Amino acid changes in conserved regions of the β -subunit of *Escherichia coli* RNA polymerase alter transcription pausing and termination

Robert Landick,¹⁻³ Judith Stewart,² and Donna N. Lee²

Departments of ²Biology and ³Biochemistry and Molecular Biophysics, Washington University, St. Louis, Missouri 63130 USA

Control of transcription at pause and termination sites is common in bacteria. Many transcriptional pause and termination events are thought to occur in response to formation of an RNA hairpin in the nascent transcript. Some mutations in the β -subunit of *Escherichia coli* RNA polymerase that confer resistance to the transcription inhibitor rifampicin also alter the response to transcriptional pause and termination signals. Here, we report isolation of termination-altering mutations that do not confer rifampicin resistance and show that such mutations occur predominantly in limited regions of the β -subunit polypeptide. One region is between amino acid residues 500 and 575, which encompasses the locations of almost all known rifampicin-resistance mutations. Many termination-altering mutations also occur in two other regions: between amino acid residues 740 and 840 and near the carboxyl terminus of the β -subunit (amino acid residues 1225–1342). Amino acid sequences in these three regions of the β -subunit are conserved between prokaryotic and eukaryotic β -subunit homologs. Several mutations that alter transcription termination *in vitro* affect amino acid residues that are identical in prokaryotic and eukaryotic RNA polymerase β -subunit homologs, suggesting that they alter an important function common to multisubunit RNA polymerases. We propose that these three regions of the β -subunit may contact the nascent RNA transcript, the RNA–DNA heteroduplex, or the DNA template in the transcription complex and that mutations in these regions alter transcription pausing and termination by affecting these contacts.

[Key Words: RNA polymerase; β -subunit mutations; transcription termination; transcription pausing]

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In *Escherichia coli*, transcriptional regulation occurs both at initiation and during elongation (Hoopes and McClure 1987; Landick and Yanofsky 1987a; Yager and von Hippel 1987). Two events that affect transcript elongation are pausing and termination; both are thought to occur in response to formation of an RNA hairpin in the nascent transcript (Yager and von Hippel 1987). Pausing and termination also can occur at sites where no potential RNA secondary structure is apparent ("sequence-dependent" pausing and ρ -dependent termination; see Yager and von Hippel 1987). RNA hairpins that signal termination differ from pause RNA hairpins, chiefly by the absence of the oligo(U) tract that immediately follows termination RNA hairpins (Brendel et al. 1986; Yager and von Hippel 1987). [Note: Several terms have been used to describe termination of transcription by *E. coli* RNA polymerase caused by RNA hairpin formation: ρ -independent, factor-independent, simple, and intrinsic. To avoid suggesting a particular mechanism, we will refer to transcription termination by *E. coli* RNA

polymerase in the absence of ρ -factor at the *E. coli* and *Serratia marcescens trp* attenuators simply as termination.] In general, termination RNA hairpins also contain a higher percentage of GC base pairs and fewer unpaired regions than pause RNA hairpins. The mechanisms of pausing and termination at sites where formation of an RNA hairpin in the nascent transcript can occur are thought to be related (Farnham and Platt 1980, 1981; Platt 1986; Yager and von Hippel 1987). However, the means by which nascent transcript RNA hairpins elicit transcription pausing and termination are not resolved. In particular, the structural changes in the transcription complex that must occur at pause and termination sites and the components of RNA polymerase that are involved remain unknown.

Core *E. coli* RNA polymerase consists of four essential subunits, β' , β , and two α , and one dispensable subunit, ω (Burgess et al. 1987; Gentry and Burgess 1989). β' and β show significant amino acid sequence similarities to the largest and second largest subunits, respectively, of all known multisubunit RNA polymerases in both prokaryotes and eukaryotes (Allison et al. 1985; Biggs et al.

¹Corresponding author.

1985; Falkenburg et al. 1987; Sweetser et al. 1987; Hudson et al. 1988; Patel and Pickup 1989). Kornberg and co-workers (Darst et al. 1989) have proposed a structure for the *E. coli* RNA polymerase holoenzyme that contains a 25×50 Å cleft that may bind the DNA template, much as was found for the Klenow fragment of *E. coli* DNA polymerase I (Ollis et al. 1985). Of the *E. coli* RNA polymerase subunits, most is known about β . All antibiotic-resistance mutations that block binding of the transcription inhibitor rifampicin to RNA polymerase map in *rpoB*, the gene for the β -subunit (Jin and Gross 1988). The β -subunit probably is involved in the catalytic reaction. It reacts at lysine and histidine residues between 1047 and 1066 and between 1228 and 1244 with photoreactive nucleoside triphosphate analogs (Grachev et al. 1987, 1989) and at unknown locations with a BrdU-containing DNA template (Simpson 1979) and with short nascent transcripts containing photoreactive 5' ends (Hanna and Meares 1983). Mutations in the β -subunit affect both the binding of the σ -factor (Glass et al. 1986a) and promoter recognition (Nomura et al. 1984; Glass et al. 1986b). Thus, the β -subunit has a central role in the transcription cycle.

The response of *E. coli* RNA polymerase to transcriptional pause or termination signals is altered by β -subunit amino acid substitutions that cause rifampicin resistance (Neff and Chamberlin 1980; Yanofsky and Horn 1981; Fisher and Yanofsky 1983; Jin et al. 1988). At least one Rif^r RNA polymerase (*rpoB8*) increases both transcription pausing and termination in vitro, whereas a second (*rpoB2*) decreases both (Fisher and Yanofsky 1983). Almost all Rif^r mutations occur between amino acids 500 and 575 of the β -subunit polypeptide (Jin and Gross 1988), and most show some alteration in vivo in either ρ -dependent or ρ -independent termination (Jin et al. 1988). These findings suggest that the β -subunit is involved in the response to RNA hairpin regulatory signals; however, it is unclear whether interaction with RNA hairpins is the natural function of the region of the β -subunit to which rifampicin binds or whether Rif^r mutations affect the response to nascent transcript RNA hairpins by altering some other interaction.

To increase our understanding of the role of the RNA polymerase β -subunit in termination and pausing, we wished to determine what types of termination-altering mutations could be obtained in *rpoB*, without limiting our search to rifampicin-resistance mutations. To simplify our experiment, we used the β -subunit overproducing plasmid pRL385, which allows dominant expression of a plasmid-borne mutant *rpoB* gene in a strain containing a wild-type chromosomal *rpoB* gene (Landick et al. 1990). We performed bisulfite mutagenesis of *rpoB* on gapped duplex derivatives of pRL385, thus targeting mutations to defined regions of the gene. Using a screening technique that can detect changes in termination in vivo, we identified regions of *rpoB* that could alter transcription termination when mutagenized.

We found that these mutations occur in clusters that include, but are not limited to, the Rif^r region. Many of the amino acid substitutions observed between residues

500 and 575, the Rif^r region, alter termination without producing resistance to rifampicin. Termination-altering mutations were clustered in regions that are conserved among prokaryotic and eukaryotic β -subunit homologs. We show here that for at least some of these mutations, purified mutant RNA polymerase exhibits the predicted transcriptional termination behavior in vitro. However, some of the altered enzymes respond differently to two different transcriptional termination signals. One very interesting mutation, *rpoB5101*(PS560, TI563), not only alters termination but also essentially abolishes transcription pausing in vitro. [Note: Amino acid substitutions are designated with the one-letter code for the wild-type residue, followed by the one-letter code for the mutant residue, followed in turn, by the position in the β -subunit polypeptide where the substitution occurred (i.e., PS560 is Pro $\rightarrow$ Ser at position 560).] Our findings are consistent with the interpretation that these localized mutations affect segments of the β -subunit that contact the DNA template or the nascent transcript, either as an RNA–DNA heteroduplex or in a transcript-binding site, and thus affect transcription pausing and termination either directly through these contacts or by altering the tendency for formation of RNA hairpins in the nascent transcript.

Results

Increased or decreased transcription termination by RNA polymerase containing a plasmid-encoded, mutant β -subunit can be detected in vivo

To determine which regions of the β -subunit are important for regulation of transcript elongation, we wished to detect altered termination resulting from expression of mutagenized derivatives of the pRL385 *rpoB* gene (Landick et al. 1990). To detect termination-altering mutations directly, we needed a screening method that was sensitive to increased or decreased termination. To detect increased termination, we screened for resistance to 5-methylanthranilic acid (5-MAA^r), which results from decreased synthesis of toxic 5-methyltryptophan when termination at the *trp* attenuator is increased (Yanofsky and Horn 1981). To detect decreased termination, we screened for chloramphenicol resistance (Cm^r) that resulted from decreased termination at a simple ρ -independent terminator ($\Delta trpL430$; Stroynowski and Yanofsky 1982) preceding a *cat* gene. We used a strain that allowed detection of both of these phenotypes and contained a deletion of *recA* to minimize recombination between the plasmid-borne and chromosomal *rpoB* genes (see Materials and methods).

To verify that these screening methods could detect termination-altering mutations in the *rpoB* gene on plasmid pRL385, we tested the effects of Rif^r mutations known to alter termination. We grew strains containing plasmids that carried either wild-type *rpoB*, a Rif^r mutation that decreases transcription termination [*rpoB2* (HY526); Yanofsky and Horn 1981; Jin et al. 1988], or a Rif^r mutation that increases transcription termination

[*rpoB*7 (IF572); Yanofsky and Horn 1981; Jin et al. 1988] and then replica-plated them to appropriate selective media (see Materials and methods). Only the strains carrying pRL385*rpoB*2 and pRL385*rpoB*7 grew in the presence of rifampicin, only the strain carrying pRL385*rpoB*2 grew in the presence of chloramphenicol, and only the strain carrying pRL385*rpoB*7 grew in the presence of 5-MAA (Fig. 1). Thus, enough plasmid-encoded, altered β -subunit displaced the chromosomally encoded β -subunit to produce the expected phenotypes. Based on these results, we concluded that screening for 5-MAA^r and Cm^r would allow detection of altered termination following site-directed mutagenesis of pRL385. However, these screens also would detect changes in transcription initiation.

Bisulfite-induced mutations that alter termination are localized in certain regions of rpoB

We chose bisulfite-induced cytosine deamination as the technique most amenable to targeted mutagenesis of selected regions of *rpoB*. To employ the gapped duplex approach (Shortle and Nathans 1978; Folk and Hofstetter 1983), we prepared a set of pRL385 derivatives in which 200- to 400-bp segments of *rpoB* were deleted and replaced with an *Xho*I linker. We constructed 13 deletion plasmids that divided the *rpoB* gene into 13 nonoverlapping intervals (Fig. 2). We then mutagenized with bisulfite partially single-stranded, wild-type pRL385 that had been hybridized with the individual deletion plasmids (see Materials and methods). After recovery, repair, and amplification (see Materials and methods), mutagenized plasmids were transformed into the test strain and 500 isolates from each mutagenized sample were screened for altered termination and for rifampicin resistance.

All three phenotypes (Rif^r, 5-MAA^r, and Cm^r) were recovered from samples mutagenized in some, but not all, intervals (Fig. 2). DNA sequencing and reconstruction experiments (see below) eventually revealed that several isolates either were due to changes in unintended locations (three Rif^r isolates from interval 5-mutagenized DNA) or contained no change in the expected interval (several isolates with weak phenotypes, most notably in interval 12). These samples were dropped from the distribution of mutations described here. Thus, legitimate mutations leading to Rif^r were recovered only from the interval 7-mutagenized sample. Interval 7 encompasses the locations of almost all known Rif^r mutations (Jin and Gross 1988). Mutation to 5-MAA^r (increased termination) was limited to 5 of the 13 intervals (Fig. 2), with the greatest numbers coming from samples mutagenized in interval 7 (23 isolates) and interval 13 (22 isolates). Fourteen of the 23 5-MAA^r derivatives recovered from interval 7-mutagenized samples also were Rif^r; however, the remaining nine were Rif^s. All of the 5-MAA^r colonies recovered from interval 5-, 6-, 9-, and 13-mutagenized samples were Rif^s. We recovered Cm^r colonies from samples mutagenized in 8 of the 13 intervals (Fig. 2). Again, the greatest numbers of putative termination-altering mutations were recovered from interval 7-mu-

Figure 1. Detection of increased and decreased transcription termination caused by the presence of plasmid pRL385 carrying known *rpoB* mutations. *E. coli* strain RL211 transformed with the indicated pRL385 derivatives (wild-type, *rpoB*2, or *rpoB*7) was inoculated onto an LB plate containing 100 μ g ampicillin/ml and 1 mM IPTG and incubated overnight at 37°C to form the master plate. Bacteria were transferred from the master plate to selective plates and grown, as described in Materials and methods.

tagenized (30 isolates) and interval 13-mutagenized (27 isolates) samples. Fourteen Cm^r colonies recovered from interval 7-mutagenized samples were Rif^r. We recovered no legitimate 5-MAA^r, Cm^r, or Rif^r colonies from samples mutagenized in intervals 1, 3, 10, 11, and 12.

Interestingly, several of the isolates resistant to 5-MAA also were resistant to chloramphenicol (interval 5, 5 of 7; interval 7, 4 of 23; interval 13, 4 of 22), apparently reflecting a respective increase and decrease in termination at the two terminators we used. This seemingly contradictory result may be explained by differences in the way RNA polymerase transcribes these termination sites in vivo. The wild-type *trp* leader is subject to translationally coupled attenuation, for which a transcriptional pause site is an essential component and where alternative RNA folding may complicate the response of mutant polymerases (Landick and Yanofsky 1987a), whereas Δ *trpL430* is a simple, ρ -independent terminator (Stroynowski and Yanofsky 1982). Differential effects on initiation also cannot be ruled out.

To determine the average number and distribution of C $\rightarrow$ T changes in our samples, we sequenced 48 randomly selected isolates from both interval 7- and interval 9-mutagenized DNAs. The number of changes per template in these samples was very close to that predicted by ideal single-hit kinetics (Figs. 3A and 4A). Furthermore, the distribution of changes within these two intervals was not obviously biased (Figs. 3B and 4B). To determine whether changes occurred outside of the targeted regions, we sequenced both intervals 7 and 9 in these 96 random isolates. Only one unintended change

Figure 2. Number of transformants per 500 screened that grew on selective media in each of 13 intervals of *rpoB*. Screening was conducted as described in Materials and methods. The phenotypes indicated are resistance to 100 μ g rifampicin/ml (rifampicin resistance); resistance to 100 μ g 5-MAA/ml, indicative of increased termination (increased termination); and resistance to 10 μ g chloramphenicol/ml, indicative of decreased termination (decreased termination). The height of the bar corresponds to the total number of mutants found with the indicated phenotype in the interval where the bar is positioned. The number of mutants that were sequenced is indicated by the height of the shaded region. The number of unique mutants that correspond to single amino acid changes is indicated by the height of the black area. For interval 7, isolates that contained known *Rif^R* mutations, alone or in combination with other changes, are included in statistics for total and sequenced isolates but not for those with unique or single mutations. Isolates that were both 5-MAA^r and Cm^r were included in the numbers for each of the phenotypes. Lightly shaded regions within the box labeled *rpoB* indicate the positions of known *Rif^R* mutations. Hinge (wavy lines) and nucleotide cross-linking regions (crosshatched lines) are described in the Discussion. Regions conserved among bacterial [*E. coli* (Ovchinnikov et al. 1981); *Salmonella typhimurium* (Lisitsyn et al. 1988)]; spinach chloroplast (Hudson et al. 1988)] β -subunits are indicated by the lightly shaded boxes. Regions conserved between the *E. coli* β -subunit and the second largest subunits of yeast (Sweetser et al. 1987) and *Drosophila* (Falkenburg et al. 1987) RNA polymerase are indicated by the heavily shaded boxes.

was found, suggesting that the odds of a change outside the target on any given plasmid were on the order of 1 in 10. This estimate agrees with 10 of the 107 sequenced plasmids from interval 5-, 7-, 9-, and 13-mutagenized samples that contained no sequence changes in the expected intervals. Thus, we were satisfied that the distribution of samples producing termination-altering phenotypes (Fig. 2) is a reasonable representation of the distribution of possible termination-altering mutations in *rpoB*.

Termination-altering substitutions occur at a limited number of rpoB codons

To further characterize these mutations, we chose to concentrate on the four intervals that showed the

highest frequency of termination-altering mutations: intervals 5, 7, 9, and 13. Interestingly, three of these (intervals 7, 9, and 13) are regions that contain significant sequence similarity to the β -subunit homolog in eukaryotic RNA polymerase II (see Discussion; Figs. 2–5). We sequenced the appropriate region of most of the plasmids that were derived from samples mutagenized in these four intervals and that appeared to alter termination or cause rifampicin resistance (Fig. 2). Some plasmids were not sequenced because they did not produce single-stranded DNA, perhaps owing to the properties of the altered RNA polymerase present in these isolates.

Many isolates had the same amino acid changes. For example, only 4 of the 36 *Rif^R* derivatives specified amino acid changes that had not been reported pre-

Figure 3. Sequence of amino acid changes in interval 7. (A) Number of C → T changes per plasmid. The results are given as the percentage of the 48 plasmids examined. (B) Distribution of C → T changes in a randomly selected population of pRL385 mutagenized in interval 7. The positions of C → T changes are plotted relative to the codons indicated on the sequence in C. (C) Amino acid changes and phenotypes of termination-altering and rifampicin-resistant isolates of pRL385 mutagenized in interval 7. Isolates that contained known Rif^r mutations, alone or in combination with other changes, are not shown. Phenotypes are indicated by – when no growth was detected, +/- when weak growth was detected, and + when definite growth was detected. For Cm^r, + indicates resistance to 10 μg chloramphenicol/ml and ++ indicates resistance to 20 μg chloramphenicol/ml. The *E. coli* β-subunit amino acid residues that are conserved in the yeast (Sweetser et al. 1987) and *Drosophila* (Falkenburg et al. 1987) β-subunit homologs are underlined. Possible bisulfite-induced mutations are indicated below the sequence of interval 7. (m) Amber mutation. The positions of known Rif^r mutations (Jin et al. 1988) are shown below the possible bisulfite-induced mutations. (Δ) Deletion. Overlined sequence indicates insertion.

viously to cause rifampicin resistance [*rpoB5077*(SF512), *rpoB5085*(SF512, TI563), *rpoB5091*(PL552, PL560), and *rpoB5135*(AV532, PL535, TI563); Fig. 3]. A strong Rif^r phenotype for the alleles *rpoB5091* and *rpoB5135* and a weak Rif^r phenotype for *rpoB5077* and *rpoB5085* were confirmed by growth in the presence of rifampicin of transformants that were temperature sensitive for chromosomal *rpoB* expression and by in vitro transcription assays with crude extracts (data not shown). In addition, several previously characterized Rif^r derivatives were isolated repeatedly, either as single changes or in combination with additional substitutions [e.g., *rpoB2*(HY526), 14 times, and *rpoB111*(PL564), 8 times].

Similarly, certain amino acid changes were found repeatedly in isolates from intervals 7 and 9 that exhibited altered termination phenotypes. For example, in interval 9 the SF788 change was found in five of the seven unique derivatives that gave the decreased termination phenotype (Fig. 4). Interestingly, the SF788 change alone gave a very weak decreased termination phenotype, whereas in combination with a second change it sometimes produced a stronger phenotype. We recovered only

3 single changes leading to altered termination of the possible 43 bisulfite-induced changes in interval 9.

A similar bias was observed among termination-altering mutations in interval 7 (Fig. 3). Here, single changes leading to altered termination were recovered at 10 of 43 possible sites. However, changes in certain regions and particular codons within the 500–575 region appeared repeatedly in plasmids that produced an altered termination phenotype. For instance, six of seven derivatives that gave the strongest decreased termination phenotype had changes in codons 560–565. Four of these were double or triple mutants that included the TI563 change, although this change, alone, had a weaker decreased termination phenotype.

In contrast, the distributions of unique termination-altering mutations in intervals 5 and 13 were not clustered (Fig. 5). Amino acid changes throughout these regions appeared to alter elongation, although in interval 5, only 5 of the possible 35 changes were recovered as termination-altering mutations and no change was recovered more than once. In interval 13, 10 of the possible 36 changes were recovered, but certain changes

Figure 4. Sequence of amino acid changes in interval 9. (A) Number of C → T changes per plasmid. The results are given as the percentage of the 48 plasmids examined. (B) Distribution of C → T changes in a randomly selected population of pRL385 mutagenized in interval 9. The positions of C → T changes are plotted relative to the codons indicated on the sequence in C. (C) Amino acid changes and phenotypes of termination-altering isolates of pRL385 mutagenized in interval 9. Mutations, phenotypes, and conserved regions are indicated as described in the legend to Fig. 3.

were isolated repeatedly, suggesting that we have found most of the bisulfite-inducible, termination-altering mutations in this interval that would be viable in our experiment. PL1258 was isolated five times as a single change and twice in combination with other changes; the PS1258 RC1301 change was isolated twice. Also, AV1277 was isolated six times as a single change. Because we obtained mutations only in a limited number of the bisulfite-sensitive codons, we conclude that certain amino acid residues within intervals 5, 7, 9, and 13 are particularly important for control of transcript elongation.

In intervals 5, 7, and 9, most of the plasmids that produced the strongest termination-altering phenotypes contained two amino acid changes in the affected region, even though approximately half of the randomly selected populations of interval 7- and 9-mutagenized samples contained only a single-base change (Figs. 3A and 4A). In these regions, apparently, multiple amino acid changes produced stronger termination-altering phenotypes than single amino acid changes. In contrast, we recovered single amino acid changes in 20 of the 25 plasmids mutagenized in interval 13 that we were able to sequence.

To verify that the phenotypes we observed were caused by the amino acid changes detected in the mutagenized intervals, we excised a small DNA fragment containing the mutagenized region from several plasmids and ligated it into appropriately prepared, wild-type plasmid (see Materials and methods). Reconstruction experiments were performed on *rpoB5080*(RC368) and *rpoB5074*(RC352, IT433) from interval 5, *rpoB5081*(PS806) and *rpoB5116*(SF788) from interval 9, and *rpoB5117*(SF1322) from interval 13. The reconstructed plasmids were screened as described above,

and in each case, the same termination-altering phenotype was observed. Thus, for intervals 5, 9, and 13, it is clear that the altered termination phenotype can be caused by mutations in the designated areas. We were less concerned with this control for interval 7 mutations, because analysis of Rif^r mutations already has established that altered termination can result from changes in this region (Jin et al. 1988).

Amino acid substitutions in purified RNA polymerases significantly alter transcript elongation in vitro

To confirm that the mutations we characterized cause changes in termination, we purified and tested in vitro RNA polymerases containing several of the altered β -subunits (see Materials and methods). To assure that the purified enzymes contained little or no wild-type RNA polymerase, we transformed the mutant plasmids into an *E. coli* strain with an amber mutation in the chromosomal *rpoB* gene and a temperature-sensitive *supD* (amber suppressor) gene that was inactive at 40°C (see Materials and methods). RNA polymerases were prepared from transformants grown at 40°C, thus eliminating the source of wild-type β -subunit. This approach prevented us from purifying RNA polymerase containing many of the altered β -subunits because they would not support growth at 40°C. Either these mutant RNA polymerases are temperature-sensitive, themselves, or they are recessive-lethal and cannot support growth in the absence of some wild-type β -subunit.

We used the purified, mutant RNA polymerases to measure transcription pausing and termination in vitro (Table 1; Fig. 6). Assays were performed by using DNA templates on which the very strong A1 *E. coli* RNA polymerase promoter from bacteriophage T7 was fol-

A

			340	350	360	370	380	390	400	410	420	430															
			LFTNDLDHGPYISETLRVDPNTDRLSALVEIYRMMRPGEPTREAAESLFENLFFSEDRYDLSAVGRMKFNRSLLREEIEGSGILSKDDIIDVMKKLDIRNGKG																								
			Possible Bisulfite-induced mutations	Y	L	F	I	C	L	I	C	V	C	CL	LL	CV	V	F	C	FV	C	CF	C	F	FT		
			S									S			SS												
Rif ^r	MAA ^r	Cm ^r																									
-	+	++	<i>rpoB5070</i>									I															
-	+	+/-	<i>rpoB5080</i>											C													
-	-	+	<i>rpoB5136</i>													L											
-	-	++	<i>rpoB5075</i>														V										
-	+	+	<i>rpoB5072</i>																					F			
-	+	+	<i>rpoB5137</i>										S														
-	+	-	<i>rpoB5074</i>													L									T		
-	-	+/-	<i>rpoB5138</i>																						T		

B

			1230	1240	1250	1260	1270	1280	1290	1300	1310	1320	1330	1342										
			PVTVGYMYMLKLNHLVDDKMHARSTGSYSLVLTQORLGKKAQFGGQRFGEVWVALEAYGAAYTLQEMLTVKSDDVNGRTKMYLNIIVDGNHMEFGMPESFNVLKLRIRSLGINIELEDE																					
Possible Bisulfite-induced mutations	I	Y	YVCF	I	F	IrrnL	Vm	mC	V	V	VV	I	m	FI	F	CI	Ym	L	L	F	CF			
Rif ^r	MAA ^r	Cm ^r																						
-	+	-	<i>rpoB5131</i>		Y																			
-	+	+/-	<i>rpoB5120</i>				L																	
-	+	-	<i>rpoB5124</i>						V															
-	+	-	<i>rpoB5125</i>							V														
-	-	+	<i>rpoB5127</i>								V													
-	+	+	<i>rpoB5129</i>									I												
-	-	+/-	<i>rpoB5128</i>													I								
-	-	+/-	<i>rpoB5119</i>														S							
-	+	++	<i>rpoB5117</i>															F						
-	+/-	++	<i>rpoB5126</i>																		F			
-	-	+	<i>rpoB5118</i>				L				V													
-	+	-	<i>rpoB5130</i>				L									C								
-	+/-	-	<i>rpoB5121</i>				S									C								

Figure 5. Sequence of amino acid changes in intervals 5 and 13. Mutations, phenotypes, and conserved regions are indicated as described in the legend to Fig. 3. (A) Amino acid changes and phenotypes of termination-altering isolates of pRL385 mutagenized in interval 5. (B) Amino acid changes and phenotypes of termination-altering isolates of pRL385 mutagenized in interval 13.

lowed by one of the terminators used to screen for termination-altering mutations (wild-type *trp* or Δ *trpL430*) and then the strong T1 terminator from the *rnnB* rRNA operon of *E. coli* (see Materials and methods; Fig. 6; Table 1). We found that the response to transcriptional pause or termination signals was altered for nearly all of the enzymes that we tested (Table 1). Several from 5-MAA^r isolates exhibited increased termination at 30°C at *trpL*, consistent with their in vivo phenotype [e.g., *rpoB5074*(RC352, IT433) and *rpoB5137*(PS345, PL372); Table 1]. However, most RNA polymerases from Cm^r isolates did not terminate less often at Δ *trpL430*, although many were less efficient at *trpL* [e.g., *rpoB5101*(PS560, TI563) and *rpoB5102*(PS560); Table 1]. Differences in the arrangements of promoters and terminators used in vivo (wild-type *trp* and Δ *trpL430*) and in vitro (T7 A1-*trp* and T7 A1- Δ *trpL430*) could account for some of these discrepancies. For example, termination by wild-type RNA polymerase at Δ *trpL430* in vitro was unexpectedly inefficient (Table 1), perhaps owing to an effect on termination efficiency of the promoter or early transcribed region (Golinger et al. 1989; Telesnitsky and Chamberlin 1989a). It also is possible that some of the mutations affect termination in vivo by al-

tering interactions with a termination-modulating factor, such as NusA, which was absent from the in vitro assays, or that effects of some mutations on transcription initiation are superimposed on the termination phenotypes in vivo.

The most dramatic change in transcript elongation was observed with *rpoB5101*(PS560, TI563) RNA polymerase, which nearly eliminated pausing at the *trpL* pause site (Fig. 6), decreased termination at some sites (*trpL* and *rnnB* T1 at 37°C; Δ *trpL430* at 30°C), and increased it at others (*trpL* and *rnnB* T1 at 30°C; Δ *trpL430* at 37°C; Table 1). Several other clear cases of differential response to elongation control signals were observed. At 37°C, *rpoB5108*(SF772, SF788) and *rpoB5110*(TI830) RNA polymerases terminated at the *trpL* attenuator similarly to the wild-type enzyme but transcribed through the subsequent *rnnB* T1 terminator two to three times more frequently than wild type (Table 1). Also, two mutant RNA polymerases [*rpoB5074*(RC352, IT433) and *rpoB5102*(PS560)] had increased half-lives at the *trpL* pause site yet terminated less efficiently at the *trpL* attenuator.

From these experiments we concluded that (1) the most dramatic change in elongation behavior occurred

Table 1. Transcription pausing and termination by mutant RNA polymerases

<i>rpoB</i> allele	Interval	Amino acid change(s)	Phenotype ^a		<i>trpL</i> pause half-life ^b	Termination efficiency (37°C)			Termination efficiency (30°C)		
			5-MAA ^c	Cm ^r		<i>trpL</i> ^c	<i>rrnB</i> T1 ^d	$\Delta trpL430$ ^e	<i>trpL</i> ^c	<i>rrnB</i> T1 ^d	$\Delta trpL430$ ^e
<i>rpoB</i> (wt)			–	–	44 ± 2	67 ± 2	71 ± 5	23 ± 2	40 ± 2	57 ± 6	34 ± 1
<i>rpoB5080</i>	5	Arg ₃₆₈ →Cys	+	±	40 ± 8	75 ± 2	74 ± 2	27 ± 4	49 ± 2	54 ± 10	39 ± 4
<i>rpoB5074</i>	5	Arg ₃₅₂ →Cys Ile ₄₃₃ →Thr	+	–	75 ± 7	55 ± 4	62 ± 8	24 ± 6	68 ± 3	63 ± 10	48 ± 6
<i>rpoB5137</i>	5	Pro ₃₄₅ →Ser Pro ₃₇₂ →Leu	+	+	72 ± 19	81 ± 2	91 ± 22	72 ± 20	79 ± 4	68 ± 14	42 ± 20
<i>rpoB8^f</i>	7	Gln ₅₁₃ →Pro	+	–	84 ± 10	95 ± 5	ND	ND	ND	ND	ND
<i>rpoB101^f</i>	7	Gln ₅₁₃ →Leu	–	–	ND	87 ± 5	57 ± 16	39 ± 9	76 ± 3	74 ± 15	ND
<i>rpoB2^f</i>	7	His ₅₂₆ →Tyr	–	+	18 ± 4	50 ± 5	41 ± 6	30 ± 10	26 ± 3	ND	ND
<i>rpoB7^f</i>	7	Ile ₅₇₂ →Phe	+	–	50 ± 6	81 ± 2	79 ± 8	21 ± 2	64 ± 2	73 ± 10	ND
<i>rpoB5009</i>	7	Pro ₄₉₇ →Ser	–	+	42 ± 6	91 ± 15	50 ± 20	ND	50 ± 5	ND	ND
<i>rpoB5002</i>	7	Thr ₅₆₃ →Ile	–	+	20 ± 4	58 ± 3	ND	19 ± 2	62 ± 2	ND	21 ± 3
<i>rpoB5102</i>	7	Pro ₅₆₀ →Ser	–	++	83 ± 13	44 ± 3	83 ± 6	22 ± 4	68 ± 2	66 ± 11	60 ± 3
<i>rpoB5101</i>	7	Pro ₅₆₀ →Ser Thr ₅₆₃ →Ile	–	++	X	42 ± 2	51 ± 2	40 ± 4	69 ± 3	80 ± 17	9 ± 5
<i>rpoB5108</i>	9	Ser ₇₇₂ →Phe Ser ₇₈₈ →Phe	–	+	18 ± 4	66 ± 2	19 ± 2	21 ± 2	51 ± 2	ND	32 ± 4
<i>rpoB5110</i>	9	Thr ₈₃₀ →Ile	–	±	43 ± 10	79 ± 2	42 ± 5	26 ± 5	57 ± 3	56 ± 9	51 ± 4
<i>rpoB5116</i>	9	Ser ₇₈₈ →Phe	–	±	17 ± 5	75 ± 2	60 ± 5	35 ± 7	41 ± 5	45 ± 8	29 ± 40
<i>rpoB5118</i>	13	Pro ₁₂₅₈ →Leu Ala ₁₂₈₃ →Val	–	+	30 ± 12	68 ± 3	76 ± 7	22 ± 2	48 ± 3	64 ± 8	ND
<i>rpoB5117</i>	13	Ser ₁₃₂₂ →Phe	+	++	51 ± 8	40 ± 3	87 ± 18	19 ± 3	55 ± 5	59 ± 11	62 ± 3
<i>rpoB5126</i>	13	Ser ₁₃₃₂ →Leu	±	++	22 ± 3	74 ± 2	52 ± 2	18 ± 1	46 ± 2	ND	23 ± 1
<i>rpoB5127</i>	13	Ala ₁₂₈₄ →Val	–	+	48 ± 7	72 ± 2	84 ± 6	26 ± 2	48 ± 2	ND	45 ± 5
<i>rpoB5120</i>	13	Pro ₁₂₅₈ →Leu	+	±	51 ± 5	43 ± 3	87 ± 20	22 ± 2	64 ± 4	69 ± 11	54 ± 3

(X) *rpoB5101* RNA polymerase did not pause at the *trpL* pause site (see Fig. 6).

(ND) Not determined.

^aPhenotypes caused by the presence in RL211 of pRL385 carrying the *rpoB* mutations. Symbols are as described in the legend to Fig. 3.

^b*trpL* pause RNA half-life determined as described in Materials and methods. A 2 s.d. error is from the regression analysis.

^cPercent termination at the *trpL* terminator, determined as described in Materials and methods. The template used is shown at the bottom of Fig. 6. A 2 s.d. error from the AMBIS Radioanalytic Imaging System was propagated in the calculation of percent termination.

^dPercent termination at the *rrnB* T1 terminator, determined as described in Materials and Methods. The template used is shown at the bottom of Fig. 6. A 2 s.d. error is as described for the *trpL* terminator.

^ePercent termination at the *S. marcescens* $\Delta trpL430$ terminator (Stroynowski and Yanofsky 1982). The template used was similar to that shown at the bottom of Fig. 6, except that DNA from *S. marcescens* $\Delta trpL430$ replaced the *trp* leader fragment. A 2 s.d. error is as described for the *trpL* terminator.

^f*rpoB2*, *rpoB7*, *rpoB8*, and *rpoB101* are previously identified Rif^r *rpoB* mutations that alter transcription pausing or termination [Fisher and Yanofsky 1983; Jin et al. 1988]. Data on these mutant RNA polymerases are given here to allow comparison with the directly selected, termination-altering *rpoB* mutations.

when RNA polymerase was altered in interval 7; (2) RNA polymerases altered in interval 5 exhibited the least severe effects on termination in vitro; (3) with the exception of *rpoB5080*(RC368), every altered polymerase we examined exhibited a significantly altered elongation phenotype in at least one of our in vitro assays; (4) many mutations affected pausing and termination very differently; and (5) the effect of amino acid substitutions on termination efficiency varied, depending on temperature and the termination site examined (Fig. 6; Table 1). Because we examined only those alleles that permit cell growth in the absence of a functional *rpoB* gene, they are likely to have weak termination-altering phenotypes. Mutations with drastic effects on termination are likely to be lethal and could be present in plasmids unable to support growth of the *rpoB*(Am) *supD*(Ts) strain at 40°C.

Discussion

Not all termination-altering amino acid substitutions in *rpoB* cause rifampicin resistance

The primary goal of our work was to determine whether termination-altering *rpoB* mutations could be isolated without relying on the Rif^r phenotype. The unequivocal answer is yes. Not only are such mutations found outside of the residue 500–575 region (interval 7) that con-

tains most Rif^r mutations (Fig. 2), but also many mutations within the 500–575 region are Rif^s and can alter elongation at least as dramatically [e.g., *rpoB5101*(PS560, TI563)] as Rif^r mutations characterized to date [Yanofsky and Horn 1981; Fisher and Yanofsky 1983; Jin et al. 1988]. We also detected four previously undescribed Rif^r mutations in interval 7 [*rpoB5077*(SF512), *rpoB5085*(SF512, TI563), *rpoB5091*(PL552 PL560), and *rpoB5135*(AV532, PL535, TI563)], although the first two are only modestly resistant to rifampicin. We detected no Rif^r mutations outside the 500–575 region. Our findings are strong confirmation of the localization of Rif^r mutations described by Jin and Gross (1988). Although Rif^r mutations at codon 143 (Lisitsyn et al. 1984) and at or near codon 680 (Boothroyd et al. 1983; Jin and Gross 1988) have been described, the primary β -subunit contacts to bound rifampicin must be made by amino acid residues in the 500–575 region.

The complete distribution of termination-altering mutations in *rpoB* includes at least the four regions that we have characterized (intervals 5, 7, 9, and 13; Fig. 2). Because we detected Cm^r after mutagenesis of several other intervals, it is possible that other regions of the β -subunit are involved in transcript elongation. We have not yet purified RNA polymerases containing β -subunits altered in these other regions to test this possi-

Figure 6. In vitro assays to determine transcription pausing by RNA polymerases purified from strains expressing altered *rpoB* genes. Samples were withdrawn from synchronized in vitro transcription reactions at the times indicated and electrophoresed through 10% polyacrylamide, 7 M urea gels (see Materials and methods). One example is shown of a β -subunit altered in each of the four intervals that were characterized in detail. (A) Wild-type RNA polymerase; (B) *rpoB5080*(RC368) RNA polymerase; (C) *rpoB5101*(PS560, TI563) RNA polymerase; (D) *rpoB5116*(SF788) RNA polymerase; (E) *rpoB5118*(PL1258, AV1283) RNA polymerase. (P) *trpL* pause RNA; (L) RNA terminated at the *trpL* attenuator; (T1) RNA terminated at *rrnB* T1. Times at which the reactions were sampled are as follows. For A, B, and D: (lane 1) 30 sec; (lane 2) 60 sec; (lane 3) 90 sec; (lane 4) 150 sec; (lane 5) 4 min; (lane 6) 6 min. For C, as for A, B, and D, except lane 7, 10 min. For E: (lane 1) 30 sec; (lane 2) 60 sec; (lane 3) 2 min; (lane 4) 4 min; (lane 5) 6 min. Diagram at bottom shows schematically the DNA template used for in vitro transcription reactions. (PCR1 and PCR2) Positions of primers used to amplify the DNA template; (A20) position at which RNA polymerase stalls in the absence of UTP. Arrows within boxes designate positions of the segments of RNA secondary structures that form pause and termination signals. (1, 2, 3, and 4) Traditional designations for segments of the *trp* leader RNA that can form alternative secondary structures (Landick and Yanofsky 1987a).

bility. However, except for two putative interval 6 mutations, we did not detect the increased termination phenotype in samples mutagenized in intervals other than the four that we studied in detail (Fig. 2). It is likely that these four regions of β , particularly intervals 7, 9, and 13, contain the amino acid side chains that are most important to the role of the β -subunit in the response of RNA polymerase to transcriptional termination signals.

Very strong termination-altering mutations probably were not identified in this study. We would have detected only those termination-altering mutations that either are weak enough not to be lethal or that also inhibit β -subunit assembly into holoenzyme (Landick et al. 1990). Furthermore, two clusters of mutations, between residues 490 and 505 and residues 1275 and 1295, where a significant fraction of possible bisulfite-induced changes were recovered with termination-altering phe-

notypes, were not viable at 40°C in the *rpoB*(Am)*supD*(Ts) strain. Mutations in these areas may have strong termination-altering properties that we were unable to characterize. The strongest termination-altering mutations probably await description in experiments that can analyze dominant-lethal mutations.

Termination-altering, amino acid changes occur at positions conserved in phylogenetically diverse RNA polymerase β -subunit homologs

A very interesting aspect of our results is the occurrence of termination-altering mutations in regions of the β -subunit that are conserved between prokaryotic and eukaryotic RNA polymerases (Fig. 2; Falkenburg et al. 1987; Sweetser et al. 1987; Lisitsyn et al. 1988; Leffers et al. 1989; Patel and Pickup 1989). Intervals 7, 9, and 13 all

contain significant regions of amino acid similarity to the second largest subunits of yeast and *Drosophila* RNA polymerases (Fig. 2). Furthermore, within these regions, termination-altering mutations tend to occur at or near the conserved residues. In interval 7, the most significant amino acid conservation occurs from residue 550 to 575 (Fig. 3). We observed the strongest termination-altering phenotypes from mutations in which at least one of the amino acid residues in the 550–575 region was changed. Most notable is *rpoB5101*(PS560, T1563), which dramatically altered elongation in vitro (Fig. 6; Table 1) and which changes two conserved amino acid residues (Fig. 3). Additional termination-altering mutations at conserved residues may have been missed in our experiment due to a dominant-lethal phenotype.

A similar bias can be found for *Rif^r* mutations that alter transcription termination. Most *Rif^r* mutations occur between residues 510 and 535 (Fig. 3; Jin and Gross 1988). Within this region, *Rif^r* mutations have been found at five of the six positions that are conserved between prokaryotic and eukaryotic RNA polymerases (*rpoB5077* at Ser-512, described here; *rpoB8* and *rpoB101* at Gln-513, *rpoB114* at Ser-522, *rpoB2* at His-526, and *rpoB3401* and *rpoB3402* at Arg-529; see Jin and Gross 1988). Six of these seven *Rif^r* mutations (*rpoB5077*, *rpoB8*, *rpoB101*, *rpoB2*, *rpoB3401*, and *rpoB3402*) alter transcription termination (Table 1; see Fisher and Yanofsky 1983; Jin et al. 1988).

Termination-altering mutations in intervals 9 and 13 (amino acid residues 740–840 and 1230–1342) also occurred within conserved regions of *rpoB* (Fig. 2). In interval 9, the most notable mutations occurred between Ser-788 and Thr-830, a region including 13 conserved amino acid residues (Fig. 4). Although we did not recover termination-altering mutations at any of these 13 positions, only two were susceptible to bisulfite-mutagenesis. In interval 13 (amino acids 1230–1342), there are 28 conserved amino acid residues (Fig. 5B). Nine are bisulfite-sensitive; we recovered termination-altering mutations at three (*rpoB5131* at His-1237, *rpoB5127* at Ala-1284, and five different mutations in which Pro-1258 was altered; Fig. 5B). In both intervals 9 and 13, the tendency for termination-altering mutations to occur in the same areas, and occasionally at the precise residues, that are conserved among β -subunit homologs is remarkable. Interestingly, Scafe et al. (1990b) recently described eight conditional mutations in conserved regions of yeast RNA polymerase II, all of which occur in or near regions corresponding to intervals 9 or 13 in *rpoB*.

The absence of mutations between residues 840 and 1200 also deserves comment. This region contains at least two different functionally distinct regions. From residue 965 to 1030 is a dispensable segment of the β -subunit polypeptide, which can tolerate deletion (Glass et al. 1986b) or insertions (Kashlev et al. 1989) without causing lethal changes in RNA polymerase function. Single amino acid changes in this region, which may act as a hinge between two domains of the β -subunit (hinge, Fig. 2), probably would not affect transcription termina-

tion. Between residues 1047 and 1066 is a highly conserved section of polypeptide that cross-links to nucleotide analogs (Grachev et al. 1987, 1989) and presumably forms a portion of the catalytic site (NTP x-link, Fig. 2). Mutations in the active site probably would destroy catalytic function of the enzyme and would not be recoverable in our experiment.

Mutations in the β -subunit could alter transcription termination in several ways

The mechanisms of transcription pausing and termination at sites that specify RNA hairpins remain subjects of intense debate. The explanations cited most often depend on a paradigm for the transcription complex in which the dimensions of the unwound segment of DNA (17 ± 1 bp) and the RNA/DNA heteroduplex (12 ± 1 bp) are constant during elongation (Yager and von Hippel 1987, 1990). Termination has been attributed to dissociation of the RNA–DNA heteroduplex by the combined effects of RNA hairpin formation and weak pairing by the remaining rU·dA base pairs (Farnham and Platt 1980, 1981; Yager and von Hippel 1987, 1990). Pausing has been attributed to partial disruption of the hybrid by RNA hairpin formation (Landick and Yanofsky 1987b; Yager and von Hippel 1987). Alternative models for pausing and termination are associated with the view that the RNA–DNA hybrid is much shorter than 12 bp (3 bp or less) and that the transcript is positioned in a site on polymerase by direct protein–RNA interactions (Arndt and Chamberlin 1990; M. Chamberlin and C. Kane, pers. comm.). In this view, termination results when RNA hairpin formation removes the transcript from this binding site, resulting in active rearrangement of the transcription complex and release of the RNA. No mechanistic relationship between termination and pausing is assumed, and the latter is explained solely by the effects on elongation of interactions between RNA polymerase and the template or transcript. A third class of models for pausing and termination invokes direct interactions between RNA hairpins and RNA polymerase to alter the enzyme's potential for elongation (Farnham and Platt 1980).

What aspect of transcript elongation might be affected by termination-altering mutations in *rpoB* and what implications do our findings have for these models? We offer three thoughts. First, it seems to us unlikely that the majority of the mutations alter direct RNA hairpin–RNA polymerase interactions, because the most significant mutations occur in regions and at residues conserved between prokaryotic and eukaryotic RNA polymerases. Although prokaryotic and eukaryotic RNA polymerases clearly have common functions (e.g., RNA chain synthesis), they do not respond similarly to nascent transcript secondary structures (Dedrick et al. 1987). Specifically, there is little correlation between termination by purified *E. coli* and calf thymus RNA polymerases at a variety of DNA sequences (Dedrick et al. 1987; Reines et al. 1987). Thus, it seems unlikely that direct RNA polymerase–RNA hairpin interaction is the

driving force behind conservation of sequences in intervals 7, 9, and 13. Rather, these segments of the β -subunit polypeptide must be involved in some function that both participates in the response to transcriptional pause and termination signals and is conserved between prokaryotic and eukaryotic RNA polymerases.

Second, the finding that some mutations affect different pause and termination sites in dramatically different ways (*rpoB5101*, *rpoB5074*, *rpoB5102*; Table 1) challenges simple models for termination in which pausing is an obligatory intermediate (Farnham and Platt 1981). *rpoB5101* RNA polymerase exhibited a strong defect in pausing yet terminated with near normal efficiency at some sites (Fig. 6; Table 1). Furthermore, some mutant RNA polymerases terminated at certain sites with near wild-type efficiency but transcribed through other sites much more often than the wild-type (e.g., *rpoB5108* and *rpoB5110*; Table 1) or, in one case (*rpoB5101*), appeared to terminate at new sites (Fig. 6). These findings suggest that termination is more complicated than a simple equilibrium between RNA–DNA hybrid and RNA hairpin formation that is unaffected by protein–nucleic acid interactions (Yager and von Hippel 1987, 1990).

Finally, we suggest that regardless of the precise mechanisms of pausing and termination, many of the termination-altering mutations in *rpoB* may affect contacts between RNA polymerase and the DNA template or the nascent transcript, either in an RNA-binding site or as part of an RNA–DNA hybrid. A subtle interplay between the efficiency of RNA hairpin formation and the precise nature and strength of the RNA polymerase–RNA transcript or RNA polymerase–RNA–DNA heteroduplex interactions might allow certain amino acid changes to favor RNA hairpin formation at some sites more strongly than at others. Thus, termination-altering mutations in the β -subunit may exert their effects by increasing or decreasing the stability of the normal form of the elongating ternary complex relative to forms that contain RNA hairpins and are in paused or terminating configurations (equilibrium view). Another possibility is that changes in these regions influence pausing and termination kinetically. Changes in the rate of elongation, mediated by altered contacts with the template, the transcript, or the nucleoside triphosphate could increase or reduce the time available for RNA hairpins to form and influence the transcription complex.

One attraction of this view is that it can explain the overlap between Rif^r mutations and termination-altering mutations. Bound rifampicin blocks synthesis of RNA chains longer than 3–4 nucleotides (McClure and Cech 1978; Schulz and Zillig 1981), probably because it binds in a site normally occupied by the RNA transcript or the RNA–DNA heteroduplex in an elongating transcription complex. Because RNA polymerase clearly did not evolve the capacity to bind rifampicin, a reasonable postulate is that the rifamycin antibiotic was evolved by *Streptomyces* to inactivate bacterial RNA polymerase by binding to an overlapping but nonidentical set of amino acid residues in this domain.

A complete description of the mechanisms of transcription pausing and termination is complicated by our lack of knowledge about the three-dimensional structure of RNA polymerase. In the electron diffraction image of RNA polymerase (Darst et al. 1989), the holoenzyme contains a large cleft that presumably surrounds the transcription bubble. The interpretation proposed above would place intervals 7, 9, and 13 of the β -subunit within such a cleft in the elongating form of the enzyme. We have argued that interval 7, which contains most known Rif^r mutations, probably contacts the RNA transcript or the RNA–DNA heteroduplex. Although similar contacts to intervals 9 and 13 must be considered, it is tempting to speculate that these regions contact the DNA template, because sequence changes downstream from the position occupying the active site can alter dramatically both transcription pausing (Lee et al. 1990) and termination (Telesnitsky and Chamberlin 1989b) and because a mutation in yeast RNA polymerase II implicated in control of transcription initiation occurs at a position corresponding to Gly-1282 in interval 13 of *rpoB* (Scafe et al. 1990a). Further analysis of the properties of the altered enzymes that we have described here, as well as isolation and characterization of additional termination-altering mutations, may allow us to propose a more detailed model for the complex processes of transcription pausing and termination.

Materials and methods

Bacteria, bacteriophage, and plasmids

E. coli strain RL211 [F[−] *lacI*^r λ RL12 Δ (*recA-srl*)306 *srl*-301 :: Tn10-84 *tnaA2 trpR*] is a W3110 derivative that was constructed by use of standard bacterial genetic techniques (Miller 1972). λ RL12 is *cl*⁺ Δ (β - γ) Δ *nin5* and carries between the *SalI* sites a 2-kb DNA fragment that encodes a transcriptional fusion of *S. marcescens* Δ *trpL430trpE* (Stroynowski and Yanofsky 1982) to *cat* from plasmid pACYC184 (Chang and Cohen 1978). *E. coli* strains RL583 [F[−] *leu*(Am) *tsx trp*(Am) *lacZ2110*(Am) *galK*(Am) *galE supD43,74*(Ts) Δ (*recA-srl*)306 *srl*-301 :: Tn10-84 *rpsL rpoBcl*(Am) *sueA sueC*] and RL585 (as RL583, but *sueC*⁺) are derivatives of strains MX1444 and MX1494, respectively, obtained from M. Oeschger (Louisiana State University Medical Center, New Orleans). In strains RL583 and RL585, expression of the *rpoB*(Am) gene is deficient at temperatures above 37°C (Oeschger and Wiprud 1980). pRL385 contains a minimal *rpoB* gene expressed from the *lac* promoter and overproduces the β -subunit of *E. coli* RNA polymerase ~20-fold (Landick et al. 1990).

Media and growth conditions

To screen for altered termination phenotypes, RL211 was transformed with pRL385 derivatives and grown overnight (~17 hr) at 37°C on LB plates containing 100 μ g ampicillin/ml. Colonies were transferred in regular arrays to LB Amp plates containing 0.5 mM IPTG (U.S. Biochemicals) and again grown for 17 hr at 37°C. These master plates were then replica-plated by velvet transfer (Miller 1972) to a series of selective media. The first replica from the velvet was made onto an LB Amp IPTG plate containing 100 μ g rifampicin/ml (Sigma); the

second replica was made onto an agar plate containing only Vogel–Bonner (VB) minimal salts (Miller 1972). This plate was then used to prepare a new velvet with a lowered inoculum, and the second velvet was used to transfer bacteria sequentially to LB Amp IPTG plates containing 20 μ g or 10 μ g of chloramphenicol/ml and, finally, to a VB minimal plate containing 0.2% glucose, 50 μ g of cysteine/ml, 40 μ g of leucine and methionine/ml, 100 μ g of 5-MAA/ml, 5 μ g of indole/ml, 100 μ g of ampicillin/ml, and 0.5 mM IPTG (Yanofsky and Horn 1981). Rich-medium plates were incubated for 17 hr at 37°C, and 5-MAA plates were incubated for 44 hr at 30°C (Yanofsky and Horn 1981).

DNA manipulations and plasmid construction

Plasmid DNAs were prepared on a small scale by using the alkaline lysis procedure (Maniatis et al. 1982) and on a large scale by using the Triton X-100 lysis procedure, followed by CsCl gradient centrifugation (Ausubel et al. 1989). Unless otherwise stated, all DNA manipulations were performed following standard published protocols (Maniatis et al. 1982; Ausubel et al. 1989). DNA sequencing was performed on single-stranded plasmids derived from pRL385 by the dideoxynucleotide sequencing method (Sanger et al. 1977; Ausubel et al. 1989), using modified T7 DNA polymerase (Sequenase, U.S. Biochemicals), [α -³⁵S]dATP (Amersham), and *rpoB*-specific oligonucleotide primers.

Specific deletions in pRL385 were prepared as follows. pRL385 was cleaved with the appropriate restriction enzymes (Fig. 2), and the ends were repaired with either Klenow DNA polymerase or T4 DNA polymerase. In some cases, it was necessary to perform partial restriction endonuclease digestions. Synthetic *Xho*I linkers [d(CTCGAGCTCGAG)] were ligated to the blunt ends, and the linear DNAs were isolated from low-melting temperature agarose after treatment with *Xho*I. The isolated DNAs were recircularized with ligase and transformed into JM109. The interval 7 deletion was prepared similarly except that the *Bcl*I-cleaved DNA was recircularized directly without attachment of *Xho*I linkers. Plasmid reconstruction experiments were performed by excising mutant DNA fragments with *Bst*XI and *Cla*I (interval 5), *Hpa*I and *Bst*EII (interval 9), or *Bst*EII and *Sac*I (interval 13) and ligating the mutant fragment into appropriately cleaved pRL385.

Bisulfite mutagenesis

Bisulfite mutagenesis of gapped duplex pRL385 derivatives was conducted essentially as described by Shortle and Nathans (1978) and Folk and Hofstetter (1983). Five hundred nanograms of single-stranded pRL385 was combined with 10 μ g of *Xho*I-cleaved (or *Bcl*I-cleaved for interval 7) double-stranded pRL385 in 100 μ l of 0.14 M NaPO₄ (pH 6.8) and heated to 100°C for 3 min. The samples were then allowed to cool slowly (~30 min) to 60°C and were held at 60°C for an additional 15 min. After shifting the samples to 37°C, 300 μ l of 4 M sodium bisulfite and 10 μ l of 50 mM hydroquinone were added. After incubation for 10 min, the samples were passed through 5-ml Biogel P-10 columns equilibrated with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1 M NaCl and incubated overnight at 37°C after addition of one-tenth volume of unbuffered 1 M Tris. The DNAs were ethanol-precipitated, repaired with Klenow polymerase (Folk and Hofstetter 1983), and used to transform JM109. DNA was prepared directly from the pooled transformants by alkaline lysis (Ausubel et al. 1989) and used to transform RL211 to screen for termination-altering mutations in pRL385.

Purification of RNA polymerase

Wild-type RNA polymerase was purified from MRE600 *E. coli* cells (Grain Processing Co., Muscatine, IA) by the method of Burgess and Jendrisak (1975). Small amounts of RNA polymerase were purified from RL583 or RL585 that had been transformed with pRL385 or its mutagenized derivatives. The transformed cells were grown overnight at 34°C in 5 ml of LB containing 100 μ g ampicillin/ml, transferred to 200 ml of LB containing 100 μ g ampicillin/ml and 0.5 mM IPTG, and grown at 40°C to a density of 50 Klett units. The cells were chilled rapidly in a dry-ice ethanol bath and used for RNA polymerase minipurifications following either the polyethylene glycol method of Gross et al. (1976) or the heparin–agarose method of Chamberlin et al. (1983).

In vitro transcription reactions

DNA templates were prepared by polymerase chain reaction (PCR) from derivatives of pRL418 (Lee et al. 1990) that contained, in the *Hinc*II site, either wild-type *E. coli trpL* DNA from +44 to +188 (relative to the transcription start) or *S. marcescens ΔtrpL430* DNA from –4 to +118. After 30 cycles of PCR with the M13 universal primer and a custom oligonucleotide that hybridized between T1 and T2 of *rrnB*, the amplified DNA was phenol/CHCl₃-extracted, ethanol-precipitated, and dissolved in TE buffer. To measure paused transcription complex half-lives, 2.5 pmoles of RNA polymerase and 1 pmole of DNA template were combined and incubated at 37°C for 10 min in 100 μ l of 40 mM Tris–HCl, 20 mM NaCl, 14 mM MgCl₂, 14 mM β -mercaptoethanol, 2% (vol/vol) glycerol, 20 μ g of acetylated BSA/ml, 240 μ M ApU dinucleotide, and 2.5 μ M ATP, CTP, and [α -³²P]GTP (36 Ci/mmol) (Levin et al. 1987). Elongation from A-20 was initiated by the addition of unlabeled GTP to 20 μ M, the other three nucleotides to 150 μ M, and heparin to 8 μ g/ml. Ten-microliter aliquots were removed at appropriate time intervals and mixed with 10 μ l of 2 $\times$ TBE, 0.1% bromophenol blue, and 0.1% xylene cyanol saturated with urea. RNA samples were analyzed by electrophoresis through a 10% polyacrylamide, 7 M urea TBE gel. Radioactivity in the pause RNA bands was quantitated with an AMBIS radioanalytic imaging system. The pseudo-first-order rate of pause RNA disappearance was determined from the slope by semilogarithmic regression analysis. Transcription assays to determine termination efficiencies were performed as described above, except in 25- μ l reactions. After the addition of all 4 nucleotides, the reactions were continued for 10 min at 37°C. Percent termination was calculated as mole % terminated RNA/(mole % terminated RNA + mole % readthrough RNA).

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R Landick, J Stewart and D N Lee

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