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# Identifying Interacting Regions in the β Subunit of *Escherichia coli* RNA Polymerase

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Numerous physical and genetic approaches have identified residues in the α, β, β' and σ subunits of *Escherichia coli* RNA polymerase that are involved in transcriptional processes; in contrast, relatively little data exist to demonstrate interacting regions within or between the subunits themselves. As a means of identifying regions in the β subunit that may interact, we have sought intragenic suppressor mutations of a class of elongation-defective and termination-proficient inviable *rpoB* alleles that affect highly conserved residues. We obtained intragenic allele-specific suppressors of GD566 (located in conserved region D) and AV676 (located in conserved region E). With one exception, these allele-specific suppressors also map to highly conserved regions of the β subunit. Allele specific suppression is a genetic criterion for protein–protein interaction. Moreover, the functional properties of the mutants suggests that suppression is likely to result from protein–protein interaction rather than from functional compensation. Our suppression studies provide evidence for the interaction of conserved regions B and D as well as conserved regions E and H of the β polypeptide. We suggest that these, as well as other conserved regions of the β polypeptide, may interact with each other to provide a framework for the function of the enzyme.

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## Introduction

Cellular RNA polymerase is a multisubunit enzyme that carries out the transcriptional program of the organism and also integrates the multiple inputs that regulate this process. To understand the catalytic and regulatory properties of the enzyme, we need to identify the individual functional units of the enzyme and dissect their functions. We have studied the organization of RNA polymerase in *Escherichia coli*, where core RNA polymerase ($\alpha_2\beta\beta'$) carries out elongation and termination and holoenzyme ($\alpha_2\beta\beta'\sigma$) is responsible for initiation (reviewed by Burgess *et al*., 1987).

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Abbreviations used: IPTG, isopropyl-β-D-thiogalactopyranoside; DE52, diethylamino-ethyl cellulose.

One way to dissect complex proteins is through suppression genetics analysis. Suppression genetics is based on the premise that an existing altered function allele can be restored to wild-type function by a second mutational change which restores the enzyme to a more wild-type state. Thus, suppression genetics allows specific structural and functional interactions between polypeptides or within a single polypeptide to be identified. In principle, suppressors may restore function in one of two ways. First, suppressors may restore the protein–protein contacts in the mutant enzyme to a more wild-type state. This type of suppression is generally allele-specific and provides a genetic indication of regions within a polypeptide that may interact. Second, suppressors may provide an independent activity that compensates for the mutant defect. This type of suppression is not generally allele-specific and can provide an indication of the crucial functional defect of the original mutant class (reviewed by Hartman & Roth, 1973).

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Suppression genetic studies have successfully identified a variety of interactions in a diversity of organisms, including the identification of proteins that interact in biochemical pathways and in multi-subunit complexes (Adams *et al.*, 1989; Hollingsworth & Johnson, 1993; Lissemore *et al.*, 1993; Luck, 1984; Nomura *et al.*, 1977) More particularly, suppression genetics has been applied to an understanding of RNA polymerase structure and function. Three regions of the largest subunit of *Saccharomyces cerevisae* RNA polymerase II have been demonstrated to interact structurally or functionally with the carboxyl terminal region of the second largest subunit (Martin *et al.*, 1990). In addition, intragenic suppression studies of a mutant *rpoB* allele have demonstrated that two regions of the β polypeptide, each of which can be altered to confer resistance to rifampicin, are likely to be in close proximity in RNA polymerase (Singer *et al.*, 1993).

We have recently identified five mutations in *rpoB*, which are located in regions of β that are highly conserved among all β homologues (Tavormina *et al.*, 1995). These mutant RNA polymerases were unable to support cellular growth and exhibited severe elongation and termination defects when assayed *in vitro*. In this work, we report our efforts to isolate intragenic suppressors that restore the ability of these mutant proteins to support cell growth. We successfully isolated intragenic suppressors for two of the five mutants. Almost all the suppressor mutants occur in highly conserved regions of the β subunit and several, in isolation, are very deleterious to the function of RNA polymerase. The positions and specificity of the suppressors lead us to suggest that several regions of the β polypeptide interact.

## Results

### Isolation of suppressing RNA polymerase mutants

Strain RL585 encodes a non-polar amber mutation in the chromosomal *rpoB* locus and a temperature sensitive suppressor tRNA. Because this strain produces functional β only at low temperatures, it can be used to select for *trans rpoB* alleles capable of providing all functions necessary for growth at high temperature. We used hydroxylamine to mutagenize plasmids carrying each of the five elongation and termination defective inviable *rpoB* alleles selected for suppression analysis. Since hydroxlamine causes the same transitions that led to the original alleles (C to T), this mutagenesis scheme will not produce true revertants. We screened 50,000 RL585 transformants corresponding to each hydroxylamine-mutagenized allele for growth at 42°C. Mutagenesis of plasmids containing either GD566 or AV676 resulted in RL585 transformants that grew at 42°C, whereas mutagenesis of plasmids containing the other three alleles did not (Table 1).

### Mapping and sequencing of the suppressor mutations indicates that they are located in conserved regions of *rpoB*

GD566 is located in a region of RNA polymerase that can also mutate to confer resistance to rifampicin ($Rif^R$). This region has been termed Rif Cluster II. In addition, regions of RNA polymerase termed Rif Clusters I and III, as well as the region surrounding residue 146, have also been shown to mutate to $Rif^R$ (Jin & Gross, 1988; Severinov *et al.*, 1994). As a first step towards identification of the suppressing mutations, we sequenced each of these regions. All 17 suppressors of GD566 and four of seven suppressors of AV676 revealed a mutational change using this approach. These changes were reconstructed into GD566 or AV676 and in every case found to be sufficient for full suppression (as measured by the growth of RL585 at high temperature). We used restriction fragment swap experiments to locate the remaining suppressing mutations. If the fragment being exchanged contained the original inviable allele (GD566 or AV676), it was reconstructed into a wild-type *rpoB* gene. If the fragment being exchanged did not contain the original inviable allele, it was reconstructed into an *rpoB* gene that encoded the GD566 or AV676 change. Reconstructed clones were then tested to determine

**Table 1.** Summary of mutant isolation

| Mutagenized *rpoB* allele | No. of suppressor candidaes | No. linked to haploinviable allele | Sequence change | No. of isolates |
|---|---|---|---|---|
| GD566 | 19 | 17 | EK142 | 5 |
| | | | DN516 | 1 |
| | | | HY526 | 1 |
| | | | PS564 | 1 |
| | | | GK566 | 9 |
| AV676 | 7 | 7 | PL560 | 1 |
| | | | SF621 | 1 |
| | | | EK672 | 2 |
| | | | AV679 | 1 |
| | | | GD1074 | 1 |
| | | | GS1110 | 1 |
| GS1249 | 0 | 0 | | |
| GD1266 | 1 | 0 | | |
| EK1272 | 0 | 0 | | |

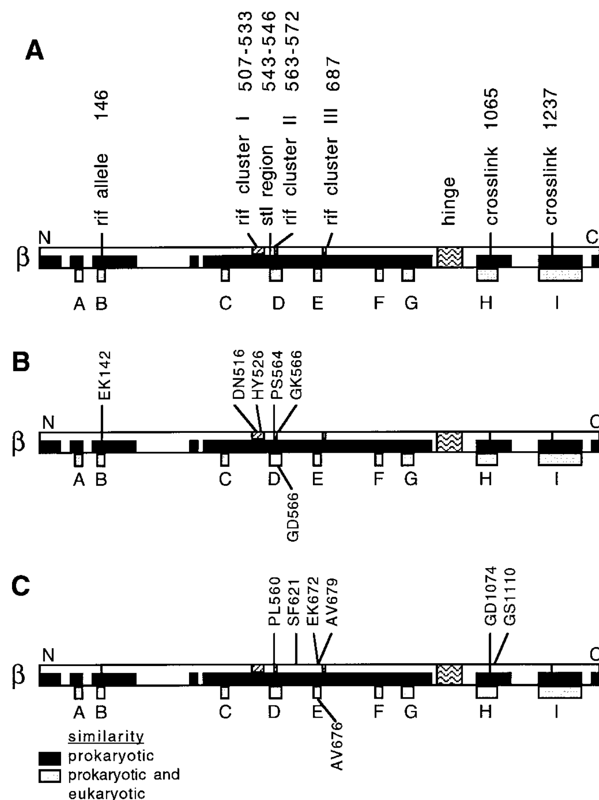


**Figure 1.** Locations of mutations within β. A, Regions of β that can be altered to confer rifampicin or streptolydigin resistance, regions that display conservation between prokaryotic and eukaryotic homologues, a dispensable region (hinge) and residues that crosslink priming nucleotides are indicated. Segments A to I are bounded by the following residues in the β subunit of *E. coli* RNA polymerase: A, 83 to 106; B, 134 to 148; C, 438 to 453; D, 548 to 577; E, 660 to 678; F, 793 to 828; G, 859 to 889; H, 1047 to 1118 and I, 1198 to 1296. Of the residues in these segments 44% are identical between *E. coli* and *S. cerevisae* homologues; conservative changes account for another 29%. B, Location of alleles that suppress GD566. C, Locations of alleles that suppress AV676.

whether they could confer suppression to GD566 and AV676. Once mapped, DNA corresponding to the entire restriction fragment exchanged was sequenced. In every case, the change identified was sufficient to confer full suppression.

Identification of the mutational changes in the 24 suppressing alleles revealed eleven different mutational alterations in the β subunit (Table 1). Three of these alleles, DN516, HY526 and PL560, have been isolated previously. EK142 and GK566, which suppressed GD566, were isolated numerous times, possibly indicating that these sites are particularly responsive to hydroxylamine mutagenesis.

The β subunit of prokaryotic RNA polymerase shares significant homology with its eukaryotic counterpart across most of the protein. Nine segments of particularly high homology have been identified and are termed segments A through I. These segments exhibit about 70% similarity and constitute about 25% of the protein (Figure 1A). Seven of our mutants alter a residue in one of the highly conserved segments B, D, E or H (Figure 1B and C, Figure 2). Many of these seven alter a residue that exhibits either identity or conservative substitution in all prokaryotic and eukaryotic homologues examined to date (Figure 2). The other four mutants are located in regions conserved among prokaryotes. The fact that the suppressor mutations are located in highly conserved regions suggests that they alter functionally important residues in RNA polymerase.

### The basis for the inviability of GD566 may be different from that of AV676

The profound elongation and termination defects exhibited by GD566 and AV676 (Tavormina *et al.*, 1996) suggest that these phenotypes are the basis for their inviability; the suppressor alleles provide a tool to test this proposal. We purified the mutant enzymes and compared their elongation rates and capacity for termination with that of the original alleles. Briefly, elongation rate is determined by measuring the time required to transcribe the 6000 nt located between the bacteriophage T7A1 promoter and the T7 terminator on the T7D111 template; termination efficiency is judged by read-through of the *his* attenuator and the T7 terminator.

For suppressors of AV676, both the elongation rate and the termination defect were significantly restored to close to wild-type levels by virtually all of the suppressors (Figure 3B). This finding suggests that the primary defect of AV676 is directly or indirectly related to the elongation and termination defects of the enzyme.

For GD566, this was not the case. Suppressors had only a minor effect on either the elongation rate defect or the termination defect of GD566 (Figure 3A). GD566 elongated at approximately 25% of the wild-type rate on the T7D111 template (Figure 3A). Two of the suppressor alleles, encoding the PS564 and GK566 alleles, did not significantly ameliorate the elongation defect of GD566. The remaining three suppressor alleles, which encode the EK142, DN516 and HY526 alleles, only slightly ameliorated (25%) the elongation rate of GD566. GD566 RNA polymerase terminated much better (10 to 50-fold more) than wild-type RNA polymerase (Figure 3A). With the exception of PS564, all of the suppressors did appear to slightly increase the read-through of the mutant RNA polymerase on both the *his* attenuator and the T7 terminator. However, the read-through in all cases but one was still significantly below that of the wild-type enzyme. In many cases, the apparent increase in read-through was not significant because it was within the error of the assay (see Materials and Methods). These studies indicate that cells can retain viability even when they terminate transcrip-

tion significantly more than the wild-type enzyme *in vitro*. In addition, these data suggest that neither the very slow elongation rate nor the striking termination proficiency of GD566 is the primary defect responsible for the inability of this enzyme to support cell growth. Further comparison of the biochemical properties of suppressed GD566 RNA polymerase with those of GD566 alone may reveal the essential function of this region of RNA polymerase.

## Pairwise reconstruction of suppressor alleles with inviable alleles

Some suppressor alleles alter residues distant from the allele upon which they were isolated and may confer suppression by restoring protein–protein interactions between distant regions of β. A demonstration of allele-specificity would provide genetic evidence consistent with this interpretation. To ask whether the suppressors specifically restore

**Segment B**

| *E. coli* β 138-146 | I | N | G | T | **E** | R | V | I | V |
|---|---|---|---|---|---|---|---|---|---|
| EUBACTERIA (7) | 7/7 | 7/7 | 7/7 | 5/7 | 7/7 | 7/7 | 7/7 | 3/7 | 7/7 |
| CHLOROPLAST (7) | 2/7 | 7/7 | 7/7 | 0/7 | 0/7 | 7/7 | 2/7 | 2/7 | 1/7 |
| ARCHAEBACTERIA (2) | 2/2 | 1/2 | 1/2 | 0/2 | 2/2 | 2/2 | 2/2 | 1/2 | 1/2 |
| EUKARYOTES (6) | 5/6 | 6/6 | 6/6 | 1/6 | 6/6 | 0/6 | 6/6 | 2/6 | 0/6 |

**Segment D**

| *E. coli* β 558-569 | V | C | **P** | I | E | T | **P** | E | ***G*** | P | N | I |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EUBACTERIA (7) | 3/7 | 7/7 | 7/7 | 7/7 | 7/7 | 7/7 | 7/7 | 7/7 | 7/7 | 6/7 | 7/7 | 7/7 |
| CHLOROPLAST (7) | 1/7 | 7/7 | 7/7 | 6/7 | 3/7 | 7/7 | 1/7 | 7/7 | 7/7 | 0/7 | 7/7 | 0/7 |
| ARCHAEBACTERIA (2) | 0/2 | 2/2 | 2/2 | 0/2 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 | 1/2 | 2/2 | 0/2 |
| EUKARYOTES (6) | 3/6 | 6/6 | 5/6 | 0/6 | 5/6 | 6/6 | 6/6 | 6/6 | 6/6 | 0/6 | 0/6 | 0/6 |

**Segment E**

| *E. coli* β 668-677 | I | P | F | L | **E** | H | D | D | A | N |
|---|---|---|---|---|---|---|---|---|---|---|
| EUBACTERIA (7) | 7/7 | 7/7 | 7/7 | 7/7 | 7/7 | 6/7 | 7/7 | 7/7 | 6/7 | 6/7 |
| CHLOROPLAST (7) | 6/7 | 7/7 | 7/7 | 2/7 | 7/7 | 7/7 | 1/7 | 7/7 | 7/7 | 7/7 |
| ARCHAEBACTERIA (2) | 2/2 | 2/2 | 0/2 | 0/2 | 2/2 | 2/2 | 0/2 | 0/2 | 0/2 | 0/2 |
| EUKARYOTES (6) | 6/6 | 6/6 | 5/6 | 0/6 | 1/6 | 6/6 | 0/6 | 0/6 | 0/6 | 0/6 |

**Segment H**

| *E. coli* β 1071-1077 | G | N | K | ***G*** | V | I | S |
|---|---|---|---|---|---|---|---|
| EUBACTERIA (7) | 7/7 | 7/7 | 7/7 | 7/7 | 7/7 | 5/7 | 5/7 |
| CHLOROPLAST (7) | 7/7 | 7/7 | 7/7 | 7/7 | 0/7 | 6/7 | 7/7 |
| ARCHAEBACTERIA (2) | 2/2 | 0/2 | 2/2 | 2/2 | 2/2 | 0/2 | 0/2 |
| EUKARYOTES (6) | 6/6 | 0/6 | 6/6 | 6/6 | 1/6 | 2/6 | 0/6 |

**Segment H (cont.)**

| *E. coli* β 1107-1113 | M | N | I | **G** | Q | I | L |
|---|---|---|---|---|---|---|---|
| EUBACTERIA (7) | 7/7 | 7/7 | 4/7 | 7/7 | 7/7 | 6/7 | 7/7 |
| CHLOROPLAST (7) | 7/7 | 7/7 | 0/7 | 7/7 | 7/7 | 2/7 | 0/7 |
| ARCHAEBACTERIA (2) | 2/2 | 0/2 | 0/2 | 2/2 | 0/2 | 1/2 | 2/2 |
| EUKARYOTES (6) | 6/6 | 0/6 | 3/6 | 4/6 | 0/6 | 0/6 | 0/6 |

**Figure 2.** Seven of the 11 alleles in this study have a mutational change in conserved segments B, D, E or H. A partial amino acid sequence comparison from conserved segments B, D, E and H. Homologues of the β subunit of *E. coli* RNA polymerase were selected for comparison if they displayed an entire open reading frame containing each of the conserved segments A through I. These homologues are: Eubacteria: *Salmonella typhimurium*, *Buchnera aphidicola*, *P. putida*, *B. subtilis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Thermotoga maritima*. Chloroplast RNA polymerase from the following species: *Chlamydomonas reinhardti*, *Heterosigma carterae*, *Spinacea oleracea*, *Euglena gracilis*, *Nicotinia tabacum*, *Oryza sativa* and *Zea mays*. Archaebacteria: *Halobacterium halobium* and *Thermoplasma acidophilum*. Eukaryotes: *Schizosaccharomyces pombe* RNA pol II, *Saccharomyces cerevisae* pol II, *Saccharomyces cerevisae* pol III, *Drosophila melanogaster* pol II, *Homo sapiens* pol II and *Arabidopsis thaliana* pol II. Identity to the *E. coli* β subunit amino acid sequence is indicated. Seven of the 11 alleles in this study alter a residue in one of conserved segments B, D, E or H. These mutational changes are indicated in bold. Of these seven, two alter residues identical in all homologues examined. These alleles are italicized.

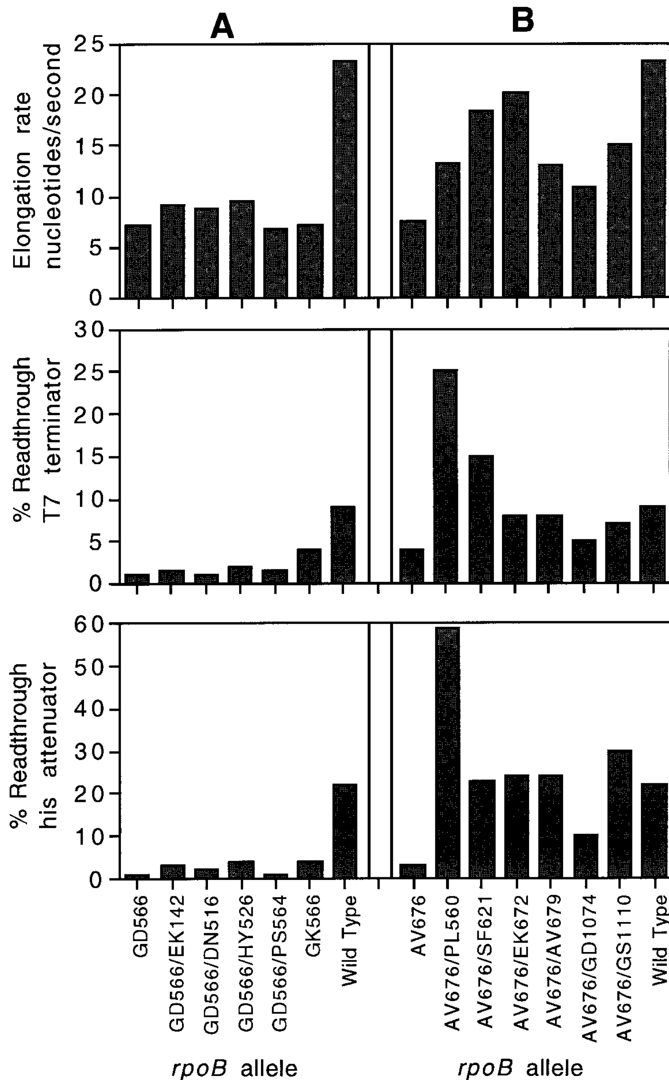


**Figure 3.** Suppression of the transcriptional defects of GD566 and AV676. The transcriptional properties of the doubly mutant alleles (suppressors + GD566 or AV676) were examined *in vitro* in order to determine whether the suppressors were restoring the elongation and termination defects of GD566 and AV676 to more wild-type levels. In brief, elongation rates were determined by measuring the length of time required for a synchronized population of RNA polymerase molecules to traverse the 6 kb between the promoter and the terminator on the T7D111 template; percentage readthrough was determined by quantifying the amount of radioactivity present in the readthrough and terminated bands generated from a single round of transcription on templates containing either the T7 terminator or the *his* attenuator. For experimental details, see Materials and Methods. A, Suppressors of GD566 do not significantly alter the elongation or termination defects of GD566 *in vitro*. B, Suppressors of the elongation or termination defects of AV676 *in vitro*.

function to only a single allele, we recombined the "distant" suppressors with each of the five elongation defective, termination proficient inviable alleles utilized in this study. In all, three suppressing alleles of GD566 (EK142, DN516 and HY526) and four suppressing alleles of AV676 (PL560, SF621, GD1074 and GS1110) were systematically tested. In addition, we reconstructed these suppressor alleles as single mutational changes in order to assess their transcriptional properties.

### Suppression of inviability is primarily limited to the allele upon which the suppressor was isolated

As a genetic means of identifying allele-specific suppressors, we asked which of the pairwise combinations of alleles could provide all functions necessary for growth as determined in the *rpoBam* strain at 42°C. Two suppressors of GD566 (EK142 and DN516) and two suppressors of AV676 (GD1074 and GS1110) exhibited allele-specific restoration of viability (Figure 4B and C). Two additional alleles, PL560 and SF621, preferentially suppressed the original allele AV676 and weakly suppressed one or two additional alleles (Figure 4C, D and E). Finally, HY526, a Rif$^r$ allele isolated many times that has enhanced read-through (Fisher & Yanofsky, 1983; Jin & Gross, 1988; Landick *et al.*, 1990b) weakly suppressed both GD566 and AV676 (Figure 4B and C).

Three of the reconstructed single suppressor alleles, PL560, SF621 and GS1110, were unable to support cell growth (Figure 3A), providing further evidence that these alleles alter functionally important residues in RNA polymerase. One of these alleles, GS1110, supported cell growth only in combination with AV676.

### Suppression of the transcriptional defects is primarily limited to AV676

The AV676 suppressors significantly ameliorated the elongation and termination defects of the original allele (Figure 3B). Having reconstructed these suppressors with all the slow alleles, we are

now in a position to ask whether these suppressing effects on elongation and termination are specific for AV676. To this end, we purified and tested mutant RNA polymerases from each of the reconstructed strains.

The PL560 and SF621 alleles did not exhibit absolute allele specificity for the restoration of growth (Figure 4C, D and E) and they also showed a wider suppression of transcriptional defects. By themselves, these mutant polymerases elongated at about the same rate as wild-type and exhibited enhanced read-through at the two terminators we have examined (Figure 5). Both PL560 and SF621 increased the elongation rate of several alleles significantly; however, a strong effect on terminator read-through was limited to AV676. Thus, as was the case with viability, PL560 and SF621 preferentially suppressed AV676 but also partially compensated for the defects of other mutant alleles. This general suppression may suggest that the enhanced read-through property of these mutants provides a function that compensates for overtermination by a variety of mutant RNA polymerase alleles.

In keeping with their allele specificity in restoring viability, GD1074 and GS1110 suppressed the elongation and termination defects of AV676 virtually exclusively. These two alleles had no significant effect on the elongation and termination defects of GS1249, GD1266 or EK1272 and consistently exacerbated the elongation and termination defects of GD566. Examination of the transcriptional properties of GD1074 and GS1110 reconstructed as single mutants revealed defects in elongation and termination qualitatively similar to, but not as extreme as, those of AV676 (Figure 5). GS1110, a very strong suppressor of AV676, is particularly striking. The GS1110 mutational change by itself resulted in phenotypes very similar to those of AV676: inviability, slower elongation and enhanced termination. Yet the double mutant strain was viable and almost wild-type in its elongation and termination properties (Figures 4 and 5). In fact, the double mutant was more wild-type in all properties examined than either single mutant. Suppression, in this case, is unlikely to involve providing a compensating function. Instead, GD1074 and GS1110 may function by restoring protein–protein contacts within β.

## Discussion

We describe 11 *rpoB* alleles that were identified because they suppress the inviability of two elongation and termination deficient *rpoB* alleles. The fact that the alleles we obtained were predominantly located in highly conserved regions

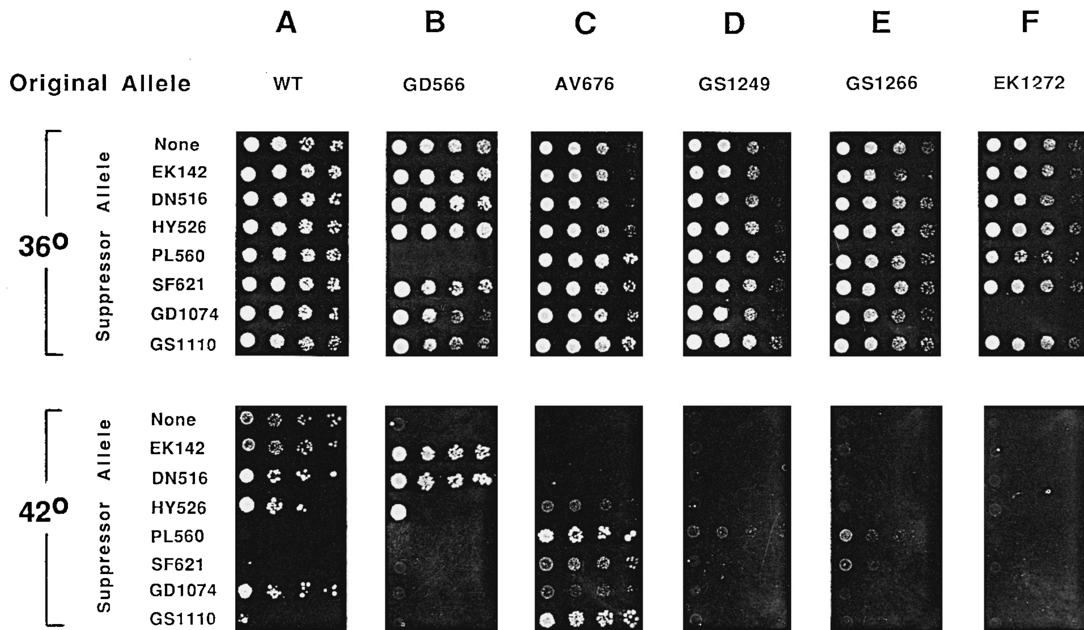


**Figure 4.** Suppression of inviability of five defective *rpoB* alleles. Pairwise combinations of each suppressor allele with wild-type and each of five elongation and termination defective, inviable alleles were constructed on the *rpoB* pRL385. Each of the reconstructed single and double alleles were transformed RL585 and serial fivefold dilutions of these transformation mixes were spotted at 36°C and 42°C to determine alleles specificity *in vivo* (as determined by restoration of growth). In each panel, each row of four spots corresponds, from left to right, to 2 μl of: undiluted transformation mix, a fivefold dilution of the transformation mix, 25-fold dilution, and 125-fold dilution. A. Suppressor alleles reconstructed in an *rpoB* + gene. PL560, SF621 and GS1110 are incapable of supporting growth. B, Suppressor alleles reconstructed in an *rpoB* (GD566) gene. The double mutant PL560/GD566 was not reconstructed. C, Suppressor alleles reconstructed in an *rpoB* (AV676) gene. D, Suppressor alleles reconstructed in an *rpoB* (GS1249) gene. E, Suppressor alleles reconstructed in an *rpoB* (GD1266) gene. F, Suppressor alleles reconstructed in an *rpoB* (EK1272) gene. The double mutant GD1074/EK1272 was not reconstructed.

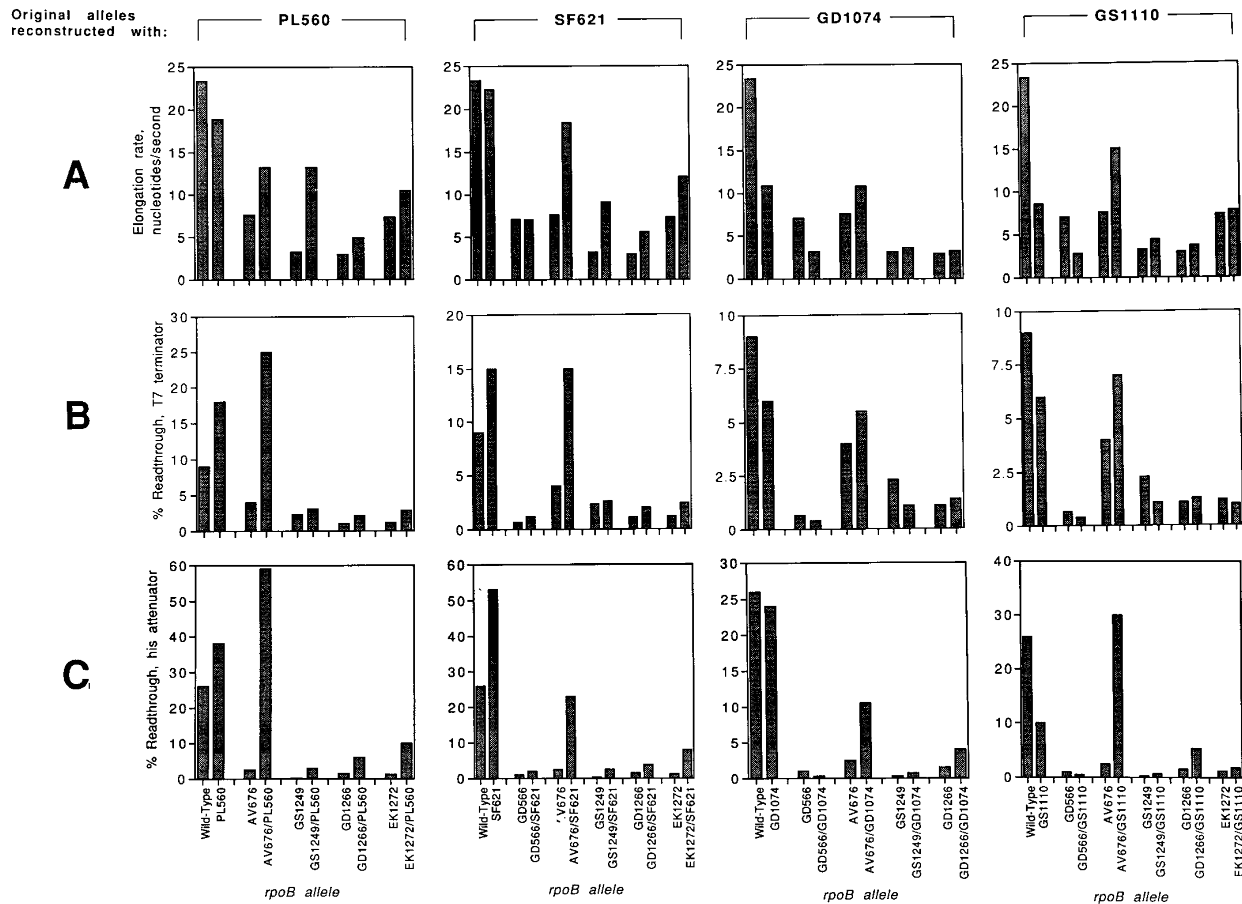


**Figure 5.** Allele specific suppression of the elongation and termination defects of AV676. The transcriptional properties of the double mutants containing pairwise combinations of alleles between the suppressors isolated on AV676 (PL560, SF621, GD1074 and GS1110) and the elongation and termination defective alleles (GD566, AV676, GS1249, GD1266 and EK1272) were examined *in vitro* to determine whether the suppressors were restoring the elongation and termination defects of AV676 in an allele-specific manner. In brief, elongation rates were determined by measuring the length of time required for a synchronized population of RNA polymerase molecules to traverse the 6 kb between the promoter and the terminator on the T7D111 template; the % readthrough was determined by quantifying the amount of radioactivity present in the readthrough and terminated bands generated from a single round of transcription on templates containing either the T7 terminator or the *his* attenuator. For experimental details, see Materials and Methods. PL560 and SF621 provide suppression of transcriptional defects to a spectrum of defective, inviable alleles, whereas GD1074 and GS1110 suppress the defects of AV676 virtually exclusively. A, Effect of PL560, SF621, GD1074 and GS1110 on the elongation defects of five inviable alleles. B, Effect of PL560, SF621, GD1074 and GS1110 on the termination defects of five inviable alleles at the *his* attenuator.

of *rpoB*, and that three are themselves incapable of supporting cellular growth, argues that these suppressors significantly alter the behavior of RNA polymerase. Analysis of the transcriptional properties of the doubly mutant enzymes relative to the original mutants suggests that the basis of the inviability of GD566 does not derive from its elongation or termination defects, whereas that of AV676 is intimately connected with its elongation and termination defects. Pairwise combination of the suppressor alleles with five elongation and termination defective inviable alleles revealed that suppression is primarily specific for the allele upon which the suppressor was isolated and identified four cases of complete allele-specific suppression. We discuss below the insights these alleles lend to a structure-function analysis of β.

## Functional units in RNA polymerase involve non-contiguous conserved segments

Accumulating evidence suggests that functional units in RNA polymerase may be comprised of segments of polypeptides that are not contiguous in the primary sequence. The best examples are the construction of the binding site for rifampicin and of the catalytic core of the enzyme. Studies of rifampicin resistant (Rif$^r$) mutants have suggested that four distinct segments of the β polypeptide interact to form the binding site of the drug (Jin & Gross, 1988). Appropriate interaction of these sites is dependent upon assembly of the subunits into core RNA polymerase since rifampicin does not bind free β and binds only weakly to the $\alpha_2\beta$ subassembly (Jin & Gross, 1988; McClure & Cech,

1978). The catalytic core also appears to be comprised of distinct segments. Crosslinking studies have identified two regions in β and one in β' that are close to either the 5' initiating nucleotide or the 3' end of the nascent RNA chain, implicating these regions in formation of the active site (Borukhov *et al*., 1991; Mustaev *et al*., 1991). The phenotypes of substitution mutations in the immediate vicinity of the crosslinks in the β subunit are consistent with the idea that these regions of the polypeptide participate in forming the catalytic center of the enzyme (Mustaev *et al*., 1991; Sagitov *et al*., 1993). The locations and phenotypes of our suppressor mutations provide further evidence for the idea that functional units of β are not contiguous in the primary structure of the polypeptide.

GD566, located in conserved region D of β, provides a unique probe for the structure and function of a region previously associated with resistance to rifampicin (Jin & Gross, 1988; Severinov *et al*., 1993, 1994). Although GD566 is located in Rif Cluster II, this mutational change does not confer resistance to rifampicin, suggesting that it is functionally distinct from the Rif$^r$ alleles. Indeed, the growth, elongation and termination phenotypes of the mutation are considerably more severe than those of any Rif$^r$ allele studied so far. In the present study, we have selected second site suppressors of GD566. Interestingly, the two allele-specific suppressors of this mutation (EK142 and DN516) identify the same regions of the protein identified by Rif$^r$ mutations. The DN516 suppressor was previously identified as a Rif$^r$ allele in Rif Cluster I (Jin & Gross, 1988) and the EK142 suppressor, located in conserved region B, is only four amino acids away from the strong Rif$^r$ allele VF146. Thus, a probe that identifies a different functional aspect of the region provides independent evidence that the Rif region is comprised of discrete segments of β that are in close proximity and act as a functional unit in RNA polymerase.

The AV676 mutation potentially identifies a new set of interactions in β. AV676 is located in conserved segment E (amino acid residues 660 to 678) of β. GD1074 and GS1110 are allele-specific suppressors of AV676, and are located in conserved segment H (amino acid residues 1047 to 1118). These results suggest that conserved segment H interacts with conserved segment E. The allele-specific suppression provided by GS1110 was particularly noteworthy. By itself, GS1110 was inviable and exhibited elongation and termination phenotypes similar to AV676. However, the double mutant supported robust cell growth and exhibited virtually normal elongation and termination phenotypes. These results provide strong genetic evidence that GS1110 in region H is providing structural compensation for the AV676 defect in region E and suggests that portions of these conserved regions may be in close proximity.

We were unable to identify any intragenic suppressors of three different alleles, GS1249, GD1266 and EK1272, located within conserved segment I. There are a number of trivial possibilities for the cause of this failure, including lack of saturation mutagenesis or structural alterations that are unable to be compensated by single amino acid changes. But our failure to identify second site suppressors of these segment I mutants could have biological meaning. In *S. cerevisae* RNA polymerase II, a conditional mutation in the conserved segment I of the β homologue was found to be suppressed by mutations in conserved segments C, G and H of the β' homologue (Martin *et al*., 1990). Although these studies did not establish allele specificity, they suggest the possibility that segment I of β may interact with regions of β'. We are planning studies to determine whether this is the case.

### An emerging view of the construction of the β subunit

This study provides further support for the view that the construction of RNA polymerase requires interactions between separate regions of the β subunit. Our results suggest that a residue in conserved region D is in close proximity to a residue in conserved region B of the β polypeptide and also provide additional evidence that the ''rif cluster'' residues form an interacting region. Moreover, these suppression studies suggest that a residue located in conserved segment E is in close proximity to a residue in conserved segment H. Thus, residues quite distant in the primary structure of β may interact in the tertiary structure. These studies raise the possibility that many of the conserved regions of the β subunit may interact to form a framework for the activities of the polypeptide.

## Materials and Methods

### Chemicals and enzymes

Hydroxylamine, isopropyl β-D-thiogalactopyranoside (IPTG), heparin, human IgG agarose and heparin agarose (Type I) were purchased from Sigma Chemicals. T4 DNA ligase, Klenow fragment and restriction enzymes were purchased from New England Biolabs., Inc. (Beverly, MA). Ultrapure nucleotide triphosphates and calf intestinal alkaline phosphatase were purchased from Boehringer Mannheim. Diethylaminoethyl cellulose (DE52) was purchased from Whatman. [$\alpha$-$^{32}$P]UTP was purchased from DuPont-NEN, Boston, MA; [$\alpha$-$^{32}$P]GTP was purchased from Amersham International Buckinghamshire, UK. Low melting point agarose and gelase were purchased from Epicentre Technologies. Amplitaq was purchased from Perkin-Elmer Cetus. Sequenase version 2 was purchased from US Biochemicals.

### Strains and plasmids used

Strains and plasmids used in this study are listed in Table 2.

### General bacterial techniques and media

Cells were grown in LB broth or SOC broth with shaking. Growth of cultures was monitored by measuring the change in *A* at 600 nm.

LB broth, SOC broth and LB agar were made as described (Maniatis *et al.*, 1982; Miller, 1972). Where indicated, ampicillin (50 μg/ml) and kanamycin (30 μg/ml) were added.

Plasmid DNA was purified using the Magic miniprep or Wizard miniprep system (Promega Corp., Madison, WI).

T7D111 phage DNA was prepared following the protocol for λ DNA preparation (Maniatis *et al.*, 1982), using MC1061 as a host.

Cells competent for transformation by calcium shock or electroporation were prepared as described (Mandel & Higa, 1970; Zabarovsky & Winberg, 1990). Calcium shock transformations and electroporations were performed as described (Morrison, 1979; Zabarovsky & Winberg, 1990) and transformants were plated directly for ampicillin selection.

### Mutagenesis and screening for suppressor alleles

All *rpoB* mutations were carried on the 7.2 kb, pUC-based plasmid pRL385. This plasmid has been described previously (Landick *et al.*, 1990a); briefly, it directs the expression of *rpoB* from the *lac* promoter and confers resistance to ampicillin. A 14 μg quantity of plasmid pRL385 DNA encoding one of five elongation and termination defective recessive lethal alleles (GD566, AV676, GS1249, GD1266 or EK1272) was subjected to 45 minutes of hydroxylamine mutagenesis *in vitro* as described with minor modifications (Oosawa *et al.*, 1988). After exposure to the mutagen, plasmid DNA was precipitated with two volumes of isopropanol once in the presence of 2.5 M ammonium acetate, then resuspended in 0.3 M sodium acetate and precipitated with three volumes of ethanol twice. The pellets were washed with 70% ethanol to remove residual salts and resuspended in TE.

To select intragenic suppressors, 500 ng of mutagenized DNA was transformed *via* electroporation into RL585. This strain encodes a non-polar amber mutation in the chromosomal *rpoB* locus and a temperature-sensitive suppressor tRNA, allowing selection for *trans rpoB* alleles that provide all functions necessary for growth at high temperatures (Landick *et al.*, 1990b). Immediately after electroporation, the transformation mix was resuspended well in 1 ml SOC media and divided into five samples. To estimate transformation efficiency, tenfold serial dilutions of one were immediately plated on selective media at the permissive temperature. To identify intragenic suppressor candidates, the four remaining samples were plated on selective media at the non-permissive temperature. Colonies that arose at the non-permissive temperature within 30 hours were retained for further analysis.

### Mapping and reconstruction

Because the mutant alleles are under control of the *lac* promoter, all mapping and reconstruction was done in a *lac*I$^{q}$ strain to prevent expression of the mutant *rpoB* alleles that might lead to the selection of reversion or suppression events on the mutant plasmids. Furthermore, because the selected phenotype reflects suppression of a deleterious allele, all mapping was performed such that the suppression event could be screened. This requires that the subclones constructed during the mapping encode the deleterious allele.

As a first step towards identifying intragenic suppressor alleles, sequences surrounding Rif Clusters I, II and III, as well as sequences surrounding position 146, which can also be altered to confer resistance to rifampicin, were sequenced on all suppressors. To verify that the changes identified in this manner were sufficient to suppress the inviability of GD566 or AV676, these changes were reconstructed by subcloning appropriate restriction fragments (described below) into the single inviable allele on which they had been isolated. For alleles near residue 146, the 800 bp *Sty*I-*Bst*XI fragment was exchanged, for alleles near rif clusters I and II, the 230 bp *Bcl*I fragment was exchanged and for alleles near Rif Cluster III, the 1190 bp *Bst*XI-*Hpa*I fragment was exchanged. The entire restriction fragment exchanged was sequenced to verify that all mutational changes had been identified.

To map the remaining suppressor alleles, the following restriction fragments, which span the *rpoB* gene, were exchanged between suppressor and wild-type or suppressor and inviable mutant alleles: *Bam*HI-*Bst*XI (1054 nt), *Bst*XI-*Hpa*I (1193 nt), *Hpa*I-*Bst*EII (1466 nt) and *Bst*EII-*Sac*I (420 nt). If the fragment being exchanged contained GD566 or AV676, it was reconstructed into a wild-type copy of *rpoB*. If the fragment being exchanged did not contain GD566 or AV676, it was reconstructed into a copy of *rpoB* that encoded GD566 or AV676. The entire fragment that provided suppression (as measured by growth of RL585 at high temperature) was sequenced to identify all mutational changes present.

**Table 2.** Strains and plasmids

| Strain or plasmid | Revelant markers | Source/reference |
|---|---|---|
| CAG 324 | *galK*$^{-}$*reA*$^{-}$ | McKenney *et al.*, 1981 |
| CAG 5200 | CAG 324; pES4 | This work |
| CAG 5270 | CAG 324; pPLT7 | This work |
| RL585 | MX 1494 *supD43; 74(Ts) rpoBcl(Am)* | Landick *et al.*, 1990a |
| RL 656 | W3110 *rpoB*5142 (Rif$^{r}$, S. Aureus Protein A insertion) | This work |
| CAG 5434 | RL 656; pPLT13 (*lac*I$^{q}$) | This work |
| MC 1061 | *hsdR*$^{-}$ *hsdS*$^{-}$ | Casadaban & Cohen, 1980 |
| pES4 | Terminators *rrn*BT1 and $t_{IS2}$ are interposed between the*rrn*AP1 promoter and the *galK* reporter in a pBR plasmid; *bla* | Jin *et al.*, 1988 |
| pPLT7 | Reporter construct from pES4 carried in pACYC; *tet* | This work |
| pRL385 | *rpoB* under *plac* control in pUC119; *bla* | Landick *et al.*, 1990a |
| pPLT2 | 9.7 kb *Nco*I-*Ssp*I fragment carrying *rpoB* and *rpoC* expressed from the upstream *rplJ*-promoter inserted in pACYC184; *tet* | This work |
| pPLT13 | MiniF supplying *lacI*$^{q}$; *kan* | This work |

To generate pairwise combinations between suppressor alleles and inviable alleles, all suppressor mutations that occurred outside the immediate vicinity of the allele upon which they were isolated were recombined onto wild-type and the remaining inviable alleles (GD566, AV676, GS1249, GD1266 and EK1272) using the restriction fragments described above. To verify that reconstructed subclones carry all expected mutational changes, subclones thus generated were sequenced in appropriate regions of *rpoB*.

### *rpoB* complementation analysis

To test for the ability of the recombined alleles to support growth in the absence of wild-type β, reconstructed alleles were transformed into RL585 *via* calcium shock (Mandel & Higa, 1970). Fivefold dilutions of the transformations were spotted onto duplicate selective plates and incubated for 30 hours at 36°C and 42°C.

### Purification of RNA polymerase

To purify the reconstructed alleles free of chromosomally encoded β, we employed strain RL656, which encodes a protein A insertion and a Rif$^r$ mutation in the chromosomal *rpoB* locus. The insertion does not interfere with the function of the resulting RNA polymerase, and allows selective removal of polymerase carrying chromosomally encoded β from polymerase carrying plasmid encoded β. Mutant alleles carried on pRL385 were transformed into a *lac*I$^q$ derivative of strain RL656. Transformants were purified on selective media, colonies inoculated directly into 500 ml of LB broth, and grown with shaking at 37°C. Upon reaching an $A$ of ∼0.15 (∼16 to 20 hours after inoculation), IPTG was added to a final concentration of 0.7 mM to induce the plasmid encoded *rpoB* alleles, and growth was continued to an $A$ of ∼0.6. Cells were harvested, quickly frozen in tubes in a dry ice-ethanol bath, and stored at −80°C. A sample of plasmid DNA from each pellet was isolated and sequenced to verify the mutant allele on the plasmid.

RNA polymerase was isolated from each cell pellet as described (Landick *et al.*, 1990b) with minor modifications. Heparin agarose was washed prior to use four times with fourfold volumes of TEGD + 0.3 M NaCl; human IgG agarose was washed prior to use four times with fourfold volumes of TEGD + 0.6 M NaCl. The "step iv" extract was dialysed over a two hour period against two 100-fold volumes of TEGD buffer + 0.3 M NaCl, and applied in a batch to heparin agarose for one hour with gentle rotation. The resulting slurry was poured into a 12 ml dispocolumn, the buffer allowed to run out of the column, and the column bed washed with 10 ml TEGD + 0.3 M NaCl. The RNA polymerase was eluted from the heparin agarose with TEGD + 0.6 M NaCl, and the eluate from the column was incubated three successive times with 100 μl human IgG agarose with gentle rotation for one hour each incubation. DEAE cellulose chromatography and storage of product were as described. All manipulations prior to storage were performed at 4°C.

To estimate the efficiency of removal of the protein A-containing RNA polymerase, we took advantage of the Rif$^r$ marker on the allele to assay percent Rif$^r$ transcription. Reconstruction experiments using wild-type RNA polymerase and varying amounts of protein A-containing polymerase from RL656 demonstrated that as little as 1% contaminating enzyme could be detected in these RNA polymerase preparations. RNA polymerase preparations from the mutant alleles routinely displayed no detectable Rif$^r$ transcription; however, the mutant RNA polymerase preparations generally displayed 50% to 100% of the activity displayed by the wild-type preparation. This indicates that protein A-containing RNA polymerase was present at less than 2% of the total RNA polymerase in these preparations.

### Elongation assays on T7D111 template

Elongation rate measurements on the T7D111 template were performed as described with minor modifications (Chamberlin *et al.*, 1979). In brief, the assay measures the time required to incorporate ribonucleotides into a 6 kb transcript. Synchronized transcription is initiated at the single $\sigma^{70}$ promoter present on the T7D111 template, and 80% of this transcription terminates at the T7 terminator located 6 kb downstream of the promoter. RNA polymerase (1 μg) was incubated with 40 μg T7D111 DNA and other reagents for ten minutes at 37°C. Transcription was initiated by the addition of pre-warmed NTPs to 0.4 mM each, including [α-$^{32}$P]UTP (at a specific activity of 1000 to 3000 cpm/nmol) and heparin to 50 μg/ml. Samples of 20 μl were sampled onto DEAE filters over a time course such that at least six time points fell before and six after the population of polymerase molecules had traversed the T7 terminator. Estimates of elongation rate were made in triplicate and generally varied less than 8%.

### PCR amplification

Templates for assaying termination efficiency were amplified by the polymerase chain reaction as described (Weilbaecher *et al.*, 1994), using plasmids generously provided by M. Chamberlin (University of California at Berkeley).

### Termination assays

The particular T7A1 promoter used in this study encodes the following initially transcribed region: (+1AUCGAGAGGGACACGGGGGAU + 21). Thus, providing substrates ApU, ATP, GTP and CTP will cause a population of RNA polymerase molecules to stall at position + 20. These stalled complexes are formed in order to synchronize transcription for subsequent manipulations; in this case, to limit transcription of a terminator-containing template to a single round. Termination reactions were performed as specified with the following modifications (Weilbaecher *et al.*, 1994). Formation of A20 complexes was performed at the following nucleotide concentrations: 200 μM ApU, 25 μM ATP, GTP and CTP and [α-$^{32}$P]GTP (36 Ci/mmol). A20 complexes were extended under the following nucleotide concentrations: 125 μM ATP, CTP and GTP and 100 μM UTP. Radioactivity present in terminated and read-through bands was quantitated with a Molecular Dynamics phosphorimager. Percent read-through was calculated by correcting for length and specific activity in the terminated and read-through bands, and using the following formula: percent readthrough = (moles read-through transcript)/(moles readthrough transcript + moles terminated transcript). Estimates of percent read-through were made in triplicate and generally varied less than 2%.

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