The β′ Subunit of Escherichia coli RNA Polymerase Is Not Required for Interaction with Initiating Nucleotide but Is Necessary for Interaction with Rifampicin

Received for publication, December 7, 2000, and in revised form, January 16, 2001
Published, JBC Papers in Press, January 22, 2001, DOI 10.1074/jbc.M011041200

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Using a modification of a highly selective affinity labeling protocol, we demonstrated that the αβ subassembly of Escherichia coli RNA polymerase efficiently and specifically interacts with the initiating purine nucleotide. Isolated β is also active in this reaction. In contrast, neither β nor αβ is able to interact with a chimeric molecule composed of rifampicin attached to an initiation substrate. Based on these results, we conclude that the RNA polymerase initiation site, specific for purine nucleotides, which ultimately become the 5′-end of the transcript, is essentially complete in the absence of the largest subunit, β′. However, the rifampicin binding center is formed only in the αββ′ core enzyme. We interpret our results in light of the high resolution structure of core RNA polymerase from Thermus aquaticus.

DNA-dependent RNA polymerase (RNAP) is a multisubunit, multifunctional molecular machine. RNAP from Escherichia coli (subunit composition αββ′ω) is the most studied enzyme of its class. Apart from its ability to catalyze phosphodiester bond formation, the partial biochemical functions of E. coli RNAP include the binding of single- and double-stranded DNA, limited melting of double-stranded DNA, binding of single- and double-stranded RNA, binding of nucleoside triphosphates, and binding of transcription inhibitors rifampicin and streptolydigin. Most of the functions common to all RNAPs are carried out by the two largest subunits, β and β′. The two largest subunits are the most evolutionary conserved and constitute >70% of the enzyme mass. Together, β and β′ contain 17 segments conserved from bacteria to man (1, 2). It is likely that the conserved segments participate in the formation of the enzyme functional sites as well as in intersubunit interactions stabilizing the core assembly.

Because the biochemical functions of the enzyme are lost upon the separation of RNAP subunits (3), the assignment of partial functions to a particular subunit or subunit segment is done indirectly by a combination of genetic and biochemical approaches. To directly elucidate the roles of different subunits in transcription, new approaches need to be developed. In this report, we used a modification of the highly selective affinity labeling technique (4) to study the ability of RNAP subassemblies to interact with the transcription initiation substrate and the transcription initiation inhibitor Rif. We present evidence that the purine-specific initiation site of RNAP (5, 6) is essentially complete in the physiological αβ subassembly (7) and is also present in the isolated β subunit. In contrast, we found that the Rif-binding site is present only in the complete RNAP core, suggesting that β′ plays an unexpectedly critical role in the assembly of this site. We interpret our data in light of the high resolution three-dimensional structure of the RNAP core from Thermus aquaticus (8).

MATERIALS AND METHODS

Preparation of RNA Polymerase Subunits and RNAP Reconstitution—RNAP subunits were overexpressed and purified as described elsewhere (9). RNAP reconstitution was performed essentially as described by Borukhov and Goldfarb (10). Inclusion bodies containing overexpressed β or β′ subunit were solubilized in denaturing buffer (10) to a final concentration of 0.25 mg/ml and dialyzed against two changes of a 30-fold excess of reconstitution buffer (10). The supernatant after dialysis was aliquoted and stored at −80 °C until further use. RNAP was reconstituted in 25-μl reactions containing 2.5 μg of β, 2.5 μg of β′, and 0.6 μg of hexahistidine-tagged α prepared as described (9). RNAP was assembled by incubating the reactions for 30 min at 30 °C.

Coupled Affinity Labeling and RNA Polymerase Reconstitution—The synthesis of the affinity labeling reagents, AMP- and ATP-based aldehydes and derivatized Rif-A, was as described (4, 11). 10 μl of individually renatured RNAP subunits or subunit combinations were incubated for 15 min at 30 °C in renaturation buffer (10) in the presence of varying concentrations of cross-linking reagent followed by the addition of 10 mM borohydride and an additional 15 min incubation at 37 °C. Missing RNAP subunits were then added, and reactions (total volume 25 μl) were incubated for an additional 30 min at 30 °C. Reactions were supplemented with 0.5 μg of recombinant ω (9), 50 ng of a DNA fragment containing the -17 A1 promoter (12), and 0.5 μM α[32P]UTP (3000 Ci/mmol). Reactions proceeded for 3 h at 25 °C and then were terminated by the addition of an equal volume of Laemmli loading buffer. Proteins were resolved on polyacrylamide gel electrophoresis on 8% SDS-gels. Labeled subunits were visualized by autoradiography and quantified by phosphorimager. In Vitro Transcription—Coupled RNAP reconstitution and affinity labeling reactions and product separation were performed as above, and affinity labeled β subunits were extracted from the SDS gels by crushing the gel chips and soaking them in 0.2% SDS overnight. Extracted subunits...
were dried in vacuo and dissolved in a small volume of water, and HCl was added to 50 mM. Cleavage was initiated by the addition of CNBr to a final concentration of 50 mM and allowed to proceed for 5 min at room temperature. Reactions were terminated by the addition of a double volume of Laemmli loading buffer. The reaction products were resolved by 20% denaturing urea-PAGE and revealed by autoradiography.

**RESULTS**

**Rationale**—Available cross-linking and mutational data indicate that the RNAP β subunit makes extensive contacts with the initiating purine nucleotide, which will ultimately become the 5′-end of the transcript (4, 14, 15). Thus, β appears to be principally responsible for the formation of the 5′-face of the catalytic center. We were interested to know whether the RNAP subassembly makes extensive contacts with the specific initiation site (i-site) present even in the free RNAP core (5, 6), so it should be possible to carry out the first step of the affinity labeling protocol in the absence of promoter DNA, followed by promoter complex formation and the template-dependent extension of cross-linked nucleotide. One can extend this approach even further and perform the cross-linking step in the absence of one or two RNAP subunits. To visualize the cross-link, one would then have to add the missing RNAP subunit(s) to reconstitute the active enzyme. The standard RNAP reconstitution protocol involves mixing the RNAP subunits under denaturing conditions followed by dialysis into reconstitution buffer. However, active RNAP can also be assembled from individually renatured subunits (3, 13). Therefore, after the addition of σ and template DNA, the original cross-link can be developed with radioactive nucleoside triphosphate specified by position +2 of the promoter.

**RNAP β and the αβ Subassembly Can Be Affinity Labeled with an Initiation Substrate Analogue Specific for βLys1065**—We used the modified affinity labeling protocol described above to ask whether the RNAP subassembly αβ, and even β alone, can be affinity labeled with initiating AMP derivatized with a cross-linkable aldehyde group (4). In this experiment, as shown in Fig. 1, derivatized AMP was added to the indicated renatured RNAP subunits or subunit combinations (Crosslinking to), and the cross-linking reaction was induced by the addition of 10 mM sodium borohydride for 15 min at 37 °C. The RNAP core was then assembled by the addition of individually renatured subunits omitted in the first step (Addition of). The cross-link was then developed by the addition of σ, the T7 A1 promoter-containing DNA fragment, and α-[32P]UTP specified by the +2 position of the template. As expected, robust labeling of β was observed in
the complete reaction, which contained all the RNAP core subunits at the initial cross-linking step (lane 10). Approximately 5% of β was radioactively labeled in the complete reaction, which represented ~40% of the labeling achieved in the standard affinity labeling protocol when RNAP open complex was used at the cross-linking stage (data not shown). Surprisingly, a high level of β labeling was observed when the mixture of α and β was modified in the absence of β′ (lane 9). The labeling efficiency dropped to ~20% of that seen in the complete reaction when β alone was used at the cross-linking step (lane 7). Similar results were obtained with initiating ATP derivatized with a cross-linkable aldehyde group positioned at the γ-phosphate (data not shown). Importantly, no labeling occurred in reactions when no extra core subunits were added in the development step, indicating that the preparations of renatured β and α were free of contaminating RNAP or β′, which would have revealed themselves by affinity labeling of β in Fig. 1a (lanes 6 and 8).

Because the assembly of active RNAP is required for β labeling to occur, the amount of radioactive labeling depends not only on the binding of the initiating nucleotide analogue by the i-site at the cross-linking step but also on the amount of active RNAP present at the final stages of the reaction. To determine the efficiency of the RNAP assembly, aliquots of reactions from lanes 7, 9, and 10 of Fig. 1a were supplemented with NTPs and in a steady-state transcription assay (Fig. 1b). RNAP activity in this assay correlated with the affinity labeling results; the amount of full-sized transcript produced was the highest when the αβ mixture was used for cross-linking, followed by the addition of β′ (lane 2), or when individually renatured α, β, and β′ were combined simultaneously (lane 3). Only ~20% of RNAP activity could be recovered when β alone was cross-linked to the initiating substrate analogue followed by the addition of α and β′ (lane 1). Thus, the relative cross-linking efficiency of the initiating AMP analogue to β in reactions containing the isolated subunit or in the αβ mixture is comparable with that achieved in the complete RNAP labeling reaction.

Additional control experiments are presented in Fig. 1c. When the initiating AMP analogue alone was reacted with 10 mM sodium borohydride for 15 min, followed by the addition of RNAP core subunits, no labeling was observed (Fig. 1c, compare lanes 5 and 6). This order-of-addition experiment establishes that the cross-linking reaction was complete by the time the missing subunits were added at the second stage of the reaction. Further, no labeling was observed when the initiating AMP analogue was incubated with individually renatured β′ followed by the reaction with 10 mM sodium borohydride for 15 min and the addition of the missing α and β (Fig. 3c, lane 3). Thus, the reagent used is strictly specific for the β subunit, as expected from previous work (4).

We used several controls to establish that the observed labeling of β resulted from the specific binding of the purine nucleotide analogue to the RNAP i-site. The presence of unlabelled ATP during the cross-linking step abolished labeling (Fig. 2a). This suggests that the affinity labeling of β reflects the interaction of the derivatized AMP with a specific nucleotide-binding site rather than a nonspecific interaction of the cross-linker with the lysines of the protein. Interestingly, the addition of UTP did not interfere with the labeling, suggesting as expected that the pyrimidine nucleotide poorly competes for the purine-specific i-site (data not shown).

To map the initiating nucleotide cross-link sites, purified radiolabeled subunits were cleaved at Met residues under “single-hit” conditions to yield families of nested, easily identifiable fragments (Fig. 2b). The pattern of radioactive CNBr-generated fragments was the same whether β alone, the αβ mixture, or the complete reaction containing α, β, and β′ was used at the cross-linking step (compare lanes 4–6 and 7–9). Previous work demonstrated that standard affinity labeling with the AMP-based reagent resulted in exclusive cross-linking to universally conserved Lys1065 in the β conserved segment H (14); the ATP-based reagent cross-linked to βLys1242 in the universally conserved segment I.2 The experiment of Fig. 2b reveals that the ATP-based reagent also cross-links to Lys1242 in our protocol because the smallest radioactive fragment visible on the gel corresponded to cleavage at βMet1242 whether β, the αβ mixture, or the complete mixture of RNAP core subunits was modified. The smallest radiolabeled CNBr fragment observed with the AMP-based reagent corresponded to cleavage at βMet1065 not to Met951 as has been previously reported for this reagent (14). Thus, with our labeling protocol at least a fraction of the cross-links occurred to a lysine between βMet1066 and Met1085, leaving Lys1073 and/or Lys1078 as possible candidates. Lys1073 is the most likely target because structural analysis of

2 M. Kozlov, I. Bass, K. Severinov, and A. Mustaev, unpublished observations.
the T. aquaticus RNAP core (8) reveals that the T. aquaticus Lys846 (E. coli Lys1073) is located within 5 Å of Lys838 (E. coli Lys1065), which is the site of AMP reagent cross-linking at standard affinity labeling conditions (4, 14). In contrast, the T. aquaticus Lys851 (E. coli Lys1078) is more than 17 Å away from Lys838. The shift in the cross-link site can be explained by conformational changes, which occur during the holoenzyme and/or promoter complex formation. We also note that affinity labeling of the E. coli RNAP carrying the K1065A mutation results in Lys1073 labeling even when the standard affinity labeling protocol is used.3

The apparent $K_m$ values for initiating ATP analogue binding to $\beta$, $\alpha/\beta$, or $\alpha/\beta/\beta'$ are calculated from the extent of affinity labeling as a function of the analogue concentration (data not shown). The calculated values were similar (650 $\mu$M $\beta$, 500 $\mu$M $\alpha/\beta$, and 575 $\mu$M $\alpha/\beta/\beta'$) and close to the published $K_m$ value for the RNAP i-site determined by independent methods (5, 6). We conclude that the observed affinity labeling is the result of the specific interaction of the initiating nucleotide with the $\alpha/\beta$ subassembly or isolated $\beta$ and that this interaction occurs in the i-site.

The $\beta'$ Subunit Is Required for the Formation of the Rif-binding Site—Rifampicin forms a tight complex with RNAP and prevents promoter escape (3, 16). Mutations in $\beta$ abolish or weaken Rif binding to RNAP, suggesting that $\beta$ forms at least a part of the Rif binding center (17, 18). To test this idea directly and to study the binding of Rif to RNAP subassemblies, we used a chimeric compound comprising Rif covalently attached to ATP (Rif-A, as reviewed in Ref. 11). The chimeric molecule interacts with RNAP core with high affinity because of the strong Rif-RNAP interaction. The molecule also serves as a primer for the transcription reaction because the linker between Rif and the nucleotide moiety allows Rif-A to interact with RNAP in a bifunctional manner such that each ligand occupies its natural binding site (11). Rif-A was derivatized with the cross-linkable aldehyde group positioned next to the $\alpha$-phosphate of the nucleotide and specific for $\beta$Lys1065 (11). RNAP $\beta$, $\alpha_2\beta$, or a mixture of $\alpha$, $\beta$, and $\beta'$ was preincubated with varying concentrations of derivatized Rif-A, and cross-linking was induced by the addition of borohydride. After the cross-linking reaction was complete, the missing subunits were added, followed by the template-dependent development of the cross-link using radioactive UTP (Fig. 3a). As a control, affinity labeling reactions were also performed with various concentrations of the AMP-based reagent (Fig. 3b). In reactions containing all RNAP subunits, comparable levels of labeling were achieved with both Rif-A and AMP. However, the concentration dependence of labeling was dramatically different, as expected. Reactions containing Rif-A saturated at low concentrations of the reagent because of the very high affinity of Rif for its RNAP-binding site. In contrast, $\beta$ labeling with the AMP-based reagent increased linearly over the whole concentration range tested because of the lower affinity of AMP for the i-site. Strikingly, no labeling with the Rif-A reagent was observed when cross-linking was performed with isolated $\beta$ or in the mixture of the $\alpha$ and $\beta$ subunits (Fig. 3a, lanes 1–6 and 1’–6’). We conclude that the $\beta'$ subunit is required for the formation of the high affinity Rif-binding site. The complete lack of labeling of RNAP subassemblies by Rif-A at 50 $\mu$M concentrations contrasts with observable levels of modification with 50 $\mu$M derivatized AMP (compare lanes 4 and 4’ in Fig. 3, a and b). Thus, Rif binding may inhibit AMP binding in the i-site, consistent with earlier kinetic data (16).

**DISCUSSION**

The principal result of this work is the demonstration that the purine-specific RNAP initiation i-site is essentially complete in the $\alpha_2\beta$ subassembly and is even present in the isolated $\beta$ subunit. In contrast, the Rif-binding site requires $\beta'$ for its formation. The critical role of $\beta$ in the formation of the i-site is supported by affinity labeling data, which demonstrate that the initiating substrate makes extensive contacts with several regions of the $\beta$ subunit (14, 15). Here, we demonstrated that $\beta$ residues in conserved segments H and I can specifically interact with the initiating nucleotide even in the absence of other RNAP subunits. Preliminary experiments demonstrate that the assembly-competent C-terminal module of $\beta$ (amino acids 643–1342), which contains conserved segments F, G, H, and I, can also be affinity-labeled by the derivatized initiating nucleotides in our assay, albeit with low efficiency.4

In contrast, our results reveal an absolute requirement of $\beta'$ for the formation of the Rif-binding site as we were unable to detect an interaction between the $\alpha_2\beta$ subassembly and Rif-A. Extensive genetic data indicate that mutations that cause Rif

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3 A. Mustaev, V. Sagitov, and A. Goldfarb, unpublished observations.

4 K. Severinov, unpublished observations.
resistance and decrease Rif binding to RNAP are found exclusively in the *rpoB* gene, coding for β. Early biochemical experiments demonstrated that, in agreement with our data, rifampicin derivatives cross-link to β nonspecifically (20). On the other hand, it has been reported that αβ weakly binds Rif as judged by spectral, gel filtration-based, and proteolytic assays (21, 22). However, in these later studies the authors did not determine whether their αβ preparation was free of traces of the RNAP core and whether the observed interaction was specific because no controls using αβ containing the β subunit carrying a Rif resistance mutation were used. In addition, there is a formal possibility that the difference between our results and those reported by previous researchers is attributable to differences between Rif and Rif-A interactions with RNAP. However, the absence of Rif-A binding and cross-linking to RNAP harboring known Rif resistance mutations makes this possibility unlikely (11).

Recently, an atomic structure of the RNAP core enzyme from the Eubacterium *T. aquaticus* was determined (8). The structure revealed a molecule with a “crab-claw” shape. In the view presented in Fig. 4, the upper jaw of the claw is made mostly of β and is bilobal; the lower jaw is made mostly of β'. The deep cleft that separates the jaws harbors the catalytic magnesium ion and binds the DNA template (8, 23). Analysis of the *T. aquaticus* RNAP structure validates the biochemical data obtained in this work with the *E. coli* enzyme. As can be seen, the Rif-binding site (defined by sites that, when mutated, prevent Rif binding and result in Rif resistance (*green, yellow, blue, and white spheres on Fig. 4*)) and the i-site (as defined by cross-link points in conserved segments H and I (*red spheres in Fig. 4*)) come from clearly different “domains” of the structure. 5

The i-site lies on the face of the β sheet domain made up from the β subunit conserved segments F, G, and H and a portion of segment I. Proteolytic studies, in vitro reconstitution, and two-hybrid analysis indicate that in *E. coli*, this domain is capable of independent folding and specific binding to RNAP α subunit (24, 25) and that evolutionary conserved residue Asp1084 in segment H is particularly important for αβ formation (25). Our results show that this domain is also capable of interacting with the initiating nucleotide α-phosphate through βLYS1073. On the structure, αi (white in Fig. 4) is immediately behind this domain, consistent with the observed stimulatory effect of α on β labeling. It should be noted that the i-site residues revealed by affinity labeling cannot be essential for catalysis because detecting cross-linking at these residues in the affinity labeling protocol requires catalysis to attach the radioactive label (14, 26). Unfortunately, the 5’-phosphate cross-links do not allow to model the position of the initiating nucleotide on the structure with confidence. However, the highlighted residues of β must be very close to the initiating nucleotide bound in the i-site to be cross-linked to the derivatized nucleotide.

Mustaev et al. (27) used selective affinity labeling of RNAP promoter complexes to study the extension of initiating nucleotides cross-linked to the *E. coli* βLYS1065* and His1237. They demonstrated that the initiating nucleotides cross-linked to LYS1065 could be extended by only one nucleotide specified by the second position of the template. In contrast, initiating nucleotides cross-linked to His1237 could be extended by as much as nine nucleotides. These results were taken as strongly supporting the inchworming model of transcription. This model attempts to explain the high processivity of transcription elongation by postulating that the catalytic center of RNAP can move with respect to the RNAP mainframe (28). In contrast, rigid body models postulate that no extensive movements of the catalytic center occur and that the addition of a single nucleotide to the nascent RNA 3’-terminus is coupled to the translocation of the whole complex along the DNA. Structural analysis indicates that the data of Mustaev et al. (27) can be explained without invoking the inchworming model. In the *T. aquaticus* RNAP structure LYS838, which is homologous to the *E. coli* LYS1065, is located in an inner strand of the β sheet domain and is firmly anchored on the RNAP mainframe. In contrast, His999, which is homologous to the *E. coli* His1237, is located on an unstructured loop that traverses the main channel of the enzyme and appears to be flexible. Thus, the extension of the cross-link at this position may simply reflect the flexibility of the cross-link site rather than the movement of the catalytic center.

The Rif pocket, as defined by Rif resistance mutations, is farther away from the catalytic center, at the interface between several structural elements of β. Mutations toward Rif resistance occur at the base of and in between the two lobes formed by the β jaw (Fig. 4). Residues defining the N-terminal-most

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5 K. Severinov and A. Mustaev, unpublished observations.
cluster of Rif mutations (E. coli position 146, T. aquaticus position 137 (white sphere)) and the C-terminal-most cluster (E. coli position 687, T. aquaticus position 566 (blue sphere)) occur at the place where the two lobes meet. Cluster I mutations (E. coli positions 507–534, T. aquaticus positions 387–414 (green spheres)) affect both lobes. Cluster II mutations (E. coli positions 563–574, T. aquaticus β positions 443–454 (yellow spheres)) affect the downstream lobe only. Cluster II amino acids lie at the base of a hairpin-like structure (blue in Fig. 4). This hairpin is directly supported by the β’ segment F helix (running vertically on the left in Fig. 4 (red and magenta)). In particular, evolutionary conserved T. aquaticus segment F residues 1085–1088, corresponding to E. coli β’ positions 786–789, make van der Waals contacts with cluster II amino acids 447–451, corresponding to the E. coli β amino acids 567–571. In addition, the T. aquaticus β’Arg1077 (E. coli Arg780) is in direct contact with the T. aquaticus βPro444. The corresponding residue in E. coli, βPro564, can be mutated toward Rif resistance (18). Thus, it appears that cluster II would be unable to fold properly in the absence of β’, which may explain the requirement for β’ for the formation of the Rif site.

In E. coli, the segment F amino acid Ser793, which is only three amino acids away from the β’ amino acids involved in the stabilization of the Rif-cluster II structure, could be mutated toward streptolydigin resistance (19). The main cluster of streptolydigin resistance mutations is in β’ between Rif cluster I and II. Our biochemical and structural analysis suggests that mutations toward Rif resistance can also be obtained in the β’ segment F. We are currently performing targeted searches for such mutations. If found, such mutants may explain the nature of infrequent Rif-resistant bacterial isolates that do not contain any changes in the known Rif resistance sites in β.

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doi: 10.1074/jbc.M011041200 originally published online January 22, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011041200

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