T311K (R-5, +++)							−	N, +++		−	−		N, +++	N, +++
T455I (R-5, +)								N, ±				N, ±	N, ±	R-4, ±
T471R (R-4, +++)						N, ±		N, ++	N, ±				N, ++	
E478D (R-5, +++)						N, ±		I-2, ++	R-4, ++				R-3, ++	
K310T (I-5, +++)	I-6, ++	−	I-6, ++	I-6, ++	I-6, ++				I-6, ++		I-6, ++			−
T471S (I-5, +++)						−		I-6, +	I-5, +				I-6, +	
E478K (I-5, ++)						−		−	−				−	
G495V (I-5, +++)							I-5, +	I-6, +		I-1, ++	I-6, ++		−	I-6, +

[a] The letters R, I, and N stand for reduced termination phenotype, increased termination phenotype, and neutral phenotype, respectively. The growth properties of the mutants are indicated as +++ (good growth), ++ (slow growth), + (poor growth), ± (very poor growth), and − (recessive lethal). The numbers next to I and R indicate the index of suppression. The index of suppression is a numerical scale that we have used to rank the mutants relative to each other in terms of color intensity that they bring about in strain RT for the reduced termination mutants and in strain IT for the increased termination mutants: 0, dark red; 1, red; 2, dark pink; 3, pink; 4, medium pink; 5, light pink; 6, white.

TABLE 5. Additional double-mutant phenotypes[a]

First mutation (phenotype, suppression index)	Phenotype and suppression index with second mutation (phenotype, suppression index):													
	T455I (R-5, +)	Q476L (R-5, +++)	Q476R (R-6, +++)	E478D (R-5, +++)	S492Y (R-5, +++)	E506A (R-4, +++)	K512T (R-5, +++)	K512N (R-5, +++)	K512E (R-1, +++)	T518S (R-3, +++)	T518N (R-4, +++)	T518A (R-5, +++)	H519P (R-6, +++)	T521A (R-4, +++)
T311K (R-5, +++)						R-6, +	R-6, +	R-6, +	R-6, +++	R-6, +	R-6, +	−	R-5, +	R-6, +
T455I (R-5, +)							R-2, ±						−	N, ±
T471R (R-4, +++)							R-6, +			R-6, +			R-3, +	R-6, +
E478D (R-5, +++)							R-6, +			R-6, +			R-4, +	R-5, ±
Q490L (R-3, +++)						R-6, ++	R-6, ++	R-6, ++	R-5, ++	R-6, ++	R-6, ++	R-6, ++	R-6, ++	R-6, ++
K310T (I-5, +++)	R-6, ++	R-2, +++	R-4, +++	−	R-2, ++		R-2, +++					R-2, +++	R-3, +++	
T471S (I-5, +++)							N, ±			R-3, ++			R-5, ++	N, ±
E478K (I-5, ++)	R-6, +++						R-2, ++			R-2, ++			N, ±	R-3, ++
Q493H (I-3, +++)						R-3, +++	R-2, +++	N, +++	N, +	R-2, +++	R-2, +++	−	−	N, +++

[a] See Table 4, footnote *a*.

the Klenow fragment of DNA polymerase, for which the chemical step is 30- to 100-fold faster than the rate-limiting conformational changes that precede it and follow it. Therefore, a 1,000-fold reduction in the rate of the chemical step is required to bring about a 10-fold reduction in k_{cat} (38).

Mutations affect termination but not initiation. Because the phenotypic color assay that we utilized responds to the absolute level of full-length *SUP4* product in the cell, a gain in suppression phenotype might conceivably result from increased transcription initiation. Such a mutant RNA polymerase allele should cause a gain in suppression when present in yeast strains with either of the *SUP4* tester alleles. In contrast, a mutant having increased ochre suppression by virtue of an altered termination phenotype would show an increase in suppressor tRNA level with one *SUP4* allele [*SUP4-U(IV)* or *SUP4-Δ94*] and a decrease with the other allele. In order to clearly categorize our *ret1* mutants by these criteria, we constructed two new tester strains. Strain SA27-2 has two integrated copies of *SUP4-Δ94* and is light pink. Reduced-termination *ret1* mutant alleles isolated from the screen in strain RT should lower the level of suppressor tRNA in strain SA27-2 and cause a shift toward red color, while increased-initiation mutant alleles should shift it towards white. Strain SA26 has multiple integrated copies of the *SUP4-U(IV)* allele and is white. Increased-termination *ret1* mutant alleles isolated from the screen in strain IT should reduce the level of read-through *SUP4* produced and shift the color from white toward red. Indeed, all the mutants isolated from strains RT and IT and shown in Fig. 5 to 8 behaved like bona fide termination mutants when tested in these two strains. That is, each of them gave color shifts that were in opposite directions in the two new strains (Fig. 2).

Properties of double mutants. Single altered-termination mutations from the first three regions were combined pairwise, and the termination phenotypes of the double mutants were examined (Tables 4; and 5). As a general rule, each double mutant showed a phenotype that was the additive result of the phenotypes of the single mutants. Double mutants with very strong increased- or reduced-termination phenotypes grew quite slowly, presumably because the termination phenotype affects production of essential polymerase III transcripts. Slow growth was also observed for some double mutants that had weak or neutral termination phenotypes. Many other combinations resulted in a recessive lethal phenotype.

Two double mutants containing the L514F change (L514F plus either G495V or E506A) showed particularly interesting behavior (Table 4; Fig. 7). The weak increased-termination phenotype of L514F was epistatic to the strong increased-termination phenotype of G495V and antagonistic to the reduced-termination phenotype of E506A. The L514F E506A double mutant had a stronger increased-termination phenotype than the L514F mutant. The dependence of the phenotypes caused by the G495V and E506A mutations upon the amino acid present at position 514 is indicative of interactions between these regions of the protein, either involving these residues directly or transmitted through conformational changes.

The results from the double-mutant combinations revealed two interesting points (Tables 4 and 5). First, all the viable double mutants combining either the K310T or the T311K mutation with mutations that map between positions 455 and 521 invariably show additivity of the termination phenotypes. This suggests that the domains including amino acids 300 to 325 and 455 to 521 participate separately in the events leading to termination. In contrast, pairs of mutations within region 455 to 521 can show additivity but often exhibit partial additivity (H519P plus T471S, E506A plus Q493H, and T521A plus E478K) or antagonistic effects (T455I plus K512T or T521A and H519P plus T471R or E478D). This indicates that the contribution of some of the amino acids to the function of this region depends upon their interactions with other amino acids. Thus, we conclude that the highly conserved region 487 to 521 and the moderately variable region 455 to 486 participate in the same aspect of termination.

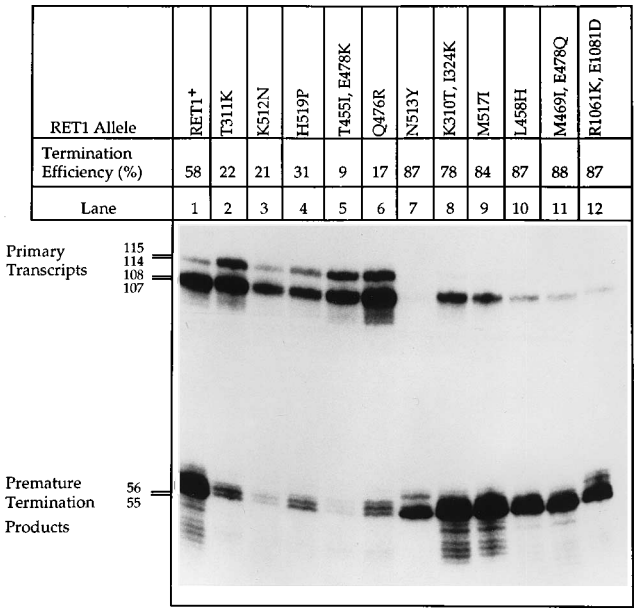

FIG. 9. In vitro termination efficiencies for the *SUP4-U(IV)* template. Shown above the autoradiograph are the *ret1* mutant alleles whose cell extracts were used in the in vitro transcription experiment and the calculated values of termination efficiency for each of them. The first five mutants from the left are reduced-termination mutants, while the following six are increased-termination mutants. Sizes are shown in nucleotides.

In vitro termination phenotypes. Transcription extracts were prepared from some of the strongest mutants from each of the four regions investigated. These extracts were then used to transcribe the *SUP4* allele *SUP4-U(IV)*. The termination efficiency on this template was calculated as the molar ratio of the RNA product terminating at the sequence of five T's in the intron to the sum of both long and short RNA products (T_5 + T_7GT_6 termination). All of the mutants tested showed an altered efficiency of termination that correlated with their in vivo termination phenotype (Fig. 9). Detailed examination of the long *SUP4* transcripts made by polymerase III from several of the strongest reduced-termination mutants showed that they terminate within the T_6 element in T_7GT_6 rather than in T_7, as does wild-type polymerase III. All of these transcripts, whether they end within T_7 or within T_6, are processable to mature suppressor tRNA and can affect colony color correspondingly.

To further characterize this large set of altered-termination mutations, we have carried out single-cycle elongation kinetic measurements and measured mutant polymerase termination efficiencies under a variety of conditions (unpublished data). Several of the increased termination mutants show impaired elongation, much like that seen for *E. coli* RNA polymerase bearing the B8 mutant allele of *rpoB* (20). These properties indicate that the concept of kinetic competition, invoked for *E. coli* RNA polymerase (51), may also apply to eukaryotic RNA polymerase III. Other of the polymerase III mutant alleles that we describe here exhibit more complex patterns of in vitro

elongation and termination, reinforcing the inferences that we draw here from in vivo mutant phenotypes. Termination occurs by a multistep pathway that involves different regions of the RET1 protein in its various stages.

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