Termination-altering amino acid substitutions in the β′ subunit of Escherichia coli RNA polymerase identify regions involved in RNA chain elongation

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To identify regions of the largest subunit of RNA polymerase that are potentially involved in transcript elongation and termination, we have characterized amino acid substitutions in the β′ subunit of Escherichia coli RNA polymerase that alter expression of reporter genes preceded by terminators in vivo. Termination-altering substitutions occurred in discrete segments of β′, designated 2, 3a, 3b, 4a, 4b, 4c, and 5, many of which are highly conserved in eukaryotic homologs of β′. Region 2 substitutions (residues 311-386) are tightly clustered around a short sequence that is similar to a portion of the DNA-binding cleft in E. coli DNA polymerase I. Region 3b (residues 718-798) corresponds to the segment of the largest subunit of RNA polymerase II in which amanitin-resistance substitutions occur. Region 4a substitutions (residues 933-936) occur in a segment thought to contact the transcript 3′ end. Region 5 substitutions (residues 1308-1356) are tightly clustered in conserved region H near the carboxyl terminus of β′. A representative set of mutant RNA polymerases were purified and revealed unexpected variation in percent termination at six different p-independent terminators. Based on the location and properties of these substitutions, we suggest a hypothesis for the relationship of subunits in the transcription complex.

[Key Words: rpoC gene; β′ subunit; RNA polymerase; transcriptional termination]

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Escherichia coli RNA polymerase contains a core of four subunits: β′, 155 kD; β, 150 kD, and two α, 43 kD each. These subunits form a scaffold and catalytic center for synthesis of RNA on a double-stranded DNA template that appear to be conserved from bacteria to humans. The two largest subunits, β′ and β in E. coli, display significant sequence similarity to homologous subunits found in all multisubunit RNA polymerases (Allison et al. 1985; Jokerst et al. 1989, Young 1991 and references therein): Each contains eight to nine conserved, colinear segments, although no sequence conservation is evident between β′ and β. However, we currently lack a clear picture of how these conserved primary sequence motifs are positioned in the three-dimensional structure of RNA polymerase.

In the transcription elongation complex (for review, see Das 1993; Chamberlin 1994; Chan and Landick 1994), RNA polymerase contacts the DNA over a 25- to 40-bp region, 12–20 of which are separated into a single-stranded “transcription bubble”. The forward contact envelopes 5–15 bp of double-stranded DNA, whereas the lagging contact appears to channel the single-stranded DNA template out of the complex, where it reanneals with the nontranscribed strand. Approximately eight 3’-proximal nucleotides of the RNA chain may be paired to or positioned near the DNA template, with the 3’ end near the leading edge of the transcription bubble. An additional transcript-segment of at least 10 nucleotides remains tightly bound to the enzyme, perhaps in a distinct transcript exit channel. Low-resolution structures of E. coli RNA polymerase holoenzyme (Darst et al. 1989), and Saccharomyces cerevisiae polymerase I [Pol I] (Schultz et al. 1993) and polymerase II [Pol II] (Darst et al. 1991) reveal contoured features of dimensions appropriate for binding these single- and double-stranded nucleic acids. However, the paths of nucleic acids within the transcription complex and the arrangement of subunits within RNA polymerase are currently unknown.

An additional RNA polymerase contact to a nascent RNA hairpin structure may occur at certain template
positions (Amdt and Chamberlin 1990), including ρ-independent terminators (Reynolds et al. 1992) and some types of pause sites (Chan and Landick 1993, 1994). Pause and termination signals also include special sequences in the duplex DNA that contact polymerase downstream from the active site (Telesnitsky and Chamberlin 1989; Lee et al. 1990) and in the 3’-proximal region of RNA (or corresponding DNA) between the stem–loop structure and the transcript 3’ end. Because many bacterial genes (Landick and Turnbough 1992) and at least some genes in eukaryotes (Wright 1993) are regulated by identification of the regions of RNA polymerase that contact these different segments of nucleic acid and elucidation of the ways that pause and termination signals elicit changes in them.

One approach to finding these segments of the RNA polymerase subunits is to identify amino acid substitutions in RNA polymerase that alter RNA chain elongation or termination, because many presumably will affect the key nucleic acid contacts. Substitutions that alter catalytic activity or affect chain elongation indirectly also may be identified by this approach. Thus, we have studied the effects on transcript elongation and termination of amino acid substitutions in the E. coli RNA polymerase subunits (Landick 1990b).

Most genetic studies of potential RNA polymerase contacts to date have focused on β, owing principally to the ease of obtaining substitutions in this subunit that confer resistance to the antibiotic rifampicin (Rif). Substitutions in six conserved regions of β, C, D (the “Rif” region), E, F, H, and I, affect the behavior of RNA polymerase at pause and termination sites (Yanoysky and Horn 1981; Jin et al. 1988, Landick et al. 1990b; Jin and Gross 1991; Sagitov et al. 1993). However, there are good reasons to suspect that β’ also plays a role in transcript elongation and its regulation. Several studies have documented cross-linking between β’ and both the DNA template (Okada et al. 1978; Chenchick et al. 1982) and the nascent RNA transcript (Hanna and Meares 1983; Dissinger and Hanna 1990; Borukhov et al. 1991). Substitutions in the Pol II homolog of β’ confer resistance to α-amanitin (Ama), a fungal toxin that blocks transcript elongation (Sentenac 1985). At least one Ama’ substitution slows transcription by purified Drosophila Pol II (Coulter and Greenleaf 1985). Finally, the rho201 allele, which produces a defective Rho termination factor, is suppressed by a mutation in rpoC, the β’-subunit gene (Jin and Gross 1989).

To locate regions of β’ potentially involved in chain elongation and termination, we screened for amino acid substitutions that altered expression of reporter genes transcribed after a ρ-independent terminator in the bacterial chromosome. Termination, rather than pausing, provides the best target for screening because it alters expression of genes downstream from the control site in a predictable fashion. Furthermore, some, if not most, interactions between RNA polymerase and the RNA and DNA at pause and termination sites are likely to share common features, even if their consequences for transcription differ.

**Results**

**rpoC was overexpressed from a specialized plasmid**

Because amino acid substitutions in the β’ subunit of RNA polymerase that alter termination might drastically inhibit growth or prove lethal in isolation, we attempted to overproduce β’ from a plasmid to a level sufficient to compete for assembly into RNA polymerase with the chromosomally encoded, wild-type β’ subunit. For the β subunit, this was accomplished by expression from a high-copy number plasmid with only modest regulation (Landick et al. 1990a,b). When we attempted to overproduce wild-type β’ in similar fashion, we found it was toxic. Therefore, we constructed a specialized β’-expressing plasmid, pRW308, that maintained a lower copy number and allowed tight regulation via the lac repressor also encoded on the plasmid (see Materials and methods).

**rpoC was mutagenized in five discrete intervals**

We mutagenized pRW308 DNA with hydroxylamine (allowing both C → T and G → A substitutions), recovered restriction endonuclease-generated DNA fragments from the mutagenized plasmid and then ligated the fragments into appropriately cut, unmutagenized pRW308 (see Materials and methods). These preparations, which targeted substitutions to each of five different ~1-kb intervals in rpoC (Fig. 1), were used to transform strain RL211 and screen for altered termination phenotypes (Landick et al. 1990b). Strain RL211 becomes resistant to 5-methylanthranilic acid (5-MAA) when termination increases in the trp operon leader region (thus decreasing synthesis of toxic 5-methyltryptophan) and becomes resistant to chloramphenicol (Cm) when termination decreases at a ρ-independent terminator (derived from the Serratia marcescens trp leader region) that precedes a cat gene and is carried on a λ prophage in the RL211 chromosome (Landick et al. 1990b).

**All but one termination-altering substitution occurred in four of the five β’ intervals**

We screened 1500–2000 colonies from each of the five mutagenized pools for resistance to Cm or 5-MAA. As we had observed in experiments on rpoB, some colonies displayed both Cm' and 5-MAA' (Landick et al. 1990b). After discarding those that failed to give a reproducible phenotype, we obtained 16 candidates mutagenized in interval 1, 17 in interval 2, 40 in interval 3, 21 in interval 4, and 28 in interval 5.

All but one (SF263, weak Cm') of the interval 1 candidates proved to be deletions that removed much or all of rpoC. In the remaining four intervals, 25–50% of the candidates proved to be large deletions or to have lost the mutations during workup of the plasmids. Whether these plasmids generated a phenotype by expression of
fragments of β', rearranged or converted to eliminate a particularly deleterious mutation, or had other consequences for cell growth that have not yet been determined. The remaining 50–75% encoded single, double, or triple amino acid substitutions in the expected intervals. For each intact mutant plasmid, the entire ~1-kb interval in which substitutions were expected was sequenced.

Eight different termination-altering amino acid substitutions were located in interval 2 (Fig. 2). All the substitutions were clustered between residues 311 and 386, a 75-amino-acid region that constitutes only 30% of the region mutated and that corresponds to conserved region C in the Pol II largest subunit (Fig. 1). Six of the eight were single substitutions between residues 311 and 352, two were double substitutions, one of which, rpoC3201, included one of the single substitutions [SF350], whereas the other, rpoC3214, was a replacement of Leu-385 and Glu-386 with Gln-Lys.

Of the 40 candidates tested in interval 3, 24 contained single amino acid substitutions; 21 were unique, two were double substitutions, and one was a triple substitution (Fig. 3). The four substitutions that were isolated more than once [rpoC3329(A1726), rpoC3325(SF733), rpoC3302(A1748), and rpoC3303(RC799)] were independent isolates of the same substitution, because they were recovered directly from the mutagenized DNA after transformation into RL211 (see Materials and methods). The 27 interval-3 substitutions fell into two subclusters: one (3a) between residues 612 and 671 and a second (3b) between residues 726 and 799. These segments correspond to conserved regions E and F in Pol II (Fig. 1).

We found 14 single and 3 double substitutions in interval 4 (Fig. 4). All but one [rpoC3414(TM934)] were unique. Although these substitutions were spread across interval 4, they could be subdivided into three subclusters: 4a, from 923 to 936; 4b, from 933 to 1076; and 4c, from 1118 to 1188 (Fig. 4). Cluster 4a occurs in the carboxy-terminal portion of conserved region G (Fig. 1). Sequences in clusters 4b and 4c display only weak similarity to the yeast Pol II subunit. Region 4b contains the most poorly conserved sequences between β' and its close relative from Pseudomonas putida (~50% iden-
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Cluster 2

E. coli DNA Polymerase I:
S. cerevisiae Pol II:

Figure 2. Sequence of amino acid changes in interval 2. Termination-altering amino acid changes, aligned with their allele numbers and phenotypes, are indicated below the relevant sequence of E. coli β'. The horizontal broken lines are drawn to clarify the alignment. [*] Alleles found to contain two or more substitutions. The phenotypes are indicated as follows [see Materials and methods]. Under C (for complementation of rpoC[Am] supD[Ts]) in strain RL602 at 37°C: (+) growth at 37°C; (±) weak growth at 37°C; (-) no growth at 37°C. Under #, the number of times the change was isolated. Under Cm: (+) ability to grow in the presence of 20 μg of Cm/ml; (+) ability to allow growth of RLI11 in the presence of 10 μg of Cm/ml; (-) unable to allow growth of RLI11 in the presence of 10 μg of Cm/ml. Under MAA: able (+) or unable (-) to allow growth of RLI11 in the presence of 100 μg/ml 5-MAA. The sequence of the corresponding region of the largest subunit of yeast Pol II is shown above the 3' sequence (vertical lines). The horizontal broken lines are drawn to clarify the alignment. The position of the substitutions occurred just upstream of the region of highest sequence conservation in this interval (Fig. 5).

We conclude that termination-altering substitutions occur in clusters in β' that are coincident with regions conserved between prokaryotes and eukaryotes (Fig. 1). Of the 55 single substitutions that we characterized in β', 7 occurred in conserved region C, 3 in E, 12 in F, 4 in G and 12 in H. Only 11 occurred outside these regions.

Many termination-altering substitutions in β' were recessive lethal

Because the β' substitutions were identified in a merodiploid strain that contained a wild-type rpoC gene on the chromosome, we next tested whether the mutant plasmids could support growth of an E. coli strain unable to synthesize β' at 37°C (see Materials and methods). One substitution in interval 2, 2 of the 3 single substitutions in interval 3a, 7 of the 14 single substitutions in interval 3b, all substitutions in 4a, only the multiple substitutions in intervals 4b and 4c, and 4 of the 8 single-substitutions in interval 5 proved lethal in this test (Figs. 2–5). These recessive-lethal substitutions may have survived in strain RLI11 because the residual amount of wild-type RNA polymerase is sufficient for growth, because they affect folding or stability of β' in a manner that inhibits assembly into RNA polymerase [although enough mutant subunit must assemble to confer the phenotype], or because they increase expression of the chromosomal rpoC gene. Similar results were observed with some β-subunit substitutions (Landick et al. 1990b). All the recessive-lethal substitutions occurred in conserved regions C, E, F, G, or H and not among the 11 substitutions that we found outside these conserved regions, further confirming the importance of these segments for RNA polymerase function.

Purified RNA polymerases containing termination-altering amino acid substitutions in β' displayed unexpected specificity intermination efficiency

Our screen for altered termination in E. coli might also detect substitutions that affect the rate of transcriptional
initiation or alter reporter gene expression indirectly. Therefore, we wished to examine effects on termination in vitro for a representative set of the mutant RNA polymerases. To allow recovery of haploid-lethal enzymes, we purified the mutant RNA polymerases containing plasmid-encoded β' from a strain that encoded β' fused to the IgG-binding domain of *Staphylococcus aureus* protein A on its chromosome (see Materials and methods). We obtained mutant RNA polymerases containing 15 different single amino acid substitutions, including at least 2 from each interval and 8 that were haploid lethal (Table 1).

We performed two types of tests on the mutant RNA polymerases. To determine whether they altered transcript elongation, we measured the elongation rate in an 892-bp segment of DNA from the *E. coli* P14 gene at 5 μM NTP (Table 1; see Materials and methods). Defects in any step of elongation, including NTP binding and translocation, should be detectable in this assay. Half the mutant enzymes exhibited near wild-type elongation rates. The remainder differed significantly, ranging from ~60% of the wild-type rate (GD333 in interval 2) to threefold faster than wild type (SF1321 in interval 5).

To test for altered termination directly, we measured the efficiency of termination for each mutant RNA polymerase at six different p-independent terminators. Wild-type RNA polymerase gave termination efficiencies that ranged from 90% to 10% at these six terminators. To simplify comparison of the mutants, we plotted the termination efficiencies for each of the 15 altered RNA polymerases in order of the strength of the terminators for wild-type RNA polymerase and included the wild-type termination efficiencies on each plot (Fig. 6).

Two types of behavior were evident. Some mutant
RNA polymerases exhibited consistently increased (GD333, AV632, PL1022, HY1252, SL1324) or decreased termination (AT730, MI747) at all six terminators. Others displayed significantly decreased or increased termination at certain terminators, although they showed wild-type termination efficiencies or even opposite effects at others (e.g., SF350, RH780, SF1321, and AV1322). In some cases, contradictory effects on termination efficiency were dramatic (e.g., RH780), whereas in others the distinction between consistent and mixed was marginal (e.g., HY1252 and SL1324 vs. SF1321 and AV1322; Fig. 6).

Three conclusions are striking from comparison of the phenotypes of mutant RNA polymerases in the different assays (Table 1). First, we observed only weak correlation of increased termination in vitro with 5-MAA\(^\text{r}\) and decreased termination in vitro with Cm\(^\text{r}\) (e.g., compare the phenotypes of MI725 and AT730). Second, some mutants conferred both Cm\(^\text{r}\) and 5-MAA\(^\text{r}\) in vivo (see also Figs. 2–5). Some substitutions in \(\beta\) also exhibited these contradictory phenotypes in vivo and in vitro (Landick et al. 1990b; G. Feng and R. Landick, unpubl.). Third, although the elongation rates of some mutants (e.g. GD333, PL1022, AT730) were consistent with their effects on termination (decreased elongation rate with increased termination and vice versa), in some cases (e.g. SF1321 and AV1322), significant changes in elongation rates were not correlated with a consistent effect on termination. We consider these findings at the end of the Discussion.

**Discussion**

We isolated mutations in a plasmid-borne copy of \(rpoC\) [e.g., compare the phenotypes of MI725 and AT730]. Second, some mutants conferred both Cm\(^\text{r}\) and 5-MAA\(^\text{r}\) in vivo (see also Figs. 2–5). Some substitutions in \(\beta\) also exhibited these contradictory phenotypes in vivo and in vitro (Landick et al. 1990b; G. Feng and R. Landick, unpubl.). Third, although the elongation rates of some mutants (e.g. GD333, PL1022, AT730) were consistent with their effects on termination (decreased elongation rate with increased termination and vice versa), in some cases (e.g. SF1321 and AV1322), significant changes in elongation rates were not correlated with a consistent effect on termination. We consider these findings at the end of the Discussion.
which, when overexpressed relative to the wild-type chromosomal gene, altered expression of genes downstream from a p-independent terminator. The 66 amino acid substitutions we characterized form clusters in β' that, by themselves, define amino acid sequences important for RNA chain elongation.

The discovery of these clusters is the most important result of this study. Several features suggest that they identify functionally important parts of β'. First, they are not the consequence of a limited set of codons susceptible to mutagenesis. Hydroxylamine can change 982 of the 1407 rpoC codons, and these are distributed uniformly throughout the gene. Second, all but the least significant clusters, 4b and 4c, correspond to regions of the β' subunit that are highly conserved in the eukaryotic homologs of β' [Fig. 1]. In this regard, they are similar to the conditional-lethal substitutions characterized in S. cerevisiae RPB1 [Scafe et al. 1990; Archambault et al. 1992; Archambault and Freisen 1993] and Drosophila RpII215 [Chen et al. 1993]; the lack of clustering among the eukaryotic substitutions may simply reflect the limited number examined. Finally, some clusters of substitutions that we detected correspond to segments of the β' polypeptide which, based on other results, are likely to be important for RNA chain elongation or termination [see below].

Table 1. Relative elongation rates and phenotypes of mutant RNA polymerases

| Region | Amino acid substitution | Haploid viability | Elongation Rate a in vitro | Phenotypes b
|--------|-------------------------|------------------|---------------------------|----------------
|        |                        |                  |                           | Cm'            | 5-MAA' |
| Interval 2 (292–544) | wild type | + | 100 | – | – |
|       | GD333 | ± | 60 | increased | + + + |
|       | SF350 | – | 100 | mixed | + |
| Interval 3 (544–877) | AV632 | ± | 80 | increased | + |
|       | MI725 | ± | 100 | mixed | + |
|       | SF728 | – | 100 | mixed | + |
|       | AT730 | – | 220 | decreased | – |
|       | MI747 | – | ND | decreased | – |
|       | RH780 | ± | 110 | mixed | + |
| Interval 4 (877–1213) | TM934 | – | 110 | mixed | – |
|       | PL1022 | ± | 70 | increased | – |
|       | RC1075 | ± | ND | mixed | + |
| Interval 5 (1213–1407) | HY1252 | ± | ND | increased | – |
|       | SF1321 | – | 300 | mixed | + |
|       | AV1322 | – | 290 | mixed | + |
|       | SL1324 | – | ND | increased | + |

*Relative elongation rate (wild-type = 100; 0.65 nucleotides/sec) on a P14 DNA template [Reynolds et al. 1992] was estimated by the change in mean chain length during elongation at 5 μM NTPs and 37°C [see Materials and methods]. Estimates of elongation rate are necessarily approximate, as the velocity of nucleotide addition and apparent K_m for NTPs vary at different template positions. (ND) Not determined.

*Phenotypes are classified as follows: [increased] increased termination in vitro at all or most terminators [no significant decreased termination]; [decreased] decreased termination at all or most terminators [no significant increased termination]; [mixed] an inconsistent pattern of increased, decreased, or near wild-type behavior at different terminators [see text and Fig. 6]. [+] +] Resistance to 20 μg/ml of Cm; [+] resistance to 10 μg/ml of Cm or 100 μg/ml of 5-MAA; [–] sensitive to Cm or 5-MAA [see Landick et al. 1990b].

Figure 5. Sequence of amino acid changes in interval 5. (For explanation of symbols, see legend to Fig. 2). rpbl-513 [FL1410] (Martin et al. 1990) is an extragenic suppressor of rpb2-2 (see legend to Fig. 2). rpbl-551 [VF1428] is an intragenic suppressor of a deletion of all but 11 of the carboxy-terminal heptapeptide repeats from the yeast Pol II large subunit [Nonet and Young 1989]. rpbl-1 [GD1437] is a temperature-sensitive mutation that ceases mRNA synthesis rapidly at 36°C [Scafe et al. 1990].

Table 1. Relative elongation rates and phenotypes of mutant RNA polymerases.
Figure 6. Termination efficiencies of mutant RNA polymerases at six different p-independent terminators. The six terminators are [T7] early terminator from bacteriophage T7; [T3] early terminator from bacteriophage T3; [P14] intercistronic, bidirectional terminator from downstream of the E. coli tonB gene transcribed opposite to tonB; (trpL) attenuator from the E. coli trp operon containing both the pause and termination signals [segments 1–4]; (trp) trp attenuator containing only the 3-4 terminator region; and [his] attenuator from the Salmonella typhimurium his operon containing only the E-F terminator region. Transcription templates were obtained by PCR of appropriate plasmids (see Materials and methods). (Q) Termination efficiencies for wild-type RNA polymerase; (O) values obtained for the mutant RNA polymerases. Termination efficiencies are plotted from the highest to lowest observed for wild type; standard deviations (from three independent determinations) are indicated by vertical lines (not visible when smaller than the symbol). Assignment of termination phenotype is described in Table 1.

Some important regions of β′ may have escaped identification in our screen if substitutions in them were lethal. For instance, the deletions that we recovered from plasmid mutagenized in interval 1 [see Results] might have arisen because substitutions there were exceptionally toxic. M. Clerget and H.A. Wiesberg (in prep.) recently found that substitutions in the zinc finger domain in interval 1 [Fig. 1] can block factor-independent anti-termination and increase termination in coliphage HK022 DNA. Thus, the zinc finger is a candidate for a nucleic acid–polymerase contact important for termination. Nonetheless, several of the substitution clusters that we found almost certainly affect portions of the enzyme important for chain elongation and termination. We will describe these regions individually and then consider implications of our results for the structure of the transcription complex and the mechanism of transcriptional termination.

Interval 2—similarity between β′ and E. coli DNA polymerase I

Interval-2 substitutions occurred within a portion of conserved region C that bears weak sequence similarity to β-sheet segments 7 and 8 and a loop separating segment 8 from helix J in the crystal structure of the Klenow fragment of DNA polymerase I (Pol K; Fig. 2). These segments form portions of the floor (β-7 and β-8) and inside surface of the “thumb” domain [α-J] of the DNA-binding cleft in Pol K (Ollis et al. 1985). This sequence conservation is more evident in the Pol II largest subunit (Allison et al. 1985), but the strong conservation between bacterial and eukaryotic homologs in the region makes alignment with β′ reasonable. Two conditional-lethal substitutions and one extragenic suppressor of a temperature-sensitive substitution in RPB2 also occur in this region of S. cerevisiae RPB1 (Fig. 2).

Interestingly, rpoC214, the suppressor of rho-201, recently was found to encode RC352 in interval 2 and also to alter elongation and termination in vitro (L.M. Heisler, G. Feng, R. Landick, D.J. Jin, W.A. Walter, and C. Gross, in prep.). Thus, termination-altering substitutions occur at Ser-350, Gly-351, and Arg-352 in β′, which correspond to Thr-666, Gly-667, and Arg-668 in the loop between β-7 and β-8 in Pol K. Substitution of Ala for Arg-668 reduces the $k_{cat}$ of Pol K to 0.25% of wild type,
Termination-altering mutations in rpoC

Termination-altering mutations in rpoC

principally by increasing the $K_d$ for DNA 20-fold (Polesky et al. 1992). In a cocrystal of Pol K and DNA, Asn-675 and Asn-678 contact phosphate oxygens on adjacent nucleotides in the primer strand just upstream from the active site (Beese et al. 1993). Although a recent crystal structure of rat DNA polymerase β suggests a different orientation of DNA (Pelletier et al. 1994), Pol β lacks the β7–β8 region; it remains likely that Asn-765 and Asn-768 in Pol K interact with the primer or template during replication. Thus, interval 2 substitutions probably identify a portion of β’ that contacts the DNA template, RNA transcript, or both immediately upstream from the active site in RNA polymerase.

Interval 3—similarity to amanitin-resistance region of Pol II

Subcluster 3a, which corresponds to conserved region E, contained only three single substitutions, two of which were recessive lethal [Fig. 3]. AV632 displayed only weak effects on termination in vitro. However, the two recessive-lethal substitutions, GD640 and ED656, await biochemical characterization.

Cluster 3b corresponds to conserved region F, which is the location of all known amanitin-resistance substitutions in Pol II (Fig. 3). One of these, RH741 in Drosophila [Chen et al. 1993], slows transcript elongation by purified RNA polymerase II [Coulter and Greenleaf 1985]. Like the Rif-binding site in β, which appears to overlap the transcript-binding channel so that Rif prevents synthesis of RNAs that are greater than trimers [Mustaev et al. 1994], the amanitin-binding site in the Pol II homolog of β’ probably is an important functional site on the enzyme that was targeted during antibiotic evolution. The large number of cluster-3b substitutions supports this view. Because α-amanitin allows at least one [DeMercyrol et al. 1989, Archambault and Freisen 1993], and sometimes up to eight (D. Luse, pers. comm.), rounds of nucleotide addition before blocking chain extension, this site must be involved in a translocation step, rather than in phosphodiester bond formation. Two attractive possibilities are that this site either is the leading edge of the 3’-proximal RNA-binding site or a portion of the downstream DNA contact. If this site is only transiently occupied by nucleic acid during the translocation cycle, amanitin could block a crucial step or step(s) in transcription and amino acid changes at the site might alter the step(s) during transcript elongation and termination.

Interestingly, region 3b can be subdivided further based on the location of an ~20-amino-acid hydrophilic insertion in the yeast Pol II subunit [Fig. 3b]. Amanitin-resistance substitutions are amino-terminal of this insertion, whereas the strongest region of conservation between the E. coli and yeast subunits as well as one conditional-lethal, Ama+ substitution in yeast Pol II occur on the carboxy-terminal side of the insertion [Fig. 3b]. In a prediction of surface probability [Emini et al. 1985], the insertion in the yeast Pol II subunit forms a highly significant spike not observed for β’. Thus, as has been sug-

gested recently for the Rif region in β (conserved region D3; Heisler et al. 1993; Severinov et al. 1993), segment 3b of β’ may fold in two distinct segments that are separated by a surface-exposed region; these segments could interact to form a functional site on the enzyme.

Interval 4—coincidence of some substitutions with a site of transcript cross-linking

Of the three subclusters that we detected in region 4, 4a is the most interesting. All substitutions in 4a were recessive lethal and occurred in conserved region G [Figs. 1 and 4]. TM934 was isolated twice independently and confers variable effects on termination in vitro [Fig. 6]. Three different substitutions in yeast Pol II also have been isolated within conserved region G [Fig. 4]. Borukhov and co-workers (1991) report that 8-azido AMP present at the 3’ end of the nascent transcript cross-links to one or more amino acid residues in the sequence RT-FHIGG, within which all four of the region-4a substitutions occurred [Fig. 4]. Thus, region-4a substitutions probably affect termination through interactions with the 3’ end of the transcript.

Assigning important functions or contacts to regions 4b and 4c is made highly questionable by the finding that nearly all of these sequences are deleted in M. leprae β’ [Honore et al. 1993]. However, five different insertions and one substitution in or near the corresponding region of the yeast Pol II largest subunit confer sensitivity to 6-azauracil and can be suppressed by overproduction of the TFIIIS elongation factor [Fig. 4; Archambault et al. 1992]. Hence, this part of β’ could be a target for elongation-factor binding. Because the single substitutions that we tested from regions 4b [PL1022 and RC1075] both exhibited strong effects in vitro at some terminators, they must either influence termination directly or cause retention of residual transcription factors (such as NusA) during purification that preserve the mutant phenotype. Further work will be required to clarify this issue.

Interval 5—tightly clustered substitutions in a region similar to Pol II

Our collection of substitutions in conserved region H of β’ are the first data linking it to a role in transcript elongation. However, one conditional-lethal and two suppressor substitutions in the yeast Pol II homolog of β’ occur in conserved region H [Fig. 5]. The suppressor substitutions complement a temperature-sensitive substitution located in conserved region I of the yeast Pol II homolog of β [Fig. 1]. Conversely two extragenic suppressors of the GD1347 conditional-lethal substitution in interval H of the yeast β’ homolog [Fig. 5] occur in conserved region I of yeast β homolog [rpb2-510(SL1145) and rpb2-513(DN1125); Martin et al. 1990]. Thus, the two carboxy-terminal regions of β and β’ appear either to interact structurally or to affect the same mechanistic
step in transcription. Interestingly, many termination-altering substitutions in *E. coli* β map to conserved region I (Landick et al. 1990b), and the yeast Pol II substitutions correspond to positions surrounding the most significant cluster of them. Thus, these carboxy-terminal regions probably play a central role in RNA chain elongation.

**β and β’ contain several homologous features**

The locations of termination-altering substitutions and several other properties of β and β’ (Fig. 1) suggest to us five distinct arguments for potentially homologous features in the two subunits. First, termination-altering substitutions were obtained in the carboxy-terminal 75% of both subunits but not in the amino-terminal 25%. Interestingly, Ohnishi (1985) reports weak sequence similarity in the first 330 amino acids of β and β’. Second, both subunits contain significant clusters of termination-altering substitutions adjacent to their carboxyl termini (Fig. 1, region 13 for β, Landick et al. 1990b; Fig. 5, region 5 for β’). Third, both subunits contain highly conserved segments near amino acid 1000 that cross-link to nucleotide analogs located in the primer site on the enzyme (Grachev et al. 1989; Borukhov et al. 1991) and in which substitutions alter elongation and termination (region 11 in β, Fig. 1; Sagitov et al. 1993; region 4a in β’, Figs. 1 and 4). Fourth, both subunits contain central segments that give rise to antibiotic-resistance substitutions (to Rif for β, to Ama for β’ homologs) and that play central roles in transcript elongation. Both of these regions tolerate small, surface-exposed insertions (Severinov et al. 1993; Fig. 3B). Fifth, both subunits can be divided into three functional domains, corresponding roughly to the amino-terminal, central, and carboxy-terminal third of the proteins, based on the positions of known insertions, deletions, and cases where β or β’ is split into two proteins (Fig. 1). The tri-domain structure of β recently has been confirmed by reconstitution of active enzyme containing amino-terminal, middle, and carboxy-terminal fragments of the subunit (K. Severinov, A. Mustaev, E. Severinova, I. Bass, V. Nikiforov, R. Landick, A. Goldfarb, and S. Darst, in prep.).

**Implications for structure of the transcription complex**

Despite the lack of obvious sequence conservation between the two subunits, these arguments suggest that β and β’ may exhibit some form of symmetry in the structure of the transcription complex. A priori, this is a reasonable hypothesis, because *rpoB* and *rpoC* probably arose by gene duplication (Ohnishi 1985; Armaleo 1987). How might this partial symmetry be manifest? Perhaps the two RNA contact sites and the two DNA contact sites are formed from some of the partially symmetrical features of β and β’ subunits (Fig. 7). Because the sequences of β and β’ have evolved beyond recognizable similarity, it is conceivable that some features diverged to form single-strand-specific or double-strand-specific sites. Catalysis and positioning of the DNA template strand and transcript 3’ end could be accomplished at the interface of the two subunits. This would account for the apparent role of both subunits in the active site and in contacts to nearby RNA and DNA. The various clusters of termination-altering substitutions may occur primarily in these distinct sites of nucleic acid binding.

In addition to explaining the weak symmetry of β and β’, this model is attractive because it suggests a possible structural basis for inchworm movement of polymerase. At least in vitro near some promoters and at certain regulatory sites, changes in RNA polymerase–nucleic acid contacts during nucleotide addition appear to be discontinuous. Chamberlin (1994) proposes that in these cases, inchworm-like movement of the two DNA contacts on the template occurs during a discontinuous cycle of chain extension in a loose, 3’-proximal RNA-binding site and alternate locking and sliding of the DNA and RNA contacts (see also Das 1993; Chan and Landick 1994, Johnson and Chamberlin 1994; Nuessler et al. 1994). Rotation or translation of β and β’ relative to one another at their interface could move the active site within the proximal RNA-binding channel and also alter the relative positions of RNA and DNA contacts. These changes could produce the different DNA footprints (Krummel and Chamberlin 1992), transcription bubble sizes (Lee et al. 1990, Chamberlin 1994), and transcript cleavage sites (Surratt et al. 1991; Borukhov et al. 1993, Feng et al. 1994) that are observed in different transcription complexes and that led to formulation of the inchworm model. Other explanations for such movements are possible in this view of transcription complex structure.
(such as movement of domains within β or β'), without contradicting the basic notion of a pseudosymmetric relationship between the two large subunits.

Although speculative, this hypothesis is consistent with available structural data. Electron microscopy of E. coli core RNA polymerase suggests the two subunits form separate arms of a bifurcate structure [Tichelaar et al. 1983]. The three-dimensional features of yeast Pol II and E. coli RNA polymerase deduced from electron microscopy of two-dimensional crystals [Darst et al. 1989, 1991], although not obviously bifurcate, could be adapted to this model.

Implications for the mechanism of termination

We have argued that some intervals in β and β' defined by termination-altering substitutions may contact the DNA template or RNA transcript near the active site (notably 7 in β and 2 and 3b in β'; see Fig. 1), whereas others may play more direct roles in catalysis [4a in β', 11 in β, see also Sagitov et al. 1993]. Given the effects on termination that we observed and the hypothetical structure for the transcription complex described above, what can we conclude about the mechanism of termination?

The most striking effects of β' substitutions were the numerous cases in which termination increased at some sites but decreased at others [Fig. 6]. Three explanations for these variable effects on termination are apparent to us: (1) different RNA or DNA sequences present at the different terminators could make contacts to the substituted amino acids that are more or less favorable for transcript release, (2) a substitution could increase or decrease the rate of chain elongation at different sites, and thus the window of opportunity for transcript release, by differentially affecting alignment of particular transcript sequences in the active site, or (3) termination could be a multistep process with different rate-limiting steps at different terminators, so that a particular substitution might affect these steps in opposite ways. If the mechanism of termination is linked to a discontinuous translocation cycle (see Chan and Landick 1994), release at different points in the cycle could explain how substitutions in an apparently dispensable region (interval 4b, see above) might give mixed effects on termination. If this region provides conformational flexibility required for subunit-subunit movement and resulting translocation, substitutions might alter the dynamics of translocation and increase or decrease termination, depending on its relationship to nascent RNA release. Regardless of the details, our results suggest that termination efficiency is governed in part by contacts of RNA polymerase to the DNA or RNA, a conclusion also drawn in a study of 13 ρ-independent terminators by Reynolds et al. [1992].

The discrepancies between effects on termination in vivo and in vitro (Table 1) may result from the absence of transcription factors like NusA, NusB, NusE, and NusG, GreA, and GreB in the in vitro assays. Just as amino acid substitutions might affect different terminators differently, interaction of one or more of these proteins with RNA polymerase might alter the translocation cycle or the configuration of particular RNA or DNA contacts so that the effects of substitutions on termination in vivo and in vitro are reversed. Alternatively, changes in cellular physiology caused by altered transcription in a particular mutant may change levels of the trp operon or cat gene products indirectly and thereby produce the in vivo phenotype.

Much work remains before we can unambiguously assign functions or particular contacts within the transcription complex to the different regions of β' described here, and those described previously for β [Jin et al. 1988; Landick et al. 1990a; Sagitov et al. 1993]. Our substitution analysis of β and β' offers targets about which to ask more detailed questions that ultimately should make conclusive assignments possible.

Materials and methods

Bacteria, bacteriophage, and plasmids

E. coli strain RL211 is derived from strain W3110 and is F- lacF- lacI112 trpC325(Am) lacZ2110(Am) galK(Am) supD43,74[Ts] ΔrecA-srl306 srl-301::Tn10-841 tnaA2 trpR. ΔRL12 encodes a transcriptional fusion of S. marcescens ΔtrpL430trpE to cat. Construction and properties of this strain were described previously (Landick et al. 1990b). E. coli strain RL602 [F- lacI112 tsx trp(Am) lacZ2110(Am) galK(Am) supD43,74[Ts] ΔrecA-srl306 srl-301::Tn10-841 rpsL ΔtrpC325(Am) sueA sueC] was derived from strain MX782 [Ridley and Oeschger 1982]. To test for halophilid viability of mutant β' subunits, derivatives of pRW308 were transformed into strain RL602 [itself unable to grow at 37°C], struck on LB plates containing 50 μg/ml of ampicillin and 0.5 mM IPTG, and incubated at 37°C. Strain RL676 [W3110 trpR tnaA2 rpsL ΔtrpC325(Am) sueA sueC] was derived from strain MX782 [Ridley and Oeschger 1982]. To test for halophilid viability of mutant β' subunits, derivatives of pRW308 were transformed into strain RL602 [itself unable to grow at 37°C], struck on LB plates containing 50 μg/ml of ampicillin and 0.5 mM IPTG, and incubated at 37°C. Strain RL676 [W3110 trpR tnaA2 rpsL ΔtrpC325(Am) sueA sueC] was derived from strain MX782 [Ridley and Oeschger 1982].

DNA manipulations and plasmid construction

Unless otherwise stated, all DNA manipulations were performed following standard published protocols [for description, see Landick et al. 1990a and Feng et al. 1994]. DNA sequencing was performed on single-stranded plasmids derived from pRW308 by the dideoxynucleotide sequencing method [Sanger et al. 1977] using modified T7 DNA polymerase (Sequenase, U.S. Biochemical), [α-35S]ATP (Amersham), and ρoC-specific oligonucleotide primers.
Mutagenesis of pRoC and phenotypic screening

pRW308 was mutagenized by incubation with 0.4 μl hydroxylamine at pH 6.0 for 24 hr at 37°C. The plasmid then was dialyzed against 10 mM Tris-HCl at pH 8.0, 1 mM EDTA and digested with appropriate restriction endonucleases (Fig. 1). After electrophoresis through low-melting agarose these fragments were ligated to the appropriate complementary large fragments from unmutagenized plasmid. To screen for altered termination phenotypes, 95 μl of frozen-competent RL211 was transformed with appropriate restriction endonucleases (Fig. 1). After elution against 10 mM Tris-HCl at pH 8.0, 1 mM EDTA and digestion with 5 μl of a ligation mixture by electroporation at 27 μF, 200 ohms, and 1.8 kV in 0.1-cm cuvettes (18 kV/cm) using a Bio-Rad Electroporator. RL211 was prepared for electroporation by growth in Luria broth (LB) to an OD₆₀₀ of 0.7, centrifuged, washed once with an equal volume of ice-cold H₂O, and then resuspended in 0.002 volume of 10% glycerol. The transformants were recovered by growth overnight (~17 hr) at 37°C on LB plates containing 100 μg/ml of ampicillin. Colonies were transferred in regular arrays to LB–Amp plates containing 0.5 mM IPTG (U.S. Biochemical) and again grown for 17 hr at 37°C. These master plates then were replica-plated by velvet transfer to a series of selective media containing 0.5 mM IPTG and screened for termination-altering phenotypes as described previously (Landick et al. 1990b).

Sequence analysis

All sequence comparisons and analyses were conducted using programs from the Genetics Computer Group at the University of Wisconsin (Devereux et al. 1984), version 7.1, running on a VAXstation cluster.

Purification of RNA polymerase

Mutant RNA polymerases were purified from strain RL676 transformed with derivatives of pRW308. Cells were grown in 200 ml of LB containing 50 μg/ml of ampicillin and 0.5 mM IPTG to a density of ~70 Klett units, chilled rapidly in dry-ice–ethanol, and lysed and subjected to polyethyleneimine precipitation and elution according to Burgess and Jendrisak (1975). RNA polymerase was purified by binding to and elution from heparin–agarose (Chamberlin et al. 1983) followed by FPLC on Mono Q (Hager et al. 1990). Wild-type RNA polymerase prepared by this method appeared to be free of exogenous transcription factors because it exhibited termination and elongation behavior identical to highly purified E. coli holoenzyme prepared from MRE600 E. coli cells (Grain Processing Co., Muscatine, IA) by the standard method of Burgess and Jendrisak (1975), followed by FPLC on Mono Q.

In vitro transcription reactions

DNA templates were prepared by PCR (Landick et al. 1990b) from plasmids carrying various ρ-independent terminators downstream from the strong A1 E. coli ρ70 promoter from bacteriophage T7. The T7, T3, and P14 templates were obtained from plasmids pAR1707, pCPG T3Te, and pCPG P14, respectively (Reynolds et al. 1992). The trpL template was obtained from plasmid pRL407 as described previously (Landick et al. 1990b). The trp and his templates were obtained from plasmids pRL522 and pGF104, respectively (Feng et al. 1994).

To measure termination efficiencies, ~0.6 pmole of RNA polymerase and 0.25 pmole of DNA template were combined and incubated to form “A20” complexes at 37°C for 10 min in 25 μl of 40 mM Tris-HCl, 20 mM NaCl, 14 mM MgCl₂, 14 mM β-mercaptoethanol, 2% (vol/vol) glycerol, 20 μg/ml of acetylated BSA, 240 μM ApU dinucleotide, and 2.5 μM ATP, CTP, and [α-32P]GTP (36 Ci/mmmole). Elongation from position A-20 was initiated by addition of unlabeled GTP to 20 μM, the other three nucleotides to 150 μM, and heparin to 80 μg/ml. After 10 min the samples were combined with 25 μl of 2× TBE, 0.1% bromophenol blue, and 0.1% xylene cyanol saturated with urea. RNA samples were analyzed by electrophoresis through a 15% polyacrylamide, 7 M urea–TBE (88 mM Tris-borate at pH 8.3, 2.5 mM Na₂ EDTA) gel. Radioactivity in the RNA bands was quantitated with an AMBIS radioanalytic imaging system. Percent termination was calculated as mol% terminated RNA/mol% terminated RNA + mol% readthrough RNA(A).

The elongation rate (Table 1) was measured using essentially the same method, except that the reaction volume was raised to 50 μl and 5 μl samples were removed at successive times out to 10 min after elongation from A-20 was initiated by addition of NTPs to a final concentration of 5 μM. After electrophoresis of the samples, the weight average of the distribution of RNA chain lengths past the P14 termination site [2][RNAₙ]: n/2[RNAₙ₋₁] where n = chain length was determined using a Molecular Dynamics PhosphorImager and appropriate size standards. The change in the average chain length per second in a time interval after all RNA polymerase molecules had passed the terminator and before any reached the end of the DNA template was used as the average rate of chain elongation.

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