Mutations in and Monoclonal Antibody Binding to Evolutionary Hypervariable Region of Escherichia coli RNA Polymerase β′ Subunit Inhibit Transcript Cleavage and Transcript Elongation*

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A 190 amino acid-long region centered around position 1050 of the 1407-amino acid-long β′ subunit of Escherichia coli RNA polymerase (RNAP) is absent from homologues of eukaryotes, archaea and many bacteria. In chloroplasts, the corresponding region can be more than 900 amino acids long. The role of this hypervariable region was studied by deletion mutagenesis of the cloned E. coli rpoC, encoding β′: Long deletions mimicking β′ from Gram-positive bacteria failed to assemble into RNAP. Mutants with short, 40–60-amino acid-long deletions spanning β′ residues 941–1130 assembled into active RNAP in vitro. These mutant enzymes were defective in the transcript cleavage reaction and had dramatically reduced transcription elongation rates at subsaturating substrate concentrations due to prolonged pausing at sites of transcriptional arrest. Binding of a monoclonal antibody, Pyn1, to the hypervariable region inhibited transcription elongation and intrinsic transcript cleavage and, to a lesser degree, GreB-induced transcript cleavage, but did not interfere with GreB binding to RNAP. We propose that mutations in and antibody binding to the hypervariable, functionally dispensable region of β′ inhibit transcript cleavage and elongation by distorting the flanking conserved segment G in the active center.

DNA-dependent RNA polymerases from eubacteria share a common subunit composition (1). The core RNAPβ′ enzyme (subunit composition α2β′) is catalytically proficient but is unable to initiate transcription on promoters. Binding of a σ subunit converts the core enzyme into a holoenzyme, which can recognize a specific set of promoters (2). The β′ and σ subunits together constitute more than 80% of the core RNAP mass and jointly form the catalytic center of the enzyme (3, 4). RNAPs from eukaryotes and archaea have subunits that are homologous to β′ and β′ of eubacterial enzymes (5–7). The evolutionary conservation within the β′β′ lineages is limited to relatively short segments of primary sequence; each subunit has 8–10 highly conserved segments. The amino- to carboxyl-terminal order of the conserved segments is invariant.

The spacing between the conserved segments can vary even when subunits from closely related species are compared, due to an accumulation of insertions and deletions. There are several reasons why studies of such evolutionarily variable regions can shed light on RNAP structure and function. First, these regions may form docking sites for species-specific regulators of transcription (8, 9). Second, variable regions are likely to be surface-exposed and can therefore be used for affinity tagging of RNAP and transcription complexes (10). Third, variable regions often tolerate splits, allowing preparation of functional RNAP with relatively short β′ and/or β′′ subunit fragments, dramatically facilitating mapping of protein-protein and protein-nucleic acid contacts during transcription (11, 12).

The focus of this report is an evolutionary hypervariable region in the C-terminal portion of Escherichia coli β′ (amino acids 1141–1131). This region is highly variable in proteobacteria and is absent from homologs from most other bacteria, archaea, and eukaryotes. Despite this apparent redundancy, numerous point mutations that altered the termination and elongation properties of E. coli RNAP were localized in the hypervariable region (25, 37, 38). In addition, mutations in the largest (β′-like) subunit of yeast RNAP II that occurred very close to the hypervariable region dramatically decreased interaction of the enzyme with transcript cleavage factor TFIIS (33). Here, we probed the role of the hypervariable region and adjacent segments of E. coli β′ by deletion mutagenesis of the cloned rpoC gene. Mutant RNAPs were assembled in vitro, and their elongation and termination properties, as well as their abilities to interact with the TFIIS analog GreB, were determined. A long deletion that completely removed the hypervariable region blocked enzyme assembly in vivo and in vitro. Short (40–60 amino acid) deletions, which together span the entire hypervariable region, did not prevent RNAP assembly and basic transcription function in vitro; however, the mutant enzymes had a dramatic defect in transcript elongation at low substrate concentrations. At high substrate concentrations, the mutants elongated and terminated transcription normally. The mutant enzymes were also defective in GreB-induced transcript cleavage, but the nature of the defect was complex, because both the ability to interact with GreB and the ability to support intrinsic transcript cleavage by the RNAP catalytic center were altered by mutations. A binding epitope for an inhibitory mAb, Pyn1, was mapped in the hypervariable region. Binding of Pyn1 to RNAP efficiently inhibited both the RNA synthesis and transcript cleavage reactions.
Our data thus demonstrate that the evolutionary hypervariable region of β’, which is completely absent from homologues from eukaryotes, archaea, and many eubacteria, is unexpectedly important for E. coli RNAP assembly and is involved in transcript elongation and cleavage. Because this region is missing from β’ homologues from most organisms, however, this involvement is probably indirect.

MATERIALS AND METHODS

Deletion Mutagenesis of the Cloned rpoC Gene—The pUC18-based pMKA2015793F rpoC expression plasmid was used to generate deletions in the rpoC gene. The plasmid is a derivative of pMKA201 (13) and carries a spectinomycin-resistant streptomycin-resistant mutation, conferring β’ Ser793 to Phe (14). To generate nested Bal31 deletions in pMKA2015793F, a unique BamHI linker was inserted in the EcoRI site at codon 987 of the structural gene. pMKA2015793F was underdigested with EcoRI (cut twice at codons 175 and 987 of the structural gene and once in the vector), and ligated to the EcoRI fragment from phage mp17Km carrying the kanamycin resistance gene (11). Kanamycin-resistant transformants were screened for the appearance of inducible truncated fragments of the β’ polypeptide using SDS-PAGE. Recombinant plasmids containing kanamycin cassette inserted at rpoC position 987 was treated with BamHI to remove the cassette and recircularized. The resultant plasmid pH1987 overproduced β’ with 6 amino acids (IPHKD) compared to between amino acids 786 and 988. To generate deletions, pIB1987 was linearized with BamHI, treated with Bal31 for various times, and recircularized. After transformation in E. coli XL1-Blue cells, transformants were screened for the appearance of inducible truncated fragments of the β’ polypeptide using SDS-PAGE. Recombinant plasmids containing kanamycin cassette inserted at rpoC position 987 was treated with BamHI to remove the cassette and recircularized. The resultant plasmid pH1987 overproduced β’ with 6 amino acids (IPHKD) compared to between amino acids 786 and 988.

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2 A. Mustaev and A. Goldfarb, manuscript in preparation.
shown in Fig. 1) deleted conserved segment G. \( \Delta(1145-1198) \) removed 54 amino acids immediately C-terminal to G and thus must have destroyed the binding epitope. In contrast, \( \Delta(877-948), \Delta(1131-1155) \), and \( \Delta\text{Asu} \), which are 90 amino acids apart, disrupt Pyn1 binding, the antibody epitope may be conformational rather than linear. Thus, we cannot exclude that \( \beta' \) sequences outside the hypervariable region may also participate in the binding of this inhibitory antibody.

\( \Delta\text{Asu} \) was the only deletion that abolished the binding of Pyn4. We conclude that \( \beta' \) amino acids 1145–1198 contribute to Pyn4 binding epitope.

Properties of rpoC Mutants in Vivo—All rpoC deletions were obtained in pMKa201S693F rpoC expression plasmid. In this plasmid, a transdominant streptolydigin-resistant S693F allele of rpoC is placed under the control of the inducible lac promoter (14). Therefore, the function of mutant, plasmid-borne rpoC can be tested in vivo by registering its ability to confer streptolydigin resistance to the sensitive host cells CAG 14064 (13). When CAG 14064 cells overproducing the WT and

3 A. Lebedev and V. Nikiforov, unpublished results.
mutant β′ subunits were plated on a medium containing 12.5 μg/ml streptolydigin, only cells overproducing parental S693F β′, and the linker insertion t987 formed colonies (Table I).

In another test of the in vivo function of mutant rpoC genes, the RL602 E. coli cells were transformed with pMKa201S693F and its derivatives, and plated at 42 °C. RL602 cells harbor an amber mutation in the chromosomal copy of rpoC that is suppressed by a temperature-sensitive suppressor (23). RL602 grows at 30 °C but fails completely to grow at 42 °C. Cells overproducing S693F β′, as well as t987, Δ(1042–1091), Δ(941–1100), Δ(987–1042), ΔKpn, and ΔAsu formed colonies at the elevated temperature (data not shown). Cells overproducing S693F β′, t987, and ΔAsu grew robustly at 42 °C, whereas the others formed minute colonies (Table I).

We do not know whether the mutant β′ subunits were the only source of β′ at high temperature. Landick et al. (24) encountered similar “partial” phenotypes when testing in vivo function of mutant, plasmid-borne rpoD genes. These authors suggested that growth at restrictive temperature may occur when the plasmid-borne rpo gene provides enough partially functional RNAP to allow the growth of the host, which has a low level of the wild-type, chromosomally encoded RNAP even at restrictive temperature. In contrast, administering streptolydigin completely inhibits wild-type, chromosomal RNAP, and thus prevents the cell growth.

In Vitro Reconstitution of Mutant RNAPs—Because the results of functional assays in vivo were inconclusive, we assembled mutant β′ into RNAP in vitro. Assembled RNAP forms characteristic peaks during chromatography on Superose-6 and Mono Q columns (17). No such peaks were observed in the course of purification of reconstitution mixtures containing Δ(877–948), Δ(1131–1155)S, and Δ(941–1130) and WT α, β, and β′ subunits; neither crude reconstitution mixtures nor chromatographic fractions contained any RNAP activity (Table I and data not shown).

The β′ subunits expressed from pMK201 plasmid are fused to the C-terminal His-tag, allowing easy purification of RNAP harboring plasmid-borne β′ (13). Our repeated attempts to purify RNAPs harboring the two largest β′ deletions from cells using affinity chromatography failed (data not shown). We conclude that deletions of conserved segments G and G′, as well as removal of the entire hypervariable region, completely abolish the ability of β′ to enter E. coli RNAP core in vitro and in vivo.

5 smaller deletions (Δ(941–1000), Δ(987–1042), Δ(1042–1091), ΔKpn, and ΔAsu) assembled into RNAP in vitro as judged by the appearance of characteristic chromatographic peaks in the course of purification. The catalytic proficiency of RNAP mutants was demonstrated by template-dependent affinity labeling of shortened β′ polypeptides with derivatized nascent RNA. In this reaction, Ni2+-NTA agarose-immobilized RNAP was used to form open complexes with the T7 A1 promoter-containing DNA fragment. Transcription was primed with a derivatized, photoactive ApUPc, complimentary to positions +1−3 of the promoter. In the presence of unlabeled GTP and ATP, transcription proceeded to position +11 and was halted because of the absence of CTP, specified by the position +12 of the template. After extensive washing, elongation complexes stalled at position 11 (EC11) were extended with radioactive CTP to obtain EC12, containing a radioactive RNA product. Further irradiation of RNAPWT elongation complexes resulted in radioactive labeling of both β′ and β with equal efficiency (Fig. 2A). As can be seen from the autoradiogram presented on Fig. 2A, labeled, full-sized β′ is absent from lanes containing mutant enzymes, establishing that little or no contaminating RNAPWT was present. Instead, lanes containing mutant enzymes have a labeled band with the mobility of β′, which represents a mixture of labeled full-sized β and mutant β′ subunits (full-sized β′ and β are 1407 and 1342 amino acids long, respectively; deletions in β′ make it impossible to separate the two proteins by SDS-PAGE). Control experiments established that (i) subunit labeling was template-dependent, and (ii) subunit labeling did not occur when photolabile, radioactive RNA 12-mer was added to RNAP in trans. Furthermore, cleavage of the RNA-product adduct with formic acid demonstrated that the RNA moiety of the adduct was 12 nucleotides in length (data not shown). On the basis of these results, we conclude that the mutant enzymes are assembled, active, and free of contaminating RNAPWT.

Transcription by Mutant RNA Polymerases—We decided to concentrate on RNAPΔKpn and RNAPΔAsu because preliminary experiments showed that RNAPΔ(941–1000), RNAPΔ(987–1042), and RNAPΔ(1042–1091) are very similar to RNAPWT.

Weilbaecher et al. (25) reported that point mutations in the β′ region under study altered the elongation and termination properties of RNAP in vivo and in vitro. Accordingly, we checked the transcription elongation and termination properties of mutant RNAPs. Immobilized elongation complexes stalled at position 20 of the T7 A1 promoter-driven transcription unit were prepared. To determine elongation rates, transcription was resumed by the addition of NTPs at subsaturating (2.5 μM) concentration. At various time points, reaction aliquots were withdrawn, and the products were analyzed by denaturing PAGE (Fig. 2A). As can be seen from Fig. 2, RNAPΔAsu did not differ significantly from RNAPWT in this assay, but RNAPΔKpn was dramatically “slower.” The apparent slow rate of transcript elongation by RNAPΔKpn was caused by extended (tifrax:1/2−2.5 min) pausing at template positions 26 and 27, and later at positions 37 and 56. The same pausing pattern was obtained when transcription by immobilized RNAPΔKpn was resumed from positions 12, 23, and 25 (data not shown).

Extended pausing by RNAPΔKpn at positions 26, 27, and 56 is intriguing, because transcription complexes artificially stalled.
at these positions are efficiently converted into an arrested conformation (26–30). During arrest at EC \textsuperscript{26} or EC \textsuperscript{27}, the catalytic center of the enzyme disengages from the 3'-end of the nascent RNA, and RNAP slides backward. As a result, the catalytic center repositions to about position 20. This intermediate complex is still able to continue transcription by sliding forward to the active conformation; it can also slide back even further, to position 11, and become permanently arrested. The permanently arrested, dead-end complex can only be rescued by transcript cleavage. In the presence of the transcript cleavage factor GreB, the catalytic center hydrolyses the RNA at position 11. The newly generated 3'-end of the 5'-end-proximal, 11-mer cleavage product can then be elongated by RNAP; the 3'-end-proximal cleavage products are lost from the complex.

To investigate the nature of the RNAP\textsuperscript{DKpn} pause at position 26, immobilized EC\textsuperscript{20} was incubated for 2 min in the presence of low (2.5 \textmu M) concentrations of NTPs, followed by a brief (30 s) incubation with GreB (Fig. 3A). As can be seen, addition of GreB to transcription reaction resulted in the appearance of new products. These new cleavage products were stably associated with transcription complexes and had the electrophoretic mobility of 20- and 22-mers. Control lanes of Fig. 3A demonstrate that the addition of GreB to stalled, arrested RNAP\textsuperscript{DKpn} EC\textsuperscript{27} resulted in cleavage, generating a 5'-terminal 11-mer, as expected. Based on these data, we conclude that in paused RNAP\textsuperscript{DKpn} EC\textsuperscript{26} and EC\textsuperscript{27}, the transcript has reversibly slid out of the active site, and therefore, the paused complexes correspond to intermediate of the arrested complex formation pathway (28–30).

Increased pausing by RNAP\textsuperscript{DKpn} could be explained by its increased ability to slide backward at certain parts of the template. Although the ability to slide back is difficult to test, we investigated the efficiency of EC\textsuperscript{27} arrest complex formation by RNAP\textsuperscript{DKpn}. In this experiment, shown in Fig. 3B by the extent of transcription arrest by artificially stalled, purified RNAP\textsuperscript{WT}. EC\textsuperscript{27} was determined by the ability to be chased into EC\textsuperscript{32} upon the addition of ATP and CTP. Freshly purified EC\textsuperscript{27} formed by both RNAP\textsuperscript{WT} and RNAP\textsuperscript{DKpn} were elongation competent (Fig. 3B, lanes 2 and 5). However, after 5 min of incubation at 37 °C in the absence of nucleotides only 10% of initial EC\textsuperscript{27} could be chased upon addition of nucleotides. Importantly, there was no difference between RNAP\textsuperscript{DKpn} and RNAP\textsuperscript{WT} in the extent of arrest (Fig. 3B, lanes 3 and 6).

Extensive pausing by RNAP\textsuperscript{DKpn} could be caused by an unusually high apparent K\textsubscript{m} for the incoming UTP (corresponding to position 27), accompanied by reversible sliding to position 20. Indeed, increasing NTP concentration decreased pausing by RNAP\textsuperscript{DKpn}. At high (250 \textmu M) substrate concentrations, RNAP\textsuperscript{DKpn} completed a single round of transcription in 1 min and terminated transcription on the \lambda \text{tR2} terminator with nearly the same efficiency as RNAP\textsuperscript{WT} and RNAP\textsuperscript{DKpn} (Fig. 3C).

Transcript Cleavage by Mutant RNAPs—A genetic screen of yeast RPO21 (an evolutionary homologue of rpo\textsubscript{C}) identified mutations that altered the ability of RNAP II to interact with
transcript cleavage factor TFIIId (31). Seven mutations were
isolated and they all clustered between conserved segments G
and H (32). It was subsequently shown that these mutations
inhibited interaction of mutant RNAP II with TFIIH by as
much as 50-fold (33). Because these mutations occurred very
close to hypervariable region studied here (see Fig. 1), we
investigated the transcript cleavage activity of mutant E. coli
RNAPs. As can be seen from Fig. 4A, EC21 formed by
RNAPΔKpn required approximately 10 times more GreB to
achieve the same extent of cleavage as complexes formed by
RNAPWT and RNAPΔAu. The same result was obtained when
the GreB homolog GreA was used to induce the cleavage reac-
tion (data not shown).

When EC20 formed by RNAPWT was incubated in pH 9.0
buffer (a condition known to stimulate the intrinsic nucleolytic
activity of RNAP (21)) transcript cleavage was observed (Fig.
4B). After 5 min incubation in pH 9.0 buffer, >75% of RNAPWT
and RNAPΔAu EC20 had undergone cleavage (Fig 4B, lanes 2
and 6, respectively). In contrast, only 10% of RNAPΔKpn EC20
had undergone cleavage after a 5-min incubation, and less than
60% was cleaved after a 20-min incubation in pH 9.0 buffer
(lanes 10 and 12). To estimate GreB binding to RNAPΔKpn, we
performed UV-photo-cross-linking of GreB to derivatized 3'-end
of nascent RNA in RNAPWT and RNAPΔKpn EC21 (Fig. 4C).
In agreement with previous data (36, 39), β’ and GreB were
efficiently cross-linked to derivatized, radioactive RNA. Lower-
ing the amount of GreB in the reaction resulted in decreased
cross-linking of GreB to RNA, but the amount of β’-RNA cross-
link remained constant. As can be seen from Fig. 4C, RNAPΔKpn complexes required approximately 10 times more
GreB to achieve the same extent of GreB-RNA cross-links as
complexes formed by RNAPWT. This effect was especially
evident at low GreB concentrations (compare Fig. 4C, lanes 6
and 12). From this result, we conclude that ΔKpn either directly
decreases GreB binding to RNAP or alters the relative position
of GreB and the RNA 3'-end, thus decreasing the efficiency of
GreB-RNA cross-linking near the catalytic center.

To estimate GreB binding to mutant RNAP directly, we used
GreB protein tagged with an N-terminal heart muscle protein
kinase (HMPK) recognition site.4 Different amounts of32P-end-
labeled HMPK-GreB were combined with RNAPWT or
RNAPΔKpn core enzymes, and after a short incubation to allow
complex formation, the reaction products were resolved by nat-
ive PAGE and GreB-containing complexes were visualized by
autoradiography (Fig. 4D). As can be seen, both enzymes
formed complexes with [32P]HMPK-labeled GreB with the
same efficiency.

Effects of mAb Binding to RNAP on Transcription in Vitro—In agreement with our previous data (15), mAb Pyn1
efficiently inhibited elongation of the nascent RNA from EC20
to EC32 by RNAPWT (Fig. 5A, top, lane 2). Transcription by
RNAPΔKpn was unaffected by Pyn1 (Fig. 5A, top, lane 7), be-
cause Pyn1 did not bind RNAPΔKpn (Fig. 5A, bottom, lane 5). In
contrast, Pyn4 had no effect on EC20 to EC25 conversion by both
enzymes, even though more than 50% of elongation complexes
were bound to mAb at the experimental conditions used (lanes
3 and 6).

Pyn1 completely inhibited intrinsic transcript cleavage by
RNAPWT at pH 9.0 (Fig. 5B, lanes 6 and 7). Transcript cleavage
by RNAPΔKpn was unaffected at these conditions (data not
shown). When GreB was added to RNAPWT, EC20 transcript
cleavage occurred even in the presence of Pyn1 (Fig. 5C). Still,
an ∼10-fold excess of GreB was required in the presence of
Pyn1 to achieve the same extent of cleavage as in its absence
(compare Fig. 5C, lanes 5 and 7). GreB did not cause disso-
ciation of Pyn1 from RNAP, as the experiment shown in Fig. 5D
demonstrates. In this experiment, different amounts of32P-
end-labeled HMPK-GreB were combined with RNAPWT core
in the presence or the absence of Pyn1. After a short incubation
to allow complex formation, the reaction products were resolved
by native PAGE, and GreB-containing complexes were visual-
ized by autoradiography. In the absence of Pyn1, [32P]HMPK-
GreB formed a complex with RNAP (Fig. 5D, lanes 1–3). Addi-
tion of Pyn1 resulted in the appearance of a radioactive
complex with a slower electrophoretic mobility (lanes 4–6). We

4 N. Loizos, unpublished results.
Transcript cleavage at elevated pH. Immobilized EC20 was transferred into a pH 9.0 transcription buffer, and reactions were incubated for indicated times, followed by electrophoresis and autoradiography. C, RNA-protein photo-cross-linking. EC21 formed by RNAPWT and RNAPΔKpn was UV-irradiated in the absence (lanes 1 and 7) or in the presence of decreasing concentrations of GreB (lanes 2 and 8, 400 ng; lanes 3 and 9, 40 ng; lanes 4 and 10, 4 ng; lanes 5 and 11, 0.4 ng; lanes 6 and 12, 0.04 ng). Reaction products were resolved on the 4–20% gradient SDS-gel and visualized by autoradiography. D, binding of RNAPWT and RNAPΔKpn to [32P]HMPK-GreB. 1 μg of RNAP of the indicated core enzymes was combined with increasing concentrations of [32P]HMPK-GreB. Reactions were incubated for 10 min to allow the complex formation, resolved by native PAGE on a 4–15% Phast gel, and visualized by autoradiography.

**Fig. 5.** Effects of mAb binding on transcription in vitro. A, EC20 containing radioactively labeled RNA were desorbed from Ni2+-NTA agarose with 50 mM imidazole (lanes 1 and 5, top panel, and lanes 1 and 4, bottom panel). Reactions were then supplemented with the indicated mAbs and 10 μM ATP and CTP (chase) to allow elongation to position 23. Reaction products were resolved by denaturing PAGE in 10% urea-gel (top panel) or by native PAGE in 4% gel (bottom panel). B, intrinsic transcript cleavage at elevated pH. Immobilized EC20 was transferred into a pH 9.0 transcription buffer, and reactions were incubated for indicated times, followed by electrophoresis and autoradiography. C, EC20 formed by RNAPWT and RNAPΔKpn was treated with increasing concentrations of GreB in the presence or in the absence of Pyn1, and reaction products were resolved on a 15% denaturing gel, visualized by autoradiography. In B and C, lanes 1 and 2 are controls, demonstrating that Pyn1 efficiently inhibited transcript elongation at the conditions used. D, binding of RNAP to [32P]HMPK-GreB. RNAP core enzyme was combined with increasing concentrations of [32P]HMPK-GreB in the presence (lanes 4–6) or the absence (lanes 1–3) of Pyn1. Reactions were incubated for 10 min to allow the complex formation and resolved by native PAGE on a 4–15% Phast gel.

**DISCUSSION**

A set of deletions in the cloned *E. coli* rpoC gene was constructed to evaluate the role of an evolutionarily hypervariable region of the RNAP β′ subunit (amino acids 941–1130) in enzyme function. This region is completely absent from β′ subunits of most bacteria and is highly divergent within proteobacteria (Fig. 1). Suprisingly, deletion of the entire hypervariable region completely prevented RNAP assembly both in *vivo* and *in vitro*. Obviously, similar deletions (relative to *E. coli* β′) naturally found in the β′ subunits from other bacteria do not prevent RNAP assembly in these organisms. Apparently, compensating differences in the α, β, and/or β′ subunits of these bacteria allow RNAP assembly to proceed.

The five functional deletions obtained in this work together remove 243 amino acids (>17%) of β′ and prove that, as in β, long regions of β′ sequence are dispensable for basic functions of *E. coli* RNAP *in vitro*. These functional deletions clearly fall into two classes. ΔAsu removes 54 amino acids N-terminal of the hypervariable region and does not change RNAP transcription in vitro. In contrast, deletions of similar size spanning the hypervariable region (Δ941–1000, Δ987–1042, Δ1042–1091, and Δ1091–1130 (ΔKpn)) cause a dramatic defect in transcription elongation at subsaturating substrate concentrations and inhibit transcript cleavage.

The functional difference between the two classes of mutants is also evident from different effects of mAb binding. Pyn1 binds to the hypervariable region (amino acids 948–1130) and efficiently inhibits RNA synthesis and intrinsic transcript cleavage. In contrast, Pyn4, the binding epitope of which is destroyed by ΔAsu, has no effect on transcription. The hypervariable region is highly immunogenic: in an independent study Luo and Krakow (34) isolated several mAbs that interacted with this region of *E. coli* β′. mAb 311G2 inhibited RNA synthesis by interfering with substrate binding, and its binding epitope was within β′ amino acids 1047–1093. Thus, 311G2 must be very similar to Pyn1.

The most striking feature of RNAPΔKpn-like enzymes is prolonged pausing at sites of transcriptional arrest (26–30), suggesting that the hypervariable region is involved in transcript elongation and cleavage. However, this involvement is proba-
bility indirect, because the hypervariable region is completely absent from RNAP of most organisms. We therefore propose that the deletions in $\beta'$ studied here act indirectly, by affecting function(s) of adjacent conserved segments. There is good evidence that segment G, which is located immediately to the N terminus of the hypervariable region, participates directly in transcript cleavage and transcriptional arrest. Borukhov et al. (35) demonstrated that an 80-amino acid-long $\beta'$ fragment containing the 8 C-terminal-most amino acids of conserved segment G and the 74 N-terminal-most amino acids of the hypervariable region contains the site that is cross-linked to the 3'-end of the nascent RNA in E. coli. It was later found that this protein-RNA contact is indicative of the arrested formation of the elongation complex (40). Further protein-RNA cross-linking experiments established that segment G and GreB proteins could both be cross-linked to the nascent RNA 3'-terminus and thus must be within several A of each other in the complex (36). Thus, it is likely that segment G can form an alternative, unproductive RNA binding site during transcript elongation. The defect in RNAP$_{Kpn}$-like enzymes could be the result of increased interactions between region G and the RNA 3'-end in the mutant complex at the potential arrest sites, causing backsliding, and elevated apparent $K_m$ for the incoming nucleoside triphosphate. This defect is only observed at subsaturating substrate concentrations, because at high substrate concentrations, addition of NMP to the 3'-end of the nascent RNA occurs faster than backsliding, and mutant enzymes elongate RNA normally. Close inspection of the gel shown in Fig. 2B reveals that RNAP$_{WT}$ also pauses at the sites of transcriptional arrest, but clears the pause sites rapidly. Thus, reversible backsliding may play a role in RNAP$_{WT}$ pausing as well.

One of the goals of this study was to obtain RNAP mutants defective in interactions with transcript cleavage factors. Data of Wu et al. (33) suggested that the region of the largest ($\beta'$-like) subunit of yeast RNAP II between conserved segments G and H may form the primary site of TFIIS interactions with its target RNAP. Seven tightly clustered linker insertion mutants studied by these authors showed little or no effect in intrinsic transcript cleavage and transcription elongation at high NTP concentrations but were defective in the TFIIS-dependent transcript cleavage, and did not form a complex with TFIIS in vitro. Thus, one of the seven yeast mutations occurred immediately to the right of conserved segment G. In E. coli, the corresponding amino acids are removed by $\Delta(1145-1198)$ (DeltaAu) mutation. Suprisingly, DeltaAu is the least defective of the five functional RNAP mutants obtained in this work. In contrast, $\Delta$Kpn-like enzymes with deletions in hypervariable region itself are defective in Gre-dependent transcript cleavage, but the defect is relatively mild. Unlike the situation with yeast RNAP, the interactions between the mutant enzymes and the transcript cleavage factor were not affected as measured by the native gel binding assay. The nature of the cleavage defect is complex, because both the intrinsic transcript cleavage activity of the catalytic center and GreB-RNA cross-linking are affected by mutations. The latter defect could be due to repositioning of segment G, which is known to be close to GreB in the elongation complex (see above). Thus, our data suggest that TFIIS and GreB, which are functional analogs, but not homologous to each other, may interact with their respective RNA polymerases differently.

Published alignments of $\beta'$-like RNA polymerase subunits (7, 24) differ significantly from the alignment presented in Fig. 1. The main feature of our alignment is the existence of an additional conserved segment, G' (E. coli positions 1031–1040). Our alignment offers clues to the striking differences in the biochemical properties of mutant enzymes obtained in this work and is supported by the pattern of evolutionary variation in this region. In archaea, eukaryotes, and most bacteria, segments G and G' are fused, forming one continuous stretch of conserved amino acids. In contrast, in proteobacteria, cyanobacteria, and chloroplasts, a long insertion occurred at the G/G' boundary (referred to as the hypervariable region in this work). The sequence of this insert appears to be unrelated between proteobacteria and cyanobacteria, and is highly divergent within each group. Interestingly, the site of the insertion relative to the G/G' boundary is the same in both groups, which are far apart from each other phylogenetically (41). Thus, insertions at the G/G' boundary may have occurred at least twice in evolution. The inserted region is highly immunogenic, and may therefore loop out at the surface of the RNAP. Deletions in or mAb binding to the hypervariable region may alter the relative position of G and G', resulting in the observed defects in transcription. The importance of G' is highlighted by the fact that the smallest deletion obtained in this work, $\Delta(1131-1155)$S, which removed G', failed to assemble in RNAP in vitro. On the other hand, it is possible that removal of approximately 50 amino acids C-terminal to G in the DeltaAu mutant did not alter the relative positions of G and G' and thus had no major impact on the transcription properties of RNAP. Ongoing site-specific mutagenesis should clarify the functional role of this segment in RNA polymerase assembly, catalytic function, and transcript cleavage.

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Dispensable Region in E. coli RNA Polymerase β' Subunit

24920

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