

Insertional Mutagenesis of a Plasmid-Borne *Escherichia coli* *rpoB* Gene Reveals Alterations That Inhibit β -Subunit Assembly into RNA Polymerase

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A plasmid was constructed that overproduces the *Escherichia coli* RNA polymerase β subunit from a *lac* promoter-*rpoB* fusion. The overproduced, plasmid-encoded β subunit assembled into functional RNA polymerase that supplied greater than 90% of the transcriptional capacity of the cells. Excess β subunit segregated into insoluble inclusion bodies and was not deleterious to cell growth. By insertion of a *Xho*I linker sequence (CTCGAG) and accompanying deletion of variable amounts of *rpoB* sequences, 13 structural alterations were isolated in the first and last thirds of the plasmid-borne *rpoB* gene. Twelve of these alterations appeared to reduce or prevent assembly of plasmid-encoded β subunit into RNA polymerase. One alteration had no discernible effect on assembly or function of the β subunit; eight others appeared to inhibit assembly but still produced detectable transcriptional activity. Three of these nine alterations produced β -subunit polypeptides that inhibited cell growth at 32°C, even though they were present in less than 50% of the cell RNA polymerase. When assembled into RNA polymerase, these three altered β subunits apparently affected essential RNA polymerase functions. Four of the recovered alterations appeared to inhibit completely or almost completely assembly of the β subunit into RNA polymerase. The results are consistent with a hypothesis that sequences in the first third of the β -subunit polypeptide are especially important for proper folding and assembly of the β subunit.

The β subunit of *Escherichia coli* RNA polymerase is an attractive target for systematic analysis of structure-function relationships in the transcription complex. The β (M_r , 151,000), β' (M_r , 155,000), and two α (M_r , 37,000) subunits constitute core RNA polymerase, an arrangement that is conserved among eubacteria (12, 72). Promoter specificity is conferred on core by a family of σ subunits (58); in *E. coli*, σ^{70} (M_r , 70,000) is the major σ factor. β and β' are encoded by the *rpoB* and *rpoC* genes, which are located contiguously at 90 min in a polycistronic operon that includes genes for four ribosomal proteins (14, 33, 37, 49, 68). α and σ^{70} are encoded by the unlinked *rpoA* (73 min) and *rpoD* (67 min) genes, respectively, which also are in operons encoding ribosomal components (22, 27, 42). All four genes have been cloned, sequenced, and verified by conditional lethal mutations to be essential for RNA polymerase function in vivo (2, 5, 11, 32, 54-56, 59).

The β subunit is intimately involved in most of the known activities of prokaryotic RNA polymerase and contains significant sequence similarities to the second-largest subunit of yeast and insect RNA polymerase II (13, 64). Mutations in *rpoB* (predominantly between codons 500 and 575) confer resistance to the transcription inhibitors rifampin and streptolydigin (28, 36, 57). Jin and Gross have determined the genetic alterations of 17 different rifampin-resistant (Rif^r) mutants (28); many of them either increase or decrease RNA hairpin-dependent transcription pausing (15), *rho*-independent termination (15, 29, 43, 70), or *rho*-dependent termination (29). Rifampin blocks transcription initiation after formation of several phosphodiester bonds (61); thus, the segment of the β subunit that contacts rifampin ordinarily may interact with a moiety, such as the RNA-DNA hetero-

duplex, that is present only in the elongating transcription complex. An analysis by Glass and co-workers of altered β subunits produced by suppression of *rpoB*(Am) mutations implicates several other regions of the β subunit either in σ binding (16, 19, 45), ppGpp (the stringent response effector) binding (18, 46), or promoter interaction (18, 47). Chemical cross-linking studies have detected interactions between the β subunit and the DNA template (63), the RNA transcript (23), and nucleotide triphosphate analogs (20). We undertook the studies reported here to develop and test a system for systematic genetic analysis of the role of the β subunit in the control of transcription elongation. To analyze amino acid substitutions in the β subunit that alter the efficiency of pausing and termination, a method to produce and systematically mutagenize the β subunit was required.

Previous work by others provided a framework for our experiments. The assembly of subunits into the core RNA polymerase, as defined by Ishihama, follows an ordered pathway: $2\alpha \rightarrow \alpha_2$, $\beta + \alpha_2 \rightarrow \beta\alpha_2$, $\beta' + \beta\alpha_2 \rightarrow \beta\beta'\alpha_2$ (25). Glass and co-workers discovered that certain β -subunit polypeptides missing ca. 100 C-terminal residues effectively compete with the wild-type subunit for assembly into core polymerase. However, these deletions prevent association of σ with core to form holoenzyme and produce a temperature-sensitive phenotype in merodiploid strains (16, 19, 45). Kashlev et al. have constructed plasmids similar to that described here (31). Some fusions of the *lac* promoter to *rpoB* did not lead to efficient incorporation of the plasmid-encoded, Rif^r β subunit into RNA polymerase. Nomura and co-workers (6, 50) have studied strains in which the entire *rpoBC* locus is under control of the *lac* promoter-operator. They found that in cells induced to coordinately overproduce β and β' , the increase in RNA polymerase content is limited to twofold by translational feedback regulation (6), whereas limitation of

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TABLE 1. Bacterial, phage, and plasmid strains

Designation	Genotype, ^a phenotype, or derivation	Source or reference
<i>E. coli</i> K-12 strains		
CY15013	W3110 <i>trpR tnaA2 trpL75 rpoB6</i> (Rif) ^b	C. Yanofsky (70)
DH5 α F'	F ⁺ <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 ΔlacU169 ϕ80dlacZΔM15</i>	Invitrogen
JC10289 (CGSC 6195)	<i>thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 rac hisG4(Oc) rfbD1 mgl-1 Δ(recA-srl)306 srl-301::Tn10-84 rpsL31 kdgK51 xyl-1 mtl-1 argE3(Oc) thi-1</i>	B. Bachmann
MX761	<i>leu</i> (Am) <i>trp</i> (Am) <i>lacZ2110</i> (Am) <i>galK</i> (Am) <i>galE rpsL tsx supD43 rpoBcl</i> (Am) <i>sueA sueC</i>	M. Oeschger
MX1444	<i>leu</i> (Am) <i>trp</i> (Am) <i>lacZ2110</i> (Am) <i>galK</i> (Am) <i>galE rpsL tsx supD43,74</i> (Ts) <i>rpoBcl</i> (Am) <i>sueA sueC</i>	M. Oeschger
MX1494	<i>leu</i> (Am) <i>trp</i> (Am) <i>lacZ2110</i> (Am) <i>galK</i> (Am) <i>galE rpsL tsx supD43,74</i> (Ts) <i>rpoBcl</i> (Am) <i>sueA</i>	M. Oeschger
RL455	From CY15013; <i>trpR tna-2 trpL75 rpoB2 Δ(recA-srl)306</i>	This work
RL514	RL455(pRL385)	This work
RL589	RL455(pUC119)	This work
RL582	As MX761 but <i>Δ(recA-srl)306 srl-301::Tn10-84</i>	This work
RL583	As MX1444 but <i>Δ(recA-srl)306 srl-301::Tn10-84</i>	This work
RL585	As MX1494 but <i>Δ(recA-srl)306 srl-301::Tn10-84</i>	This work
Phage		
AN λ 63	11-kb <i>Hind</i> III fragment (<i>rplJ'L rpoBC</i>) from chromosome of <i>E. coli</i> K-12 strain CR63 (1) in N λ 761 [λ <i>imm</i> ²¹ <i>supF Δ(att-red) gam210</i> (Am) Δ <i>nin5</i> ; <i>supF</i> derivative of N λ 742 in reference 41)	R. Hayward (49)
Plasmids		
pBR322	Ap ^r Tc ^r	7
pUC8	Ap ^r	65
pUC12	Ap ^r	65
pUC18	Ap ^r	69
pUC119	Ap ^r M13 IG ⁺ derivative of pUC19	66
pNF1346	Tc ^r <i>rplKAJL rpoB114 rpoC</i> ; 14.5-kb <i>Pst</i> I (partial) fragment from λ <i>rif</i> ^d 18 in the <i>Pst</i> I site of pBR322	14
pRL203	6.2-kb <i>Bgl</i> III fragment from pNF1346 (<i>rplA'JL rpoBC'</i>) in the <i>Bam</i> HI site of pUC8	This work
pRL262	10.8-kb <i>Bgl</i> III (partial) fragment from the CY15013 chromosome (<i>rpoA'JL rpoB2 rpoC</i>) in the <i>Bam</i> HI site of pBR322	This work
pRL264	1.1-kb <i>Sal</i> I fragment (<i>rpoB'</i> N terminus) in <i>Sal</i> I site of pUC8	This work
pRL270	440-bp <i>Eco</i> RI- <i>Kpn</i> I fragment (<i>rpoB'</i> N terminus) between <i>Eco</i> RI and <i>Kpn</i> I sites of pUC18	This work
pRL271	118-bp <i>Hpa</i> II (<i>rpoB'</i> C terminus) in <i>Acc</i> I site of pUC12	This work
pRL280	3.5-kb <i>Kpn</i> I- <i>Sal</i> I fragment (<i>rpoB'</i>) between <i>Kpn</i> I and <i>Sal</i> I sites of pRL262	This work
pRL284	Ap ^r <i>rpoB2</i> ; 123-bp <i>Sph</i> I- <i>Hind</i> III fragment (<i>rpoB'</i> C terminus) between <i>Sph</i> I and <i>Hind</i> III sites of pRL280	This work
pRL285	Ap ^r <i>rpoB</i> ⁺ ; 910-bp <i>Cl</i> aI- <i>Hpa</i> I fragment (<i>rpoB'</i>) from AN λ 63 between <i>Cl</i> aI and <i>Hpa</i> I sites of pRL284	This work
pRL385	Ap ^r M13 IG ⁺ <i>rpoB</i> ⁺ ; 4.1-kb <i>Bam</i> HI- <i>Hind</i> III fragment (<i>rpoB</i> ⁺) from pRL285 (treated with DNA polymerase I Klenow fragment) in <i>Sma</i> I site of pUC119	This work
pRL385 <i>rpoB2</i>	Ap ^r M13 IG ⁺ <i>rpoB2</i> ; from pRL385 by insertion of 234-bp <i>Bcl</i> II fragment from pRL284 between <i>Bcl</i> II sites of pRL385	This work
pRL385 <i>rpoB114</i>	Ap ^r M13 IG ⁺ <i>rpoB114</i> ; from pRL385 by insertion of 234-bp <i>Bcl</i> II fragment from pRL203 between <i>Bcl</i> II sites of pRL385	This work

^a All strains are λ^- F⁻ unless otherwise noted.

^b *rpoB6* and *rpoB2* specify the same C→T change at *rpoB* position 1576 (His→Tyr at β -subunit position 526); see reference 28.

$\beta\beta'$ synthesis can reduce RNA polymerase content to as little as 40% of the normal level without affecting cell viability (50).

To begin an analysis of the role of the β subunit in transcriptional pausing and termination, we constructed the β -subunit-overproducing plasmid pRL385, which contains the *rpoB* gene under transcriptional control of the *lac* promoter. We report here that pRL385-encoded β subunit was overproduced 20-fold and was assembled into functional RNA polymerase. Overexpression of the β subunit was not deleterious to growth of *E. coli*; the excess β -subunit polypeptide accumulated in inclusion bodies within the cells rather than being degraded. Some plasmid-encoded β subunit assembled into functional RNA polymerase that accounted for greater than 90% of the transcriptional capacity of the cell. To test the suitability of pRL385 for systematic mutagenesis of *rpoB*, we isolated 13 different insertions in *rpoB* of the sequence CTCGAG (recognition site for the

restriction enzyme *Xho*I) that did not alter the translational reading frame of the gene. Most were accompanied by deletion of variable amounts of *rpoB*, and all but one appeared to inhibit or prevent assembly of the plasmid-encoded β subunit into functional RNA polymerase. Three altered β subunits appeared partially defective for assembly but, when assembled, yielded catalytically active RNA polymerase that was detrimental to cell growth at 32°C.

MATERIALS AND METHODS

Bacteria, bacteriophage, and plasmids. All *E. coli* strains, bacteriophage, and plasmids used are listed in Tables 1 and 2. Novel *E. coli* strains were constructed by phage P1 *vir*-mediated transduction (62), using appropriate donor and recipient strains (Table 1). Δ (*recA-srl*)306 strains were constructed by cotransduction of Δ (*recA-srl*)306 and *srl-301::Tn10-84*, using P1 grown on strain JC10289. In some strains,

TABLE 2. Sequence changes in pRL385 linker insertion mutants

Plasmid isolate	Change in pRL385 DNA ^a	Change in β^b	Allele	Allele with <i>rpoB114</i> (Rif)
pRL385 <i>Xho</i> -1	A-1288-CTCGAG-A-1316	Lys-431-Thr-Arg-Glu-Lys-439	<i>rpoB5011</i>	<i>rpoB5017</i>
pRL385 <i>Xho</i> -4	T-530-CTCGAG-G-570	Pro-178-Ser-Glu-Pro-190	<i>rpoB5014</i>	<i>rpoB5046</i>
pRL385 <i>Xho</i> -5	C-569-CTCGAG-A-588	Pro-190-Ser-Glu-Val-196	<i>rpoB5015</i>	<i>rpoB5047</i>
pRL385 <i>Xho</i> -6	T-141-CTCGAG-G-154	Tyr-47-Leu-Glu-Ala-52	<i>rpoB5016</i>	<i>rpoB5048</i>
pRL385 <i>Xho</i> -16	T-984-CTCGAG-G-1045	Ser-328-Leu-Glu-Glu-349	<i>rpoB5037</i>	<i>rpoB5049</i>
pRL385 <i>Xho</i> -19	T-2652-CTCGAG-A-2668	Val-884-Leu-Glu-Lys-890	<i>rpoB5038</i>	<i>rpoB5050</i>
pRL385 <i>Xho</i> -25	G-946-CTCGAG-A-947	Met-315-Ala-Arg-Glu-316	<i>rpoB5039</i>	<i>rpoB5051</i>
pRL385 <i>Xho</i> -29	A-2742-CTCGAG-A-2740	Lys-914-Leu-Glu-Lys-914	<i>rpoB5040</i>	<i>rpoB5052</i>
pRL385 <i>Xho</i> -31	A-257-CTCGAGCTCGAGCTCAG-T-305	Cys-85-His-Ser-Ser-Ser-Ser-Val-Val-103	<i>rpoB5041</i>	<i>rpoB5053</i>
pRL385 <i>Xho</i> -36	A-72(<i>lacZ</i>)-CTCGAG-T-156	Ser-24(<i>lacZ</i>)-Leu-Glu-Phe-53	<i>rpoB5042</i>	<i>rpoB5054</i>
pRL385 <i>Xho</i> -42	T-100-CTCGAG-A-97	Ser-34-Arg-Asp-Ser-34	<i>rpoB5043</i>	<i>rpoB5055</i>
pRL385 <i>Xho</i> -54	C-3561-CTCGAG-A-3571	Phe-1187-Leu-Glu-Lys-1191	<i>rpoB5044</i>	<i>rpoB5056</i>
pRL385 <i>Xho</i> -73	A-818-CTCGAGCCGAG-T-1106	Arg-175-His-Ser-Ser-Arg-Val-Met-370	<i>rpoB5045</i>	<i>rpoB5057</i>

^a Numbers refer to nucleotide positions, with +1 being the first base in *rpoB* (or *lacZ* for the leftward endpoint of the deletion in pRL835*Xho*-36).

^b Numbers refer to codon positions, with +1 being the first codon in *rpoB* (or *lacZ* for the leftward endpoint of the deletion in pRL385*Xho*-36).

loss of Tn10 was selected by the method of Maloy and Nunn (38). The *rpoB* allele numbers used by Jin and Gross (28) are given whenever the same amino acid change is represented in the literature by more than one allele number.

Chemicals and enzymes. Chemicals were obtained from Sigma Chemical Co. or Fisher Scientific Co. Restriction endonucleases and DNA-modifying enzymes were obtained from New England Bio-Labs, IBI, or U.S. Biochemical Corp. and were used as instructed by the manufacturer. Pancreatic DNase I (grade DPFF) was obtained from Worthington Diagnostics. Oligonucleotides were prepared on an Applied Biosystems 380B oligonucleotide synthesizer by using cyanoethylphosphoramidite chemistry and were purified on Dupont NENsorb columns. *E. coli* RNA polymerase was prepared by the method of Burgess and Jendrisak (10) from MRE600 cells obtained from Grain Processing, Inc.

Plasmid DNA preparation and analysis. Plasmid DNAs were prepared on a small scale by using the alkaline lysis procedure (39) and on a large scale by using the Triton X-100 lysis procedure (62), followed by CsCl-ethidium bromide buoyant density gradient centrifugation (3). Unless otherwise stated, all DNA manipulations were performed according to standard published protocols (3, 39). DNAs were stored in 10 mM Tris hydrochloride (pH 7.9)-1 mM Na₂EDTA (TE buffer).

Construction of pRL385. pRL385 was constructed by assembly, initially into pUC18 (69), of several *rpoB* DNA fragments from different sources to yield a functional, wild-type gene (Fig. 1). To truncate *rpoB*-derived DNA immediately upstream from the coding region, a 1.1-kilobase-pair (kb) *SalI* DNA fragment, derived from pRL262, was ligated into the *SalI* site of pUC8 (65) to yield pRL264. pRL262 is a pBR322 derivative containing a 10.8-kb *Bgl*III fragment from the chromosome of CY15013 [*rpoB2*(Rif^r)] (70) that was isolated by its ability to confer resistance to rifampin from a bank of partial *Bgl*III fragments cloned into the pBR322 *Bam*HI site. A 440-base-pair (bp) *Eco*RI-*Kpn*I fragment was isolated from pRL264 and cloned between the *Eco*RI and *Kpn*I sites of pUC18 to yield pRL270. A 3.5-kb *Kpn*I-to-*Sph*I fragment from pRL262 then was ligated between the *Kpn*I and *Sph*I sites of pRL270 to yield pRL280, which encoded all but the last 23 amino acids of the β subunit. To isolate a source for the remaining *rpoB* sequences without including any *rpoC* sequences, a 110-bp *Hpa*II fragment from pRL203 was ligated into the *Acc*I site of pUC12 (64) to yield pRL271. pRL203 is a pUC8 derivative containing, in the *Bam*HI site,

a 6.2-kb *Bgl*III fragment from pNF1346 (14). Digestion of pRL271 with *Sph*I and *Hind*III yielded a 123-bp fragment that, when ligated between the *Sph*I and *Hind*III sites of pRL280 yielded a full-length *rpoB* gene in pRL284. pRL284 still contained the *rpoB2*(Rif^r; His-526→Tyr) mutation; therefore, the 910-bp fragment between *Clal* and *Hpa*I in pRL284 was replaced with the homologous wild-type fragment from AN λ 63 (49) to yield pRL285, a plasmid that expresses wild-type β subunit from the *lac* promoter originally present in pUC18. Finally, the 4.1-kb *Bam*HI-to-*Hind*III fragment from pRL285 was isolated, treated with the Klenow fragment of DNA polymerase I, and ligated into the *Sma*I site of pUC119 (66) to yield pRL385, a plasmid that contains the M13 origin for single-stranded DNA synthesis. Thus, pRL385 (Fig. 1) contains a full-length, wild-type *rpoB* gene consisting of DNA derived from several sources (in base pairs relative to the first base in *rpoB*): *Sal*I (-29) to *Clal* (1297) from the CY15013 (70) chromosome, *Clal* (1297) to *Hpa*I (2207) from AN λ 63 (49), *Hpa*I (2207) to *Sph*I (3953) from the CY15013 chromosome (70), and *Sph*I (3953) to *Hpa*II (4071) from λ d *rij*^{ad}18 (33). pRL385 contains sequences before the AUG initiation codon of *rpoB* that direct ribosomes that initiate translation of the *lacZ*' coding region to terminate eight nucleotides before the *rpoB* AUG codon and contains no sequences from the *rpoC* gene (Fig. 1B).

Insertional mutagenesis. pRL385 (100 μ g) was treated with 1 μ g of pancreatic DNase I for 20 min at 24°C in 500 μ l of 20 mM Tris hydrochloride (pH 7.5)-1.5 mM MnCl₂-100 μ g of bovine serum albumin per ml (24). After phenol extraction and ethanol precipitation, 6 μ g of this DNA was treated with T4 DNA polymerase (New England Bio-Labs) to form blunt-ended molecules. After phenol extraction and ethanol precipitation, the DNA was redissolved in 13 μ l of TE and added to a 15- μ l solution containing 1 μ g of the dodecanucleotide d(CTCGAGCTCGAG) (*Xho*I linker), 66 mM Tris hydrochloride (pH 7.6), 1 mM ATP, 1 mM spermidine, 10 mM MgCl₂, 15 mM dithiothreitol, 200 μ g of bovine serum albumin per ml, and 7 U of T4 polynucleotide kinase that had been preincubated at 37°C for 45 min. The solution was adjusted to a final volume of 40 μ l, maintaining the kinase buffer concentrations, and 2 Weiss units of T4 DNA ligase was added. After incubation for 17 h at 16°C, the solution was adjusted to 100 μ l with TE and extracted sequentially with phenol and CHCl₃. The DNA was recovered by spermine precipitation, dried in vacuo, redissolved in 60 μ l of *Xho*I buffer (New England Bio-Labs), and digested with 100

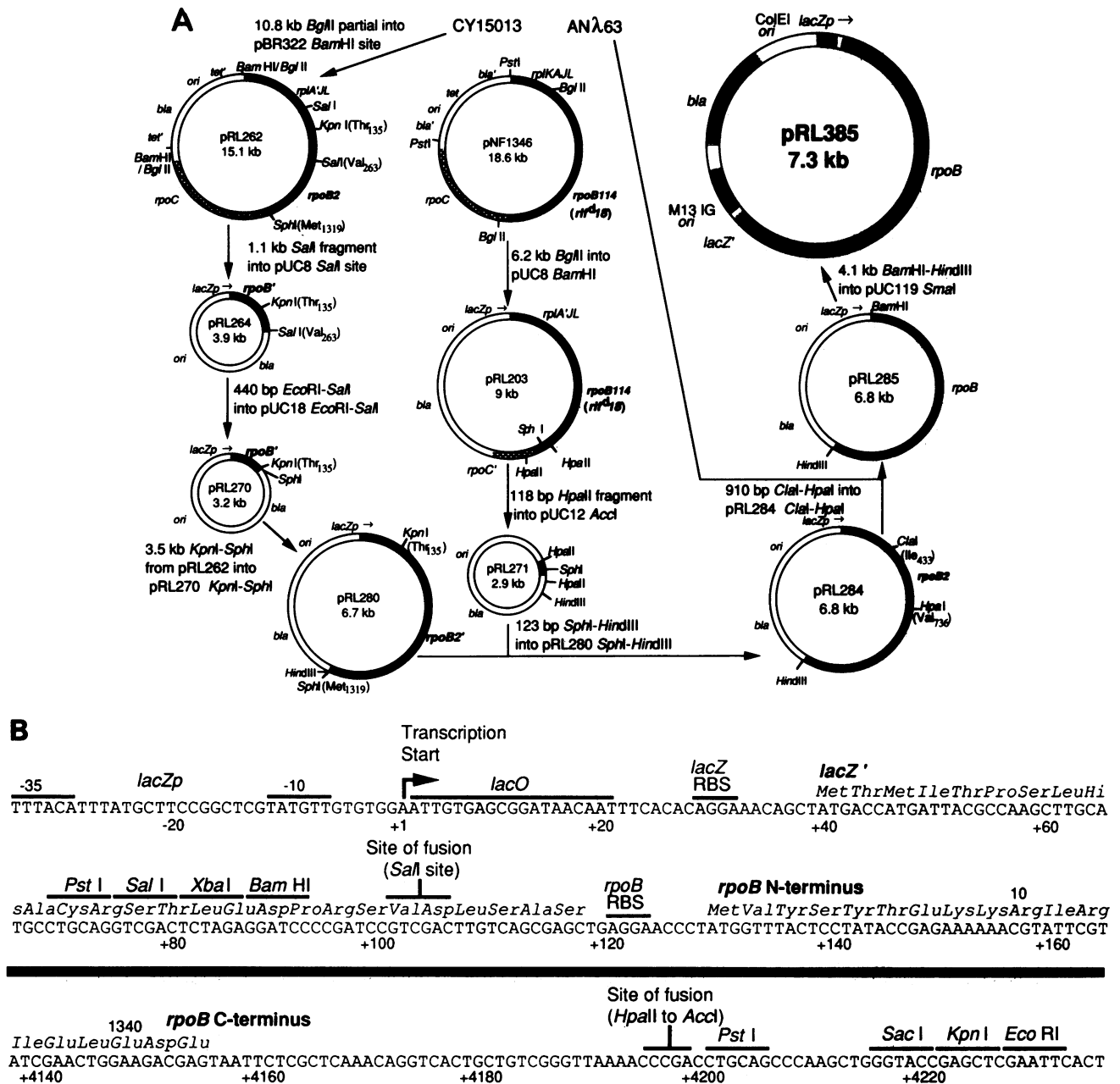


FIG. 1. (A) Scheme for the construction of pRL385. (B) DNA and protein sequences at the N-terminal and C-terminal regions of *rpoB* in pRL385.

U of *Xho*I for 3 h at 37°C. The DNA then was electrophoresed through a 0.6% low-melting agarose (SeaPlaque; FMC, Inc.) gel in 50 mM Tris-acetate (pH 8.3) at 4°C. An agarose plug containing the full-length, linear pRL385 DNA was excised and, after melting at 65°C, was extracted sequentially with phenol and chloroform. About 5 ng of linear pRL385 was recovered after spermine precipitation. This DNA was recircularized with T4 DNA ligase and subjected to a final ethanol precipitation. The ligated DNA was dissolved in 20 µl of TE and used to transform competent DH5αF' cells (Invitrogen).

Mapping of *Xho*I linker insertion sites. Transformants containing *Xho*I linker-derivatized pRL385 were screened for approximate plasmid size by direct electrophoresis of DNA from 5 µl of a lysed cell culture (4). Plasmids of the

correct size were isolated, and the approximate sites of *Xho*I linker insertion were determined by electrophoretic sizing of *Xho*I-*Eco*RI- and *Xho*I-*Pst*I-derived DNA fragments in 1% agarose gels. Accurate sites of *Xho*I linker insertions in pRL385 were determined by sizing in 8% polyacrylamide gels fragments generated with *Taq*I, *Hpa*II, and *Hpa*II-*Xho*I and labeled with ³²P by incubation with DNA polymerase I Klenow fragment (U.S. Biochemical) and [α-³²P]dCTP (3). The precise sites of *Xho*I linker insertions and the extents of deletions and insertions in pRL385*Xho* plasmids were determined by the dideoxynucleotide sequencing method (3, 60), using T7 DNA polymerase (Sequenase; U.S. Biochemical), [α-³⁵S]dATP (Amersham Corp.), and *rpoB*-specific oligonucleotide primers.

Construction of recombinants between *Xho*I linker inser-

tions and *rpoB114*. Plasmids containing *XhoI* linker insertions in the N-terminal third of *rpoB* were cleaved with *ClaI* and *BamHI*. The small fragments containing the *XhoI* linker insertions were excised after electrophoresis in low-melting agarose and ligated to a similarly treated, complementary, large fragment from pRL385*rpoB114*(Rif^r; Ser-531→Phe). Plasmids containing C-terminal, *XhoI* linker insertions were cleaved with *HpaI* and *SacI* and similarly combined with the complementary fragment from pRL385*rpoB114*. In pRL385 *Xho-1*, the *ClaI* site was destroyed by the insertion; in this case, the 234-bp *BclI* fragment from pRL385*rpoB114* was recovered and ligated to the large *BclI* fragment from pRL385*Xho-1*. The presence of the expected *XhoI* linker insertions was verified by restriction endonuclease analysis of plasmids recovered after transformation of JM109 with these ligations.

SDS-polyacrylamide gel electrophoresis. Whole-cell extracts of *E. coli* were prepared by direct solubilization of pelleted *E. coli* cells in sodium dodecyl sulfate (SDS)-gel sample buffer (34, 62) and electrophoresed on Laemmli-type SDS-polyacrylamide gels (34) consisting of a 3% polyacrylamide (acrylamide/bisacrylamide ratio, 30:0.44) stacking gel and an 8% polyacrylamide (acrylamide/bisacrylamide ratio, 33.5:0.3) running gel. Electrophoresis was continued for 30 min after the bromophenol dye had migrated off the bottom of the gels. Gels were stained with Coomassie brilliant blue R (Sigma) according to a standard procedure (62). β -Subunit polypeptide was quantitated by scanning the stained gels with a Joyce-Loebl densitometer. Areas under the peaks corresponding to the β subunit were converted to mass by interpolation between standards from known quantities of β . Total SDS-soluble protein in samples loaded to gels was determined with the Bradford assay (8) after removal of SDS with KPO₄ (71).

RNA polymerase assays. Crude extracts (fraction iv) of *E. coli* for RNA polymerase assays were prepared by the method of Gross et al. (21). RNA polymerase activity on poly d(AT) was determined as described by Gross et al. (21) except that poly r([³H]U,A) was quantitated by binding to DE81 paper as described by Weil and Blatti (67).

RESULTS

pRL385-directed synthesis of a functional β subunit. To construct a plasmid-borne *rpoB* gene that would be well suited for genetic analysis with in vitro mutagenesis techniques, we assembled pRL385 from various *rpoB* gene fragments so that minimal flanking sequences and no *rplL* or *rpoC* coding regions remained (Fig. 1). Although the three sources of these fragments were themselves mutant *rpoB* genes (CY15013, *rpoB2* [28, 70]; λ rif^d18, *rpoB114* [29, 33]; and AN λ 63, C-terminal frameshift [R. Hayward, personal communication]), the *rpoB* gene assembled in pRL385 (and pRL285) is wild type, since the known mutant regions of *rpoB2* and *rpoB114* (28) were replaced with wild-type DNA from AN λ 63 (Fig. 1A; see Materials and Methods). Although we have not verified that the entire pRL385 *rpoB* gene is identical to the published wild-type sequence (55), sequence analysis of ca. 50% of the plasmid-borne *rpoB* gene has revealed no differences from the published wild-type sequence (data not shown). Furthermore, pRL385*rpoB*⁺ caused phenotypes expected of the wild-type gene (see below) and directed synthesis of a β subunit with electrophoretic mobility identical to that of the wild-type subunit. The region upstream from *rpoB* in pRL385 was engineered to facilitate translation of *rpoB*: the *lacZ'* α -peptide-coding

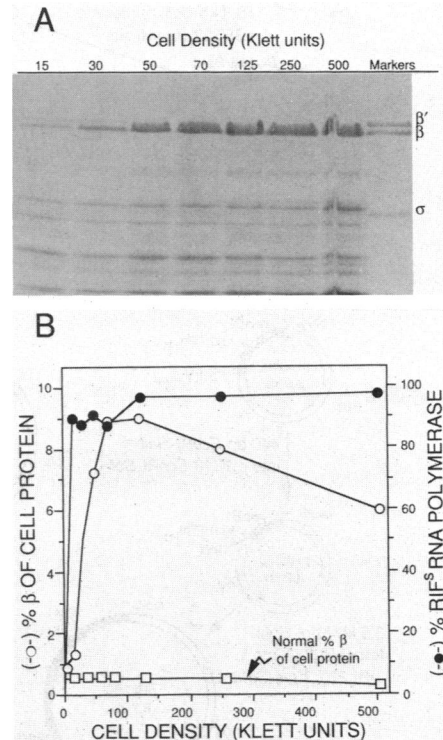


FIG. 2. Analysis of β -subunit expression from pRL385. (A) SDS-polyacrylamide gel containing solubilized proteins from strain RL514 at various stages of growth in LB medium containing 50 μ g of ampicillin per ml and 0.5 mM IPTG at 37°C. The marker lane contains (from top to bottom) the β' , β , and σ subunits of purified RNA polymerase. (B) Plots of the β subunit as a percentage of total SDS-soluble cell protein (○) determined from the gel shown in panel A and the percentage of RNA polymerase activity (●) inhibited by 10 μ g of rifampin per ml. The β subunit as a percentage of total SDS-soluble protein similarly determined for control strain RL589 containing pUC119 is shown (□). See Materials and Methods for details of the experimental procedures.

region ends in a UGA codon that overlaps the *rpoB* Shine-Dalgarno sequence (Fig. 1B; see Materials and Methods). The C-terminal region of *rpoB* in pRL385 was engineered so that no sequences from *rpoC* were present (Fig. 1; see Materials and Methods).

To determine the efficiency of *rpoB* expression from pRL385 and whether pRL385-encoded β subunit was assembled into a functional RNA polymerase, we compared β -subunit synthesis and RNA polymerase activity in cultures of the *recA* strains RL514 [chromosomal *rpoB2*(Rif^r) (pRL385*rpoB*⁺)] and RL589 [chromosomal *rpoB2*(Rif^r) (pUC119)]. Both cultures grew with doubling times of ca. 60 min and ceased log-phase growth at ca. 200 Klett units (data not shown). β -Subunit polypeptide in RL589 ranged from 0.5% of total SDS-soluble protein during log-phase growth to 0.3% after growth slowed (Fig. 2B), in good agreement with previous estimates of RNA polymerase content in *E. coli* (9). In contrast, β -subunit content in RL514 increased dramatically during growth, reaching a maximum of ca. 9% of the SDS-soluble cell protein when the cultures ceased log-phase growth (Fig. 2). We found that 90 to 98% of RNA polymerase activity in crude extracts of RL514 was inhibited by 10 μ g of rifampin per ml (Fig. 2B), whereas RNA polymerase activity from RL589 was 100% resistant to 10 μ g of rifampin per ml (data not shown). Thus, in strain RL514, essentially

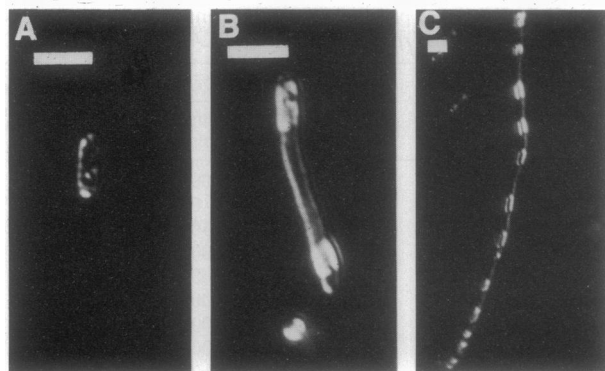


FIG. 3. Appearance of RL455 cells by differential interference contrast microscopy. Scale bars, 1 μ m. (A) Control cell containing pUC119. (B) Typical cell containing pRL385 in a late log culture. (C) Atypical cell containing pRL385, representative of 5% or less of the cells present in a late-log-phase culture.

all RNA polymerase in the cells contained β subunit encoded by pRL385.

We examined several media and cellular hosts to determine whether β -subunit synthesis from pRL385 could be repressed by the *lac* repressor. In general, we found only minimal reductions of β -subunit content in *lacI^r* strains when isopropyl- β -D-thiogalactopyranoside (IPTG) was not present. When *lacI^r* cells were grown without IPTG in the presence of 1% glucose, synthesis of the β subunit from pRL385 was reduced ca. 10-fold but could not be eliminated (data not shown). We presume that the high copy number of pUC19 derivatives was largely responsible for our inability to more completely inhibit β -subunit synthesis from pRL385.

Excess β subunit synthesized from pRL385 *rpoB* accumulated in inclusion bodies. We were surprised that accumulation of such large quantities of the β subunit did not significantly alter the growth of pRL385-transformed strains. In many experiments, using both liquid and solid media, the growth rates and appearance of cultures of strains containing

either pRL385 or pUC119 were not detectably different (data not shown). Many proteins overproduced in *E. coli* are known to form inclusion bodies (30). Furthermore, the β subunit forms insoluble aggregates when overproduced by induction of T7 RNA polymerase in strains carrying a T7 RNA polymerase promoter-driven, plasmid-borne *rpoB* gene (40). We determined that most of the β subunit present in cultures of pRL385-transformed strains could be sedimented from sonicated crude lysates at as low as $5,000 \times g$. Examination of cells from these cultures by differential interference contrast microscopy revealed highly refractile particles within cells containing pRL385 (Fig. 3). These refractile particles were similar in appearance to published photographs of other inclusion bodies in *E. coli* (30). Most of the cells we observed displayed some defect in septum formation, typically being twice the length of normal cells (Fig. 3A and B). In a small fraction of cells (less than 5%), the defect in septum formation produced very long rods containing many inclusion bodies (Fig. 3C). We concluded that β subunit overproduced from pRL385 segregated into inclusion bodies when it did not assemble into RNA polymerase.

pRL385 was mutagenized in vitro by insertion of a 6-bp *XhoI* oligonucleotide. As an initial test of pRL385 as an expression vector for systematic mutagenesis of *rpoB*, we isolated a set of pRL385 derivatives in which a 6-bp *XhoI* linker [d(CTCGAG)] was inserted at random sites. The sites were produced by cleavage of pRL385 with DNase I in the presence of Mn^{2+} , followed by treatment with T4 DNA polymerase (24). Fifty-one *XhoI* linker insertions were mapped within *rpoB*; the distribution of the insertion events recovered was reasonably uniform, with some bias toward the N-terminal region and underrepresentation of the central region of *rpoB* (Fig. 4).

By SDS-polyacrylamide gel electrophoresis of proteins from cells containing the pRL385*Xho* derivatives, we determined that 23 pRL385*Xho* derivatives contained CTCGAG insertions that did not prevent synthesis of near-full-length β subunit. All 23 derivatives caused accumulation of β subunit to essentially the same levels as caused by unaltered pRL385 (data not shown). After initial analysis by restriction endo-

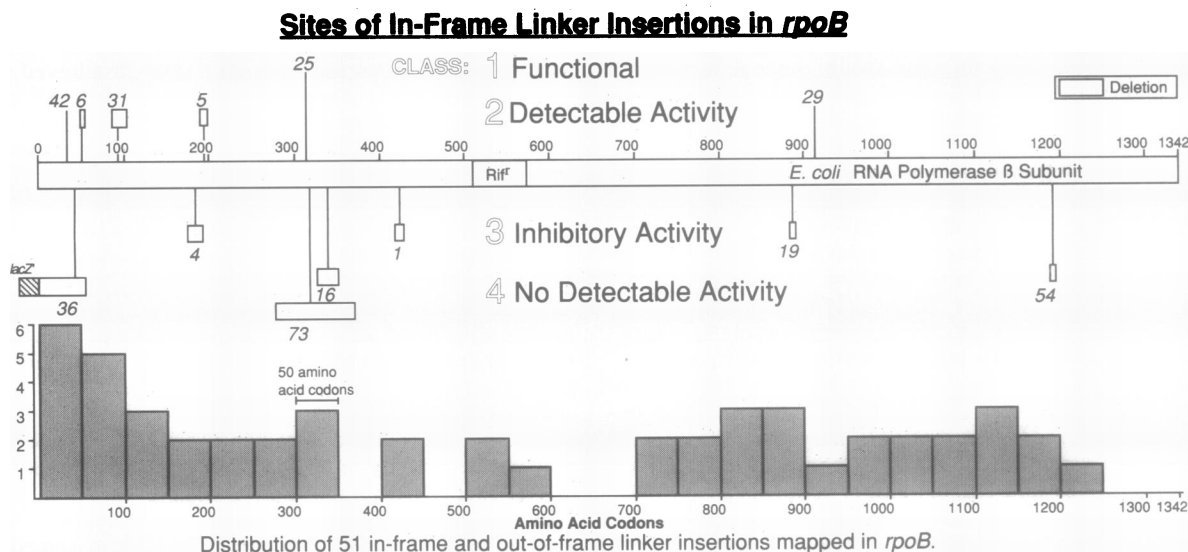


FIG. 4. Schematic representation of the locations and phenotypes of alterations recovered in the pRL385 *rpoB* gene. Rif^r indicates the region that contains all but two of the known Rif^r mutations (28). Classes 1 to 4 are defined in the text.

nuclease digestion (see Materials and Methods), we used synthetic oligonucleotide primers to determine the precise sites of CTCGAG insertion and the sizes of deletion or duplication introduced by DNase I cleavage-T4 polymerase repair (Table 2 and Fig. 4). Of the 23 that we sequenced, 7 proved to specify a shift in the translational reading frame within the last 300 codons of *rpoB*; 3 others were not unique. Most of the 13 unique plasmids with an unaltered *rpoB* reading frame contained deletions of 10 to 100 bp, although one (*Xho-25*) contained no deletion and two (*Xho-29* and *Xho-42*) contained duplications of 3 bp. Thus, DNase I cleavage in the presence of Mn²⁺ usually occurred at sites offset 3' relative to each other so that repair with T4 DNA polymerase introduced deletions. All 13 alterations occurred in the N-terminal (10 alterations) or C-terminal (3 alterations) third of the gene; no in-frame alterations were recovered in the middle third of the *rpoB*. One alteration, *Xho-36*, fused codon 25 of the *lacZ* α -peptide gene on pRL385 (Fig. 1B) to codon 53 of *rpoB* (Table 2).

Significant alterations recovered in the N-terminal and C-terminal regions of the pRL385 *rpoB* gene either inhibited assembly of β subunit into RNA polymerase or did not block β -subunit function. At least four classes of *XhoI* linker insertions in pRL385*rpoB* could be recovered in our experiment: class 1, alterations that allowed normal assembly and function of the β subunit (presumably insertions in noncritical regions and accompanied by no or small deletions); class 2, alterations that inhibited assembly of the β subunit into RNA polymerase but that, when properly assembled, produced a catalytically active RNA polymerase; class 3, alterations that inhibited assembly but that, when properly assembled, produced an RNA polymerase that, under some conditions, was deleterious to cell growth; and class 4, alterations that completely or nearly completely blocked assembly of altered β subunit into RNA polymerase. A fifth class, alterations that blocked polymerase function but not assembly, would be lethal and would not be recovered in our experiment. In some cases, classification of a particular alteration could depend on other properties of a particular strain or on growth conditions.

To classify the pRL385*Xho* derivatives according to this scheme, we performed four tests. First, we tested the ability of altered β subunit produced from the plasmids to convert a Rif^r *E. coli* strain, RL455, to rifampin sensitivity (Table 3). None of the 13 pRL385*Xho* derivatives themselves conferred rifampin resistance on a Rif^r strain. Since reduction of active polymerase content below 40% of normal levels inhibits cell growth (50), we expected that incorporation of altered β subunit into greater than 60% of the RNA polymerase in RL455 transformants would result in rifampin sensitivity; a positive result would place the alteration in class 1. Second, we prepared crude extracts of RL455 (pRL385*Xho*) strains and determined the percentage of RNA polymerase activity that was sensitive to rifampin. Rif^r activity would result from RNA polymerase containing functional, plasmid-encoded, Rif^r β subunit (Table 3). Class 1 alterations should yield a high percentage of Rif^r polymerase activity. Third, we transformed the pRL385*Xho* plasmids into strains that carried a nonpolar amber mutation in the chromosomal *rpoB* gene (*rpoBcl*) and either a temperature-resistant (*supD43*; RL582) or a temperature-sensitive (*supD43,74*; RL583 and RL585) amber suppressor and one or two enhancers of suppressor activity (*sueA sueC* [RL583] or *sueA* [RL585]). At increasing temperatures, synthesis of the β subunit decreases in these strains (51, 52). RL583 and RL585 grew at 39°C or above only with an alternative source

TABLE 3. Effects of pRL385 *XhoI* linker insertions on growth of the *rpoB2* (Rif^r) strain RL455

Plasmid	Efficiency of plating (%) ^a		Rif ^r RNA polymerase activity ^b
	Without rifampin	Plus rifampin	
pUC119	100	100	0
pRL385 <i>rpoB</i> ⁺	100	0	100
pRL385 <i>Xho-1</i>	70 ^c	130	30
pRL385 <i>Xho-4</i>	100 ^c	100	30
pRL385 <i>Xho-5</i>	100	100	20
pRL385 <i>Xho-6</i>	100	100	30
pRL385 <i>Xho-16</i>	100	100	0
pRL385 <i>Xho-19</i>	50 ^c	100	30
pRL385 <i>Xho-25</i>	100	0	90
pRL385 <i>Xho-29</i>	100	100	20
pRL385 <i>Xho-31</i>	100	100	0
pRL385 <i>Xho-36</i>	100	100	0
pRL385 <i>Xho-42</i>	100	0	80
pRL385 <i>Xho-54</i>	100	100	0
pRL385 <i>Xho-73</i>	100	100	0

^a Calculated as the percentage of colonies that grew on selective medium relative to colonies on LB plates containing no antibiotics. For pRL385 derivatives that gave plating efficiencies of other than 100% on selective medium, the numbers given are the averages of two independent experiments. Data are rounded to a single significant figure.

^b Determined by incorporation of [³H]UTP into polynucleotide, using poly(dA-dT) as a template. Each value is the percentage of activity that was inhibited by the addition of 10 μ g of rifampin per ml. Each determination was performed in duplicate, and average values were used for the calculations. Data are rounded to a single significant figure.

^c Colony size reduced by greater than 50%.

of β subunit; pRL385, pRL385*rpoB2*, and pRL385*rpoB114* allowed growth at 42°C (Table 4). Fourth, we constructed in vitro recombinants of the *XhoI* linker insertions and *rpoB114*(Rif^r) (see Materials and Methods) and tested whether the resulting plasmids allowed growth of *rpoB*(Am) strains in the presence of rifampin.

pRL385*rpoB*⁺ and two insertion derivatives, *Xho-25* and *Xho-42*, prevented growth of RL455 on solid media containing 100 μ g of rifampin per ml (Table 3). In extracts of RL455 carrying these three plasmids, 80% or more of the RNA polymerase activity was inhibited by 10 μ g of rifampin per ml (Table 3). This percentage does not necessarily correspond to the fraction of enzyme containing the altered β subunits, since the altered enzymes may have different specific activities on poly(d-A-dT). *Xho-25* allowed growth of RL583 to 42°C; *Xho-42* allowed some growth of both RL583 and RL585 to 39°C. However only *Xho-25*, when combined with the *rpoB114* mutation, was able to support growth in the presence of rifampin (Table 4). Thus, *Xho-25* alone was assigned to class 1; *Xho-42* was assigned to class 2 (Fig. 4). We do not know whether the *Xho-42* β subunit alone supported growth at 39°C of *rpoB*(Am) strains (Table 4) and that the combination of *Xho-42* and *rpoB114* resulted in an inactive or Rif^r β subunit or whether growth at 39°C of *rpoB*(Am) pRL385*Xho-42* strains occurred because the plasmid supplied enough partially functional RNA polymerase to allow the otherwise crippled strain to grow.

Four other pRL385*Xho* insertions, *Xho-5*, *Xho-6*, *Xho-29*, and *Xho-31*, produced detectable quantities of Rif^r RNA polymerase activity in RL455 (Table 3) and allowed growth of RL583 or RL585 above 37°C (Table 4). None of these insertions, when combined with *rpoB114*, could support growth in the presence of rifampin. We assigned these alterations to class 2 (Fig. 4). The results with these four derivatives lead us to suspect that the *rpoB*(Am) strains are

TABLE 4. Viability of *rpoB*(Am) *supD*(Ts) strains bearing pRL385 *Xho*I linker insertions

Plasmid	Growth ^a														
	Original isolate on plates without rifampin												Recombinant with <i>rpoB114</i> on plates with rifampin ^b		
	32°C			37°C			39°C			42°C			37°C		
	Tr ^c <i>sueA</i> ^d <i>sueC</i> ^d	Ts ^e <i>sueA</i> <i>sueC</i>	Ts <i>sueA</i>	Tr <i>sueA</i> <i>sueC</i>	Ts <i>sueA</i> <i>sueC</i>	Ts <i>sueA</i>	Tr <i>sueA</i> <i>sueC</i>	Ts <i>sueA</i> <i>sueC</i>	Ts <i>sueA</i>	Tr <i>sueA</i> <i>sueC</i>	Ts <i>sueA</i> <i>sueC</i>	Ts <i>sueA</i>	Tr <i>sueA</i> <i>sueC</i>	Ts <i>sueA</i> <i>sueC</i>	Ts <i>sueA</i>
pUC119	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-
pRL385 <i>rpoB</i> ⁺	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pRL385 <i>rpoB2</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pRL385 <i>rpoB114</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pRL385 <i>Xho-1</i>	~	-	-	+	+	-	+	-	-	+	-	-	-	-	-
pRL385 <i>Xho-4</i>	+	+	~	+	+	-	+	-	-	+	-	-	-	-	-
pRL385 <i>Xho-5</i>	+	+	~	+	+	~	+	+	~	+	-	-	-	-	-
pRL385 <i>Xho-6</i>	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
pRL385 <i>Xho-16</i>	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-
pRL385 <i>Xho-19</i>	~	~	~	+	+	+	+	~	~	+	-	-	-	-	-
pRL385 <i>Xho-25</i>	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
pRL385 <i>Xho-29</i>	+	+	+	+	+	+	+	+	+	+	~	~	~	~	~
pRL385 <i>Xho-31</i>	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
pRL385 <i>Xho-36</i>	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-
pRL385 <i>Xho-42</i>	+	+	+	+	+	+	+	+	+	+	-	-	-	-	~
pRL385 <i>Xho-54</i>	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-
pRL385 <i>Xho-73</i>	~	-	-	+	-	-	+	-	-	+	-	-	-	-	-

^a Growth of colonies of strains RL582 [*rpoB*(Am) *supD43*(Tr) *sueA sueC*], RL583 [*rpoB*(Am) *supD43,74*(Ts) *sueA sueC*], and RL585 [*rpoB*(Am) *supD43,74*(Ts) *sueA*] transformed with pRL385*Xho* plasmids. +, Normal growth; ~, significantly inhibited colony size; -, no growth. Transformed strains were struck out for single colonies on LB plates containing 100 µg of ampicillin per ml with or without rifampin and incubated at the temperatures indicated.

^b Data are from strains containing in vitro recombinants of the indicated *Xho* insertion plasmids with the *rpoB114*(Rif^r) allele (see Materials and Methods) grown on plates containing 100 µg of rifampin per ml, except the first four rows, the data in which are from strains containing the plasmids indicated in the first column.

^c Tr, *supD43*(Tr).

^d Enhancer of suppression.

^e Ts, *supD43,74*(Ts).

able to grow at 39°C when supplemented with some altered RNA polymerase activity that alone may be unable to support growth. However, we cannot rule out the alternative explanation suggested above for *Xho-42*.

We assigned three pRL385 derivatives, *Xho-1*, *Xho-4*, and *Xho-19*, to class 3. RL455 containing altered plasmids grew better when rifampin was present in the medium than when it was absent, although the effect of *Xho-4* was only on colony size (Table 3). We interpreted these findings to mean that the presence of RNA polymerase containing the plasmid-encoded, altered β subunits was detrimental to cell growth because either the catalytic or regulatory activity of the altered enzymes was impaired. *Xho-1* and *Xho-19* caused a significant inhibition of growth at 32°C when the altered plasmids were introduced into any strain carrying the *rpoB*(Am) allele. *Xho-4* gave a less severe phenotype, inhibiting only the growth of the strain RL585, in which suppression was least efficient (51, 52). We also tested growth at 32, 37, and 42°C of RL455 transformants with each of the pRL385*Xho* derivatives. None of the plasmids tested inhibited growth of RL455 transformants at 42°C; however, *Xho-1*, *Xho-4*, and *Xho-19* inhibited growth at 32°C but not at 37°C. We do not know why the effects on growth of these alterations were more severe at 32°C than at 37°C; it is possible either that assembly of the altered subunits is more efficient at low temperature or that the negative effects of the resultant enzymes are more pronounced at low temperature.

The remaining four derivatives of pRL385 were assigned to class 4 (Fig. 4). Three (*Xho-16*, *Xho-36*, and *Xho-54*) produced altered β-subunit polypeptides that did not incorporate into RNA polymerase to any significant degree. In the

rpoB2(Rif^r) strain, RL455, they produced no Rif^r RNA polymerase activity (Table 3) and had no effect on plating efficiency in the presence or absence of rifampin (Table 3). They affected growth of *rpoB*(Am) strains neither in their original form nor when combined with *rpoB114* and grown in the presence of rifampin (Table 4). The fourth derivative, *Xho-73*, met all of these criteria but one: it did inhibit growth of *rpoB*(Am) strains at 32°C (Table 4), perhaps because at this temperature the defect in its assembly was less pronounced. Since we found no evidence of catalytic activity for *Xho-73* (Table 3), we assigned it to class 4, although clearly it is a special case.

DISCUSSION

Assembly into RNA polymerase of β subunit synthesized from a plasmid-borne *rpoB* gene is inhibited by slight structural alterations. We found that the β subunit of *E. coli* RNA polymerase was overproduced ca. 20-fold from plasmid pRL385 (Fig. 1 and 2). The plasmid-encoded β subunit was assembled into functional holoenzyme that, when wild type, comprised greater than 90% of the RNA polymerase in the cell (Fig. 2). Excess β subunit was segregated into inclusion bodies within the bacteria (Fig. 3). Small changes in the primary structure of the β subunit perturbed the ability of the plasmid-encoded subunit to exhibit these phenotypes. Of 13 different in-frame alterations in the first and last thirds of the *rpoB* gene (Table 2 and Fig. 4), 12 produced some reduction in the incorporation of the altered subunit into active RNA polymerase (Tables 3 and 4). Apparently, these alterations in the first or last third of the β-subunit polypeptide reduce its

ability to compete with chromosomally encoded β subunit for assembly into RNA polymerase.

To us, it seems most plausible that the regions altered in the assembly-defective β -subunit derivatives are necessary for proper folding of the β subunit and that slow or improper folding of the altered β -subunit polypeptides leads to formation of insoluble aggregates before the altered β subunit can interact with α subunit. This would be consistent with the recovery of many alterations in the first third of the β subunit, which presumably must initiate folding of the nascent polypeptide. Such a model also would be consistent with the negative effects of class 3 insertions at 32°C, a temperature at which the effects of impaired protein folding may be less severe. Alternatively, it is possible that the altered portions of the first and last thirds of the β subunit normally contact other RNA polymerase subunits and that inhibition of assembly results from loss of these contacts. However, unless the alterations prevented $\alpha_2\beta$ formation, cell growth probably would be blocked by depletion of α .

It should be emphasized that these conclusions are based on indirect measurements of β subunit assembly into RNA polymerase. There are several possible explanations for a failure to detect wild-type levels of RNA polymerase containing the altered β -subunit polypeptides, including effects on protein folding, protein stability, protein solubility, and the specific activity on poly(dA-dT) of the altered RNA polymerase. Although not a definitive conclusion, altered folding leading to reduced assembly of the β -subunit variants is the most plausible explanation for our findings. If the altered, plasmid-encoded β subunits, which were present at ca. 20 times the wild-type β -subunit level (see Results), assembled with wild-type efficiency (comprising greater than 90% of the cellular RNA polymerase), they should have either converted a Rif^s strain to Rif^r, converted a Rif^r strain to Rif^s, or killed the cell. None of these conditions were met by the variants that we suggest are defective in the folding and assembly pathway.

Only one altered β subunit (*Xho-25*) supported growth when the chromosomal source of the β subunit was completely inactivated (Table 4); the remaining 12 alterations probably would be lethal if chromosomally encoded β subunit did not outcompete them for assembly. These 12 altered β subunits, and the 3 assigned to class 3 in particular, probably interfere with essential functions of RNA polymerase. Thus, even though pRL385 expresses the β subunit at reasonably high levels, alterations can be isolated that disrupt enzyme function, provided they also inhibit assembly. Further analysis to define the nature of the defects caused by the class 3 alterations, perhaps by *in vitro* reconstitution of RNA polymerase from overproduced β subunit purified from inclusion bodies (40), is warranted.

Several alterations in the N-terminal third of the β subunit do not eliminate catalytic activity. Several groups have postulated a functional map of the primary structure of the β subunit on the basis of the existing biochemical and genetic data (26, 35, 44, 47). A composite of these models is that the region between 500 and 575, which presumably is responsible for rifampin and streptolydigin binding, interacts with either the nascent RNA chain or nucleoside triphosphate substrates (35, 57), that the region from 750 to 900 is responsible for ppGpp binding (18, 46), that the region from 965 to 1080 is dispensable (18, 48) but may help determine promoter selectivity (18), that the C-terminal portion of the β subunit (from 1200 on) is required for binding of σ to core (16, 19), and that segments at 1063 to 1112 and 1213 to 1296 may interact with the nucleoside triphosphate substrates (20,

35). The five class 1 and class 2 alterations between 35 and 317 must not block the catalytic function of the β subunit. So far no function has been ascribed to this region, and the only reported mutant mapped to this region is a weak Rif^r mutation at Val-146 (36). This portion of the β subunit polypeptide also contains some of the most variable regions in a comparison of the β subunit of *Salmonella typhimurium* RNA polymerase (35), the corresponding eucaryotic subunits (13, 64), and the presumed β subunit of *Nicotiniana tabacum* chloroplast RNA polymerase (53). Whatever the function of the N-terminal third of the β subunit, the significant amino acid alterations in *Xho-5*, *Xho-6*, *Xho-25*, *Xho-31*, and *Xho-42* apparently do not eliminate catalytic function.

The failure to recover significant alterations in the central third of the β subunit may have occurred because they prevent proper RNA polymerase function but do not inhibit assembly. We cannot be certain why we failed to recover any in-frame alterations in the central third of the *rpoB* gene or why only 15% (4 of 26) of the *XhoI* linker insertions recovered in the C-terminal two-thirds of *rpoB* did not alter the translational reading frame. It is possible that bias in DNase I-Mn²⁺ cleavage accounts for these results. However, we believe it likely that in-frame central region alterations were present in the transformation mixture and were not recovered because of a lethal phenotype. Even though an unexpectedly low number of out-of-frame alterations (ten) were recovered in the central region (Fig. 4), the probability that all of these would shift the reading frame of *rpoB* is 1 in 58. Thus, the failure to recover central region, in-frame alterations probably occurred because alteration of this region blocks RNA polymerase function but not assembly of the β subunit into RNA polymerase. That this region also contains the interesting Rif^r region, which may interact with elongation complex-specific moieties (61), further implicates it in essential β subunit functions. This view is also supported by the fact that *Xho-1* and *Xho-19*, the two recovered alterations nearest the central region, had the most noticeably deleterious effects on the β -subunit function (Tables 3 and 4). Elucidation of the nature of the defects produced by *Xho-1* and *Xho-19*, as well as directed mutagenesis of the central region of *rpoB*, may help to define the role of this region in transcription.

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LITERATURE CITED

- Appleyard, R. K. 1954. Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. *Genetics* 39:440-452.
- Austin, S. J., P. B. Tittawella, R. S. Hayward, and J. G. Scaife. 1971. Amber mutations of *Escherichia coli* RNA polymerase. *Nature* (London) New Biol. 232:133-136.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1989. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.

4. Barnes, W. 1977. Plasmid detection and sizing in single colony lysates. *Science* **195**:393–394.
5. Bedwell, D., G. Davis, M. Gosink, L. Post, M. Nomura, H. Kestler, J. Zengel, and L. Lindahl. 1985. Nucleotide sequence of the alpha ribosomal protein operon of *Escherichia coli*. *Nucleic Acids Res.* **13**:3891–3903.
6. Bedwell, D. M., and M. Nomura. 1986. Feedback regulation of RNA polymerase subunit synthesis after conditional overproduction of RNA polymerase in *Escherichia coli*. *Mol. Gen. Genet.* **204**:17–23.
7. Bolivar, F., R. Rodriguez, P. Greene, M. Betlach, H. Heynecker, and H. Boyer. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**:95–113.
8. Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
9. Bremer, H., and P. P. Dennis. 1987. Modulation of chemical composition and other parameters of the cell by growth rate, p. 1527–1542. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
10. Burgess, R. R., and J. J. Jendrisak. 1975. A procedure for the rapid, large-scale purification of *Escherichia coli* DNA-dependent RNA polymerase involving polymin P precipitation and DNA-cellulose chromatography. *Biochemistry* **14**:4634–4638.
11. Burton, Z., R. R. Burgess, J. Lin, D. Moore, S. Holder, and C. A. Gross. 1981. The nucleotide sequence of the cloned *rpoD* gene for the RNA polymerase sigma subunit from *E. coli* K12. *Nucleic Acids Res.* **9**:2889–2903.
12. Chamberlin, M. J. 1982. Bacterial DNA-dependent RNA polymerases, p. 61–86. In P. D. Boyer (ed.), *The enzymes*, vol. 15. Academic Press, New York.
13. Falkenburg, D., B. Dworniczak, D. M. Faust, and E. K. F. Bautz. 1987. RNA polymerase II of *Drosophila*. Relationship of its 140,000 M_r subunit to the beta-subunit of *Escherichia coli* RNA polymerase. *J. Mol. Biol.* **195**:929–937.
14. Fill, N. P., D. Bendiak, J. Collins, and J. D. Friesen. 1979. Expression of *Escherichia coli* ribosomal protein and RNA polymerase genes cloned on plasmids. *Mol. Gen. Genet.* **173**:39–50.
15. Fisher, R. F., and C. Yanofsky. 1983. Mutations of the beta subunit of RNA polymerase alter both transcription pausing and transcription termination. *J. Biol. Chem.* **258**:8146–8150.
16. Glass, R. E., A. Honda, and A. Ishihama. 1986. Genetic studies on the beta-subunit of *Escherichia coli* RNA polymerase. IX. The role of the carboxy-terminus in enzyme assembly. *Mol. Gen. Genet.* **203**:492–495.
17. Glass, R. E., S. T. Jones, and A. Ishihama. 1986. Genetic studies on the beta-subunit of *Escherichia coli* RNA polymerase. VII. RNA polymerase is a target for ppGpp. *Mol. Gen. Genet.* **203**:265–268.
18. Glass, R. E., S. T. Jones, V. Nene, T. Nomura, N. Fujita, and A. Ishihama. 1986. Genetic studies on the beta-subunit of *Escherichia coli* RNA polymerase. VIII. Localisation of a region involved in promoter selectivity. *Mol. Gen. Genet.* **203**:487–491.
19. Glass, R. E., N. T. Ralphs, N. Fujita, and A. Ishihama. 1988. Assembly of amber fragments of the beta-subunit of *Escherichia coli* RNA polymerase. *Eur. J. Biochem.* **176**:403–407.
20. Grachev, M. A., T. J. Kolocheva, E. A. Lukhtanov, and A. A. Mustaev. 1987. Studies on the functional topography of *Escherichia coli* RNA polymerase: highly selective affinity labelling by analogues of initiating substrates. *Eur. J. Biochem.* **163**:113–121.
21. Gross, C., F. Engback, T. Flammang, and R. Burgess. 1976. Rapid micromethod for the purification of *Escherichia coli* ribonucleic acid polymerase and the preparation of bacterial extracts active in ribonucleic acid synthesis. *J. Bacteriol.* **128**:382–389.
22. Gross, C. A., J. Hoffman, C. Ward, D. Hager, G. Burdick, H. Berger, and R. R. Burgess. 1978. Mutation affecting thermostability of sigma subunit of *Escherichia coli* RNA polymerase lies near the *dnaG* locus at about 66 min on the *E. coli* genetic map. *Proc. Natl. Acad. Sci. USA* **75**:427–431.
23. Hanna, M. M., and C. F. Meares. 1983. Topography of transcription: path of the leading end of nascent RNA through the *Escherichia coli* transcription complex. *Proc. Natl. Acad. Sci. USA* **80**:4238–4242.
24. Heffron, F., M. So, and B. J. McCarthy. 1978. *In vitro* mutagenesis of a circular DNA molecule by using synthetic restriction sites. *Proc. Natl. Acad. Sci. USA* **75**:6012–6016.
25. Ishihama, A. 1986. Transcription signals and factors in *Escherichia coli*. *Adv. Biophys.* **21**:163–173.
26. Ishihama, A. 1988. Promoter selectivity of prokaryotic RNA polymerases. *Trends Genet.* **4**:282–286.
27. Jaskunas, S. R., R. R. Burgess, and M. Nomura. 1975. Identification of a gene for the alpha-subunit of RNA polymerase at the *str-spc* region of the *Escherichia coli* chromosome. *Proc. Natl. Acad. Sci. USA* **72**:5036–5040.
28. Jin, D. J., and C. A. Gross. 1988. Mapping and sequencing of mutations in the *Escherichia coli rpoB* gene that lead to rifampicin resistance. *J. Mol. Biol.* **202**:45–58.
29. Jin, D. J., W. A. Walter, and C. A. Gross. 1988. Characterization of the termination phenotypes of rifampicin resistant mutants. *J. Mol. Biol.* **202**:245–263.
30. Kane, J. F., and D. L. Hartley. 1988. Formation of recombinant protein inclusion bodies in *Escherichia coli*. *Trends Biotechnol.* **6**:95–101.
31. Kashlev, M. V., A. I. Gragerov, and V. G. Nikiforov. 1988. Influence of heat-shock proteins' induction on the phenotypic expression of rifampicin resistance mutations affecting RNA polymerase beta-subunit gene under the control of the lac promoter. *Genetika* **24**:1343–1352.
32. Kawakimi, K., and A. Ishihama. 1980. Defective assembly of ribonucleic acid polymerase subunits in a temperature-sensitive alpha-subunit mutant of *Escherichia coli*. *Biochemistry.* **19**:3491–3495.
33. Kirschbaum, J. B., and E. B. Konrad. 1973. Isolation of a specialized lambda transducing bacteriophage carrying the beta subunit for *Escherichia coli* ribonucleic acid polymerase. *J. Bacteriol.* **116**:517–526.
34. Laemmli, U. K. 1970. Cleavage of structure proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
35. Lisitsyn, N. A., G. A. Monastyrskaya, and E. D. Sverdlov. 1988. Genes coding for RNA polymerase beta-subunit: structure/function analysis. *Eur. J. Biochem.* **177**:363–369.
36. Lisitsyn, N. A., E. D. Sverdlov, E. P. Moiseyeva, O. N. Danilevskaya, and V. G. Nikiforov. 1984. Mutation to rifampicin resistance at the beginning of the RNA polymerase beta-subunit gene in *Escherichia coli*. *Mol. Gen. Genet.* **196**:173–174.
37. Linn, T., and J. Scaife. 1978. Identification of a single promoter in *E. coli* for *rplJ*, *rplL*, and *rpoBC*. *Nature (London)* **276**:33–37.
38. Maloy, S., and W. D. Nunn. 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. *J. Bacteriol.* **145**:1110–1112.
39. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. McKinney, J. D., J. Lee, R. E. O'Neill, and A. Goldfarb. 1987. Overexpression and purification of a biologically active rifampicin-resistant beta-subunit of *Escherichia coli* RNA polymerase. *Gene* **58**:13–18.
41. Murray, N. E., W. J. Brammer, and K. Murray. 1977. Lamboid phages that simplify the recovery of in vitro recombinants. *Mol. Gen. Genet.* **150**:53–61.
42. Nakamura, Y., T. Osawa, and T. Yura. 1977. Chromosomal location of a structural gene for the RNA polymerase sigma factor in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **74**:1831–1835.
43. Neff, N. F., and M. J. Chamberlin. 1980. Termination of transcription by *Escherichia coli* RNA polymerase in vitro. Effect of altered reaction conditions and mutations in the enzyme protein on termination with T7 and T3 DNAs. *Biochemistry* **19**:3005–3015.

44. Nene, V., and R. Glass. 1982. Genetic studies on the β -subunit of *Escherichia coli* RNA polymerase. I. The effect of known, single amino acid substitutions in an essential protein. *Mol. Gen. Genet.* **188**:399-404.
45. Nene, V., and R. Glass. 1982. Genetic studies on the β -subunit of *Escherichia coli* RNA polymerase. II. Evidence that large N-terminal amber fragments of the β -subunit interfere with RNA polymerase function. *Mol. Gen. Genet.* **188**:405-409.
46. Nene, V., and R. Glass. 1983. Relaxed mutants of *Escherichia coli* RNA polymerase. *FEBS Lett.* **153**:307-310.
47. Nene, V., and R. Glass. 1984. Genetic studies on the β -subunit of *Escherichia coli* RNA polymerase. IV. Structure-function correlates. *Mol. Gen. Genet.* **194**:166-172.
48. Nene, V., and R. Glass. 1984. Genetic studies on the β -subunit of *Escherichia coli* RNA polymerase. VI. A redundant region in the β -polypeptide. *Mol. Gen. Genet.* **196**:64-67.
49. Newman, A., and R. S. Hayward. 1980. Cloning of DNA of the *rpoBC* operon from the chromosome of *Escherichia coli* K12. *Mol. Gen. Genet.* **177**:527-533.
50. Nomura, M., D. M. Bedwell, M. Yamagishi, J. R. Cole, and J. M. Kolb. 1987. RNA polymerase and regulation of RNA synthesis in *Escherichia coli*: RNA polymerase concentration, stringent control, and ribosome feedback regulation, p. 137-149. *In* W. S. Reznikoff, R. R. Burgess, J. E. Dahlberg, C. A. Gross, M. T. Record, and M. P. Wickens (ed.), *RNA polymerase and the regulation of transcription*. Elsevier, New York.
51. Oeschger, M. P., N. S. Oeschger, G. T. Wiprud, and S. L. Woods. 1980. High efficiency temperature-sensitive amber suppressor strains of *Escherichia coli* K12: isolation of strains with suppressor-enhancing mutations. *Mol. Gen. Genet.* **177**:545-552.
52. Oeschger, M. P., and G. T. Wiprud. 1980. High efficiency temperature-sensitive amber suppressor strains of *Escherichia coli* K12: construction and characterization of recombinant strains with suppressor-enhancing mutations. *Mol. Gen. Genet.* **178**:293-299.
53. Ohme, M., M. Tanaka, J. Chungwonse, K. Sinozaki, and M. Sugiura. 1986. A tobacco chloroplast DNA sequence possibly coding for a polypeptide similar to the *E. coli* RNA polymerase β -subunit. *FEBS Lett.* **200**:87-90.
54. Osawa, T., and T. Yura. 1978. Genetic and biochemical studies of RNA polymerase mutants in *E. coli*. XVII. Amber mutants of sigma factor. *Jpn. J. Genet.* **53**:444-445.
55. Ovchinnikov, Y. A., G. S. Monastyrskaya, V. V. Gubanov, S. O. Guryev, O. Y. Chertov, N. N. Modyanov, U. A. Ginkevich, I. A. Marakova, T. V. Marchenko, I. N. Polovnikova, V. M. Lipkin, and E. D. Sverdlov. 1981. The primary structure of *Escherichia coli* RNA polymerase. Nucleotide sequence of the *rpoB* gene and amino acid sequence of the β -subunit. *Eur. J. Biochem.* **116**:621-629.
56. Ovchinnikov, Y. A., G. S. Monastyrskaya, V. V. Gubanov, S. O. Guryev, I. S. Salomatina, T. M. Shuvaeta, V. M. Lupkin, and E. D. Sverdlov. 1982. The primary structure of *E. coli* RNA polymerase, nucleotide sequence of the *rpoC* gene and amino acid sequence of the β' -subunit. *Nucl. Acids. Res.* **10**:4035-4044.
57. Ovchinnikov, Y. A., G. S. Monastyrskaya, S. O. Guriev, N. F. Kalinina, E. D. Sverdlov, I. A. Gragerov, I. A. Bass, I. F. Kiver, E. P. Moiseyeva, V. N. Igumnov, S. Z. Mindlin, V. G. Nikiforov, and R. B. Khesin. 1983. RNA polymerase rifampicin resistance mutations in *Escherichia coli*: sequence changes and dominance. *Mol. Gen. Genet.* **190**:344-347.
58. Reznikoff, W. S., D. A. Siegele, D. A. Cowing, and C. A. Gross. 1985. The regulation of transcription initiation in bacteria. *Annu. Rev. Genet.* **19**:355-387.
59. Ridley, S. P., and M. P. Oeschger. 1982. An amber mutation in the gene encoding the β' subunit of *Escherichia coli* RNA polymerase. *J. Bacteriol.* **152**:736-746.
60. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.
61. Schulz, W., and W. Zillig. 1981. Rifampicin inhibition of RNA synthesis by destabilisation of DNA-RNA polymerase-oligonucleotide-complexes. *Nucleic Acids Res.* **9**:6889-6906.
62. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
63. Simpson, R. B. 1979. The molecular topography of RNA polymerase-promoter interaction. *Cell* **20**:277-285.
64. Sweetser, D., M. Nonet, and R. A. Young. 1987. Prokaryotic and eukaryotic RNA polymerases have homologous subunits. *Proc. Natl. Acad. Sci. USA* **84**:1192-1196.
65. Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**:259-268.
66. Vieira, J., and J. Messing. 1987. Production of single-stranded plasmid DNA. *Methods Enzymol.* **153**:3-11.
67. Weil, P. A., and S. P. Blatti. 1975. Partial purification and properties of calf thymus deoxyribonucleic acid dependent RNA polymerase III. *Biochemistry* **14**:1636-1642.
68. Yamamoto, M., and M. Nomura. 1978. Cotranscription of genes for RNA polymerase subunits β and β' with genes for ribosomal proteins in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **75**:3891-3895.
69. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103-119.
70. Yanofsky, C., and V. Horn. 1981. Rifampin resistance mutations that alter the efficiency of transcription termination at the tryptophan operon attenuator. *J. Bacteriol.* **145**:1334-1341.
71. Zaman, Z., and R. L. Verwilghen. 1979. Quantitation of proteins solubilized in SDS-mercaptoethanol-Tris electrophoresis buffer. *Anal. Biochem.* **100**:64-69.
72. Zillig, W., R. Schnabel, F. Gropp, and W. D. Reiter. 1985. The evolution of the transcription apparatus, p. 42-72. *In* K. H. Schleifer and E. Stackenbradt (ed.), *Evolution of prokaryotes*. Academic Press Inc., Orlando, Fla.