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 ρ -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; Jøkerst 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 envelops 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

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positions (Arndt 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 modulating RNA chain elongation and termination, a complete understanding of gene regulation will require 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 (Yanofsky 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^r 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 $\rightarrow$ T and G $\rightarrow$ 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 $\sim$ 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^r and 5-MAA^r (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^r) 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

Figure 1. Maps of the β and β' subunits of *E. coli* RNA polymerase showing important landmarks and conserved regions. The five restriction endonuclease-generated DNA fragments used for mutagenesis are indicated by the segmented line beneath the map of β' . The *Xba*I and *Hind*III sites are not in *rpoC* but just upstream and downstream from it in pRW308. Regions of notable sequence similarity between *E. coli* β and β' and the largest subunit of yeast Pol II are shaded uniformly. Conserved regions A–H in β' were first noted by Jokerst et al. (1989) and have been widely adopted and generated for β (Young 1991; Archambault and Freisen 1993, and references therein). Less strongly conserved sequences are indicated by uniform shading without letter designations. The numbered black bars indicate regions where termination-altering substitutions have been identified in β (Jin and Gross 1988; Landick et al. 1990b; L.M. Heisler, G. Feng, R. Landick, D.J. Jin, W.A. Walter and C. Gross, in prep.) and β' (this work). Other features in β and β' are indicated with contrasting patterns and labels. In β , they are a large deletion found in several chloroplast RNA polymerases (Hudson et al. 1988), a sequence resembling one found in the RNase barnase (Shirai and Go 1991), sites of substitutions leading to streptolydigin (Heisler et al. 1993; Severinov et al. 1993) or rifampicin (Lisitsyn et al. 1984; Jin and Gross 1988; Severinov et al. 1993) resistance, location where β is divided in *Chlamydomonas* chloroplasts (Fong and Surzycki 1992), the major site of trypsin cleavage in *E. coli* RNA polymerase (Borukhov et al. 1991), a deletable hinge region in β (Glass et al. 1986), and sites where primer substrate analogs cross-link to the β subunit (Grachev et al. 1989). Features noted in β' are a putative Cys₄-Zn²⁺ motif conserved in most β' homologs (Borukhov et al. 1991), a sequence similarity to DNA polymerase I (Allison et al. 1985), a site where β' homologs are split in cyanobacteria and chloroplasts (Hudson et al. 1988; Xie et al. 1989; Igloi et al. 1990; Shimada et al. 1990; Fong and Surzycki 1992), a region in which Ama-resistance substitutions occur in Pol II β' subunit homologs (Bartolomei and Corden 1987; Chen et al. 1993), a site where the 3' end of the nascent transcript cross-links to β' (Borukhov et al. 1991), a site where an insertion occurs in the rice chloroplast β' subunit (Shimada et al. 1990), and a site where a large deletion occurs in *M. leprae* β' (Honore et al. 1993).

fragments of β' , rearranged or converted to eliminate a particularly deleterious mutation, or had other consequences for cell growth has 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 mutagenized 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*(AI726), *rpoC3325*(SF733), *rpoC3302*(AI748), 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). Together, the two regions cover 134 amino acids of the 333 in interval 3, or 40%.

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 993 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-

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 allow growth of strain RL211 in the presence of 20 µg of Cm/ml; (+) ability to allow growth of RL211 in the presence of 10 µg of Cm/ml; (-) unable to allow growth of RL211 in the presence of 10 µg of Cm/ml. Under MAA: able (+) or unable (-) to allow growth of RL211 in the presence of 100 µg/ml of 5-MAA. The sequence of the corresponding region of the largest subunit of yeast Pol II is shown above the β' sequence (vertical lines indicate identical amino acids; colons indicate similar amino acids) because the yeast subunit has been studied genetically most extensively (Young 1991; Archambault and Freisen 1993). Substitutions known in the yeast Pol II subunit are indicated above the sequence. *rpb1-5*(RC335) is a temperature-sensitive substitution that cannot survive at 36°C (Scafe et al. 1990). *rpb1-511*(RC326) suppresses the temperature sensitivity of the *rpb2-2*(GD1141) substitution in conserved region I of the yeast Pol II second largest subunit (Martin et al. 1990). A short region of sequence that appears to be conserved between *E. coli* DNA polymerase I and β' -subunit homologs is shown above the yeast Pol II sequence. Underlined amino acids indicate identities or similarities. The position of these residues in the crystal structure of DNA polymerase I Klenow fragment are indicated by the schematic above the sequence (Allison et al. 1985; see text). A single substitution (RA668) that inhibits the DNA polymerase activity of Klenow fragment is shown (Polesky et al. 1992; see text).

tity) and is missing entirely from the β' subunit in *Mycobacterium leprae* (Figs. 1 and 4). However, all of the substitutions we found in these two regions occurred at positions conserved between *E. coli* and *P. putida*.

Finally, as for region 2, substitutions in region 5 were tightly clustered. All but one [*rpoC3524*(HY1252)] were found between 1308 and 1367, which represents only 30% of the targeted region and which corresponds to conserved region H (Jokerst et al. 1989). Of the 18 characterized region-5 substitutions, 13 were unique and 8 of these were single amino acid substitutions. The majority 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 RL211 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

A

Cluster 3a

Figure 4. Sequence of amino acid changes in interval 4. (for explanation of symbols, see legend to Fig. 2). (Cluster 4a) *rpb1-501*(SF1096) and *rpb1-502*(TI1080) alter the ratio of transcripts initiated at two adjacent start sites of a Pol II promoter (Hekmatpanah and Young 1991), whereas *rpb1-510*(EA1061) is an extragenic suppressor of *rpb2-2* (Martin et al. 1990; see legend to Fig. 2). The underlined sequence is the region Borukhov et al. (1991) mapped a cross-link between *E. coli* RNA polymerase and 8-azido AMP present at the 3' end of the nascent transcript. (Cluster 4c) *rpo21-6*, *rpo21-7*, and *rpo21-9* may inhibit interaction of elongation factor TFIIS (Archambault et al. 1992; see text); two more insertions and one substitution with similar phenotypes occur just outside the segments of yeast Pol II sequence shown. Sequence alignments between β' and the *P. putida* subunit (Danilovich et al. 1988) are shown for 4b and 4c because these regions show only weak similarity to the yeast Pol II subunit (see Pühler et al. 1989). Sequences between Ala-941 in region 4a and Asp-1131 in region 4c are missing entirely in the *M. leprae* β' subunit (Honore et al. 1993; see Fig. 1).

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^r in vivo and decreased termination in vitro with Cm^r in vivo

(e.g., compare the phenotypes of MI725 and AT730). Second, some mutants conferred both Cm^r and 5-MAA^r in vivo (see also Figs. 2–5). Some substitutions in β 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*

Cluster 5

Figure 5. Sequence of amino acid changes in interval 5. (for explanation of symbols, see legend to Fig. 2). *rpb1-513*(FL1410) (Martin et al. 1990) is an extragenic suppressor of *rpb2-2* (see legend to Fig. 2). *rpb1-551*(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). *rpb1-1*(GD1437) is a temperature-sensitive mutation that ceases mRNA synthesis rapidly at 36°C (Scafe et al. 1990).

which, when overexpressed relative to the wild-type chromosomal gene, altered expression of genes downstream from a ρ -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 ^r	5-MAA ^r
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	+	+

^aRelative 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 μ M 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.

^bPhenotypes 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 μ g/ml of Cm; (+) resistance to 10 μ g/ml of Cm or 100 μ g/ml of 5-MAA; (—) sensitive to Cm or 5-MAA (see [Landick et al. 1990b]).

Figure 6. Termination efficiencies of mutant RNA polymerases at six different ρ -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). (●) Termination efficiencies for wild-type RNA polymerase; (○) 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. Note that the significance of small changes in percent termination is much greater at high values than low [e.g., a change of 95%–90% termination is a twofold increase in the number of polymerase molecules that transcribe through the terminator].

A CONSISTENT CHANGE IN TERMINATION

B SITE-DEPENDENT CHANGE IN TERMINATION

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,

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 β 7– β 8 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 (DeMerceyrol 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 translocation 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 $\sim$ 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^s substitution in yeast Pol II occur on the carboxy-terminal side of the insertion (Fig. 3b). In a prediction of surface probability (Emeni 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 D; 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.

Ascribing 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 TFIIS 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

Figure 7. Hypothesis for arrangement of β and β' subunits in a transcription complex. The relative position of β and β' could be interchanged in this model, but the short sequence similarity to the Klenow fragment (Fig. 2) and the reported DNA-binding properties of β' (Fukada and Ishihama 1974) led us to suggest this arrangement. The nontemplate strand is shown extruded to the exterior of the complex and reannealing with the template strand after it exits based on chemical and nuclease sensitivity studies (see Lee and Landick 1992; Lee et al. 1994). We emphasize that this model depicts a conceptual relationship of β and β' stripped of the three-dimensional topology that must be present in the molecular structure of the RNA polymerase (e.g., see the three-dimensional reconstructions in Tichelaar et al. (1983) and Darst et al. (1989, 1991).

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; Nudler 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. Immunoelectron 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 differ-

ently, 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^- *lacI^q* λ RL12 Δ (*recA-srl*)306 *srl*-301::Tn10-84 *tnaA2 trpR*. λ RL12 encodes a transcriptional fusion of *S. marcescens* Δ *trpL*430*trpE* to *cat*. Construction and properties of this strain were described previously (Landick et al. 1990b). *E. coli* strain RL602 [F^- *leu*(Am) *tsx trp*(Am) *lacZ*2110(Am) *galK*(Am) *galE supD*43,74(Ts) Δ (*recA-srl*)306 *srl*-301::Tn10-84 *rpsL rpoC*325(Am) *sueA sueC*] was derived from strain MX782 (Ridley and Oeschger 1982). To test for haploid 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 μ g/ml of ampicillin and 0.5 mM IPTG, and incubated at 37°C. Strain RL676 [W3110 *trpR tnaA2 rpoC*3530(β' ::*S. aureus* protein A) *zja::kan* Δ (*recA-srl*)306 *srl*-301 Tn10-84], which was used for purification of mutant RNA polymerases (see below), carries an *rpoC* gene that was fused at its carboxyl terminus to a 234-codon segment corresponding to four of the six IgG-binding domains of *S. aureus* protein A and will be described in detail elsewhere.

pRW308 was derived from pTrc99c (Amann et al. 1988) and contains a copy of *lacI^q* bearing the QK78 mutation that increases repression by *lac* repressor in the presence of inducer (Kleina and Miller 1990), an M13 origin of replication, and a *trc* promoter-expressed, minimal *rpoC* gene bearing an ATG initiation codon in place of the normal *rpoC* GTG codon. pRW308 did not overproduce β' in the absence of IPTG (estimated to be 0.5–1 times the amount produced by the chromosomal subunit) but yielded ~10-fold more β' than the chromosomal gene upon addition of IPTG.

DNA manipulations and plasmid construction

Unless otherwise stated, all DNA manipulations were performed following standard published protocols (for description, see Landick et al. 1990ab 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]dATP (Amersham), and *rpoC*-specific oligonucleotide primers.

Mutagenesis of rpoC and phenotypic screening

pRW308 was mutagenized by incubation with 0.4 M 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 μ l of frozen-competent RL211 was transformed with 5 μ l of a ligation mixture by electroporation at 27 μ F, 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, once with 0.5 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 μ g/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 μ g/ml of ampicillin and 0.5 mM IPTG to a density of ~70 Klett units, chilled rapidly in dry-ice-ethanol, and lysed and subject to polyethylenimine 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 σ ⁷⁰ 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* E σ ⁷⁰ 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 μ l of 40 mM Tris-HCl, 20 mM NaCl, 14 mM MgCl₂, 14 mM β -mercaptoethanol, 2% (vol/vol) glycerol, 20 μ g/ml of acetylated BSA, 240 μ M ApU dinucleotide, and 2.5 μ M ATP, CTP, and [α -³²P]GTP (36 Ci/mmol). Elongation from position A-20 was initiated by addition of unlabeled GTP to 20 μ M, the other three nucleotides to 150 μ M, and heparin to 80 μ g/ml. After 10 min the samples were combined with 25 μ l of 2 $\times$ TBE, 0.1% bromophenol blue, and 0.1% xylene cyanol saturated with urea. RNA samples were analyzed by electrophoresis through a 10% polyacrylamide, 7 M urea-TBE (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).

The elongation rate (Table 1) was measured using essentially the same method, except that the reaction volume was raised to 50 μ l and 5 μ l 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 μ M. After electrophoresis of the samples, the weight average of the distribution of RNA chain lengths past the P14 termination site [$\Sigma([RNA_n] \cdot n) / \Sigma[RNA_n]$, 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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