A series of active elongation complexes of the phage T7 RNA polymerase were obtained through stepwise walking of the polymerase along an immobilized DNA template. Transcripts were radiolabeled at the 16th to 18th residues, and a photocross-linkable 4-thio-UMP was separately incorporated at the 22nd, 24th, 32nd, and 38th residues. Such complexes (up to 51 nucleotides) produced by the incorporation of one nucleotide at a time were isolated and individually subjected to long wave UV cross-linking. Only when the cross-linker was positioned at the 3’-end (−1) of the elongating RNA and 8 nucleotides upstream (−9), was the RNA substantially cross-linked to the polymerase, regardless of how far it was from the 5’-end of the transcripts. Linkage of the 3’-end residue was mapped to the Thr108–Met180 region, which contains nucleotide-binding sites. The −9 residue was cross-linked to the Ala244–Met250 region rather than to the N-terminal region. These two contacts were maintained throughout the elongation complexes and reveal a route of nascent RNA through the T7 RNA polymerase in elongation complexes.

The structural organization of transcription elongation complexes has been well characterized with histidine-tagged Escherichia coli RNA polymerase arrested at specific positions on templates (1). Macromolecular interactions among DNA, RNA, and E. coli RNA polymerase in the elongation complexes have been mapped (2–5). A double clamp model has been proposed for elongating E. coli RNA polymerase where DNA entry and RNA exit sites are directed opposite to each other in the same region (5). Experimental evidence (6, 7) also is consistent with this mechanism for RNA polymerase action.

The T7 RNA polymerase, a single-subunit protein of 99 kDa, is capable of initiating transcription promoter-specifically, elongating with high processivity and terminating in the absence of additional factors. The mechanism of initiation and termination of T7 RNA polymerase has been studied extensively because of its simplicity of structure and reaction system. On the other hand, elongation has been little studied, mainly because a series of stable and active ternary elongation complexes have not been obtained with the T7 RNA polymerase.

The overall three-dimensional structures of T7 RNA polymerase complexed with an open promoter (8) and a transcribing initiation complex (9) are similar to that of free enzyme (10).

Received for publication, September 20, 2000
Published, JBC Papers in Press, October 30, 2000, DOI 10.1074/jbc.M008616200

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Some regions, including the active site, change into a more compact structure in the initiation complex. Structural information on elongation complexes with a growing transcript has not yet been determined.

Several lines of evidence have suggested that the structure of the T7 RNA polymerase ternary complex changes when it proceeds from the initiation phase to the elongation phase. Both protease sensitivity (11) and the protected region of the DNA template strand in footprinting with methidiumpropylated EDTA-Fe(II) greatly change (12, 13) during the switch from initiation to elongation. The length of the DNA-RNA hybrid also changes from 2–4 bp in the initiation complex (11) to 7 bp in an arrested elongation complex (13). However, the macromolecular interactions and the structures of the active ternary elongation complexes have not yet been examined.

In this study, we obtained a series of active and stable elongation complexes of the T7 RNA polymerase prepared by walking the enzyme along an immobilized DNA template. To probe RNA-protein interactions in ternary elongation complexes, a 4-thio-UMP was incorporated into the nascent transcripts and used to photocross-link RNA to the polymerase. Our cross-linking results suggest that the RNA runs from the active site toward a C-terminal region in the finger domain, rather than an N-terminal region near the thumb domain, in active elongation complexes of the T7 RNA polymerase.

EXPERIMENTAL PROCEDURES

DNA Template—The 216-bp BglII fragment of pET3 (14) containing a T7 promoter and the T4 terminator was inserted into the BamHI site of pUC19 (15). GGG at position 19–21 (relative to the transcription start site at +1) was mutated to TTT to avoid formation of an RNA hairpin structure. Transcription templates were obtained by polymerase chain reactions using biotinylated reverse and forward M13 primers and were immobilized by incubating with streptavidin-coated magnetic beads (Dynal, Inc.) at room temperature for 15 min.

Stepwise Walking of T7 RNA Polymerase—The biotinylated templates (30 pmol) bound to streptavidin beads were incubated with 60 μl of an equimolar T7 RNA polymerase (United States Biochemicals) in transcription buffer A of 40 mM Tris-HCl, pH 7.9, 6 mM MgCl2, 100 mM KCl, and 10 mM dithiothreitol at 37 °C for 5 min. The transcription reaction was started by adding 60 μl of a ribonucleotide mixture to final concentrations of 0.05 mM ATP, 0.05 mM CTP, and 0.005 mM GTP and continued for 20 min to produce a 15-mer RNA complex, TEC15. After five washes with 0.5 ml of transcription buffer B consisting of 40 mM Tris-HCl, pH 7.9, 6 mM MgCl2, and 100 mM KCl, the beads were resuspended in 120 μl of buffer B containing 0.33 mM [α-32P]UTP (3,000 Ci/mmol, Amersham Pharmacia Biotech) and incubated at room temperature for 2 min to obtain 32P-labeled TEC18. After four washes, the beads were resuspended in 0.8 ml of buffer B, and a 15-μl aliquot was withdrawn. The washed beads were then resuspended in 115 μl of buffer B containing 0.5 μM ATP for 2 min to obtain TEC21. TEC22 and TEC23 were obtained similarly in 110- and 105-μl reaction volumes, respectively.

The abbreviations used are: bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; TEC, transcription elongation complex.
A photo-reactive analog of UMP, 4-thio-UMP was incorporated into the +24 site with incubation in a 100-μl mixture of 5 μM 4-thio-UTP and transcription buffer C consisting of 40 mM Tris-HCl, pH 7.2, 6 mM MgCl₂, and 100 mM KCl at 37 °C for 2 min. The 4-thiouridine-incorporated radioactive TEC24 was eluted further with suitable ribonucleotides along the template sequence to TEC25, TEC26, TEC27, TEC30, TEC31, TEC32, TEC34, TEC36, TEC38, TEC40, TEC41, TEC43, TEC44, TEC47, and TEC51 in progressively smaller reaction volumes. A 5-μl portion of each 15-μl aliquot was mixed with 10 μl of gel loading buffer of 10 mM EDTA, 12 μM urea, and 0.1% (w/v) bromphenol blue and was loaded onto an 8% urea, 10% polyacrylamide gel.

**Phosphocross-linking of Elongation Complexes**—A 10-μl portion of each elongation complex aliquot was transferred to a 1.5-ml Eppendorf tube placed on ice and was then subjected to UV irradiation for 20 min using a 360-nm lamp UVGL-25 (Ultraviolet Products), as described previously (3). Each irradiated sample was mixed with 2.5 μl of gel loading buffer consisting of 60 mM Tris-HCl, pH 6.8, 2.5% (w/v) glycerol, 2% (w/v) SDS, 14.4 mM β-mercaptoethanol, and 0.1% (w/v) bromphenol blue and then heated to 95 °C for 5 min. It was then loaded onto an 8% polyacrylamide/SDS gel for separation of the cross-linked and uncross-linked polymerase. The electrophoresis buffer, pH 8.3 consisted of 25 mM Tris, 192 mM glycine, and 0.1% SDS.

**Mapping the Trypsin Cleavage Site and RNA-linked Sites**—The trypsin cleavage site of elongating T7 RNA polymerase was mapped by using the monochlonal histidine-tagged T7 RNA polymerase that was produced in E. coli from pKK-HisT7 (16). The plasmid was constructed by Hoseok Song and Changwon Kang (16) who transferred the T7 polymerase gene from pAR1219 into the XhoI site of pET-tac, derived from pET15b (Novagen). The 21-mer transcript-containing elongation complex of the His-tagged polymerase was obtained by walking and was partially digested by trypsin. The polypeptides separated by SDS-PAGE were transferred to nitrocellulose membrane by electrophoretic transfer. The membrane was probed with a monoclonal His-1 antibody against a photo-reactive analog of UMP, 4-thio-UMP was incorporated into the 45th to 47th residues. TEC44(sU38) and TEC46(sU38) could not be isolated because AMP was consecutively utilized during the elongation reaction.

**PHOTOcross-linkers at the 22nd, 32nd, and 38th positions**—The cross-linkers were incorporated at the 22nd, 32nd, and 38th positions of the transcripts. When the cross-linker was at the 22nd residue, the 22-mer and 30-mer RNA, respectively in TEC22(sU22) and TEC32(sU22), produced the most predominant cross-linking (Fig. 2A). Also, when the cross-linker was at the 32nd residue, TEC32(sU32) and TEC40(sU32) were cross-linked to the greatest extent (Fig. 2C). Also, with the cross-linker at the 38th residue, TEC38(sU38) produced the most pronounced cross-linking (Fig. 2D). TEC46(sU38) could not be isolated because AMP was consecutively incorporated into the 45th to 47th residues, TEC44(sU38) and TEC47(sU38), however, produced only a residual level of cross-linking like the other complexes. Thus, the polymerase-linking sites of transcripts were the same regardless of RNA length and cross-linker position.

**Mapping of Two RNA-linked Sites to the C-terminal One-Third Region Using Trypsin and Hydroxylamine**—The two RNA-linking sites of the elongating polymerase might be differen...
different from each other, because they are separated by 8 nucleotides on elongating transcripts. The two sites, named 3'-end and 9 contact sites here, were mapped individually by enzymatic and chemical proteolysis of the cross-linked elongation complexes.

Native T7 RNA polymerase is susceptible to proteolytic cleavage by trypsin. Initial cleavage occurs near Lys172 or Lys3180 under mild conditions and results in a nicked RNA polymerase consisting of N-terminal 20-kDa and C-terminal 79-kDa fragments (18). It was not clear, however, if the cleavage site was still the same in the elongating RNA polymerase. To address this issue, the T7 RNA polymerase that was histidine-tagged at the N terminus was used for walking to TEC24.

When such an elongation complex was digested by trypsin under mild conditions, only the 20-kDa fragment was detected on a Western blot with an anti-histidine antibody (data not shown). Thus, trypsin cleaves the T7 RNA polymerase initially at the N-terminal one-fifth location in an elongation complex similar to free enzyme under the conditions used (Fig. 3).

Partial digestion of the RNA-linked complexes TEC24(sU24) and TEC32(sU24) with trypsin yielded only one radiolabeled fragment whose size was about 90 kDa (Fig. 4A). Molecular masses of the 24- and 32-mer RNA are about 7,600 and 10,100, respectively. Thus, RNA was cross-linked to the C-terminal 79-kDa fragment of the polymerase, but not to the N-terminal 20-kDa fragment in both complexes. When a 4-thiouridine was cross-linked to the C-terminal one-third region of Gly589–Ala883 (Fig. 3). Elongation complex TEC32(sU24) was also analyzed in the same way. Only fragments of (33 + 10), (67 + 10), and (79 + 10) kDa were radioactive. (The 32-mer RNA is 10 kDa.) Thus, the 9 residue was also cross-linked to the same C-terminal one-third region (Fig. 3).

Fine Mapping of Two RNA-linked Sites Using Chemical Digestions—In mapping experiments with 2-nitro-5-thiocyanobenzoic acid, single-hit digestion conditions were used, because there are 12 Cys cleavage targets (Fig. 3). As RNA is linked to the C-terminal one-third region, only the smallest radiolabeled fragment produced under such conditions can determine the linked region. Thus, fragment complexes smaller than about 50 kDa (for Ser541–Ala883 peptide plus RNA) were closely examined.

In the case of single-hit digests of TEC24(sU24), the smallest radiolabeled fragment was of (38 + 8) kDa (Fig. 5, A and B, filled arrows), representing the size of the Ser541–Ala883 region, and the (18 + 8)-kDa fragment of Ala724–Ala883 was not observed. The fragments of (46 + 8), (60 + 8), (69 + 8), (74 + 8), and (85 + 8) kDa were also radioactive, possibly representing the Ala468–Ala883, Pro348–Ala883, Val272–Ala883, Ile217–Ala883,
and Leu<sup>125</sup>–Ala<sup>883</sup> regions, respectively. Therefore, the cross-linked fragments contained the Ser<sup>241</sup>–Cys<sup>723</sup> region. This mapping was supported by the results of more extensive digestion at a higher pH or for a longer reaction time. A group of bands around 36 kDa (Fig. 5, A and B, arrows) should not have been observed if the cross-linker were located in the other regions, Ala<sup>724</sup>–Cys<sup>839</sup> (13 kDa) or Asp<sup>840</sup>–Ala<sup>883</sup> (5 kDa). Combining the results of hydroxylamine mapping, the 3′-end contact site is in the region of Gly<sup>589</sup>–Cys<sup>723</sup> (Fig. 3).

Single-hit cleavage patterns of TEC32(sU32) were different from that of TEC24(sU24). The smallest observed fragment was of (18 + 10) kDa (Fig. 5, A and B, filled arrows), reflecting the size of the Ala<sup>724</sup>–Ala<sup>883</sup> peptide, and a (5 + 10)-kDa fragment of Asp<sup>840</sup>–Ala<sup>883</sup> was not observed. These results were consistent with those of more extensive digestion, the smallest observed fragment was of (13 + 10) kDa (Fig. 5, A and B, arrow) possibly containing the 13-kDa Ala<sup>724</sup>–Cys<sup>839</sup> peptide. Thus, the 9 contact site is located between Ala<sup>724</sup> and Cys<sup>839</sup> (Fig. 3).

There are 26 potential Met sites of cyanogen bromide cleavage (Fig. 3). When the denatured TEC24(sU24) was treated with cyanogen bromide under single-hit digestion conditions (Fig. 5C), the smallest radioactive fragment was of (28 + 8) kDa for the Thr<sup>636</sup>–Ala<sup>883</sup> region. Thus, the 3′-end contact region is in the Thr<sup>636</sup>–Met<sup>750</sup> region. Likewise the smallest fragment of TEC32(sU24) was of (21 + 10) kDa for Asn<sup>697</sup>–Ala<sup>883</sup> (Fig. 5C), suggesting that the 9 contact site is in the Ala<sup>724</sup>–Met<sup>750</sup> region. Fragments larger than that were also observed as expected, except for those of (24 + 10) kDa (for Phe<sup>667</sup>–Ala<sup>883</sup>) and (23 + 10) kDa (for Ala<sup>678</sup>–Ala<sup>883</sup>). Sites at 666 and 677 were not accessible.

Cross-links in elongation complexes TEC32(sU32) and TEC40(sU32) that contained the photocross-linker at the 32nd residue were also mapped in the same way. The mapping results were the same as above; the Thr<sup>636</sup>–Met<sup>666</sup> and Ala<sup>724</sup>–Met<sup>750</sup> regions, when located at −1 and −9 positions, respectively (data not shown).

**DISCUSSION**

Transcription elongation complexes of the T7 RNA polymerase have been obtained either by placing psoralen cross-link site specifically downstream from a promoter (13) or by withholding a ribonucleotide from a transcription reaction mixture (21). These arrested or stalled ternary elongation complexes were not subjected to further elongation. In this study, several series of active ternary complexes of the T7 RNA polymerase were obtained by the polymerase walking method. It was achieved here by immobilizing biotinylated DNA templates with streptavidin beads rather than by immobilizing the RNA polymerase. Elongation complexes of both intact and N-terminal histidine-tagged polymerases halted because of missing nucleotides were capable of extending transcripts with replenishment of nucleotides.

A 4-thio-UMP was incorporated separately at four different sites (22nd, 24th, 32nd, and 38th residues) in transcripts, and four series of elongation complexes were obtained by walking. Major photocross-links between RNA and polymerase are ob-
served when the cross-linker is positioned at the 3'-end (−1) of growing transcripts and 8 nucleotides upstream (−9), regardless of how long RNA is and how far the cross-linker is from the 5'-end (Fig. 2). Thus, two separate residues of elongating transcripts closely interact with RNA polymerase. 4-Thiouridine appears to be indiscriminate when cross-linking to amino acids in a protein because of its high photoreactivity (22, 23). Thus, photoreactive groups of the other residues may not be in close contact with the polymerase, although the nearby amino acids, if there are any, may not have been reactive.

The upstream (−9) nucleotide that is near to the protein is also near the DNA-RNA hybrid of ~7 bp, as determined from the work of Sastry and Hearst (13). When the movement of T7 RNA polymerase was blocked by psoralen cross-link at +36, the bottom strand of DNA between +30 and +36 was resistant to single-strand-specific T7 endonuclease. Elongating transcripts were previously found to interact with E. coli RNA polymerase also at two sites (5). RNA distance between the two sites (8–9 residues) in E. coli is similar to that in T7, possibly reflecting the similar length of DNA-RNA hybrid within elongation complexes. The upstream site, however, is much broader in E. coli (about 9 nucleotide residues) than in T7 complex (about 1 residue). Although the hybrid length is apparently similar in T7 and E. coli RNA polymerase, stability of elongation complexes to high salt in each case differs significantly, suggesting that the hybrid itself does not play the major role in complex stability. The shorter RNA binding site in T7 polymerase and/or unlocked DNA-binding site could explain the difference in stability.

The two residues at the 3'-end and −9 were found to cross-link to different regions of elongating T7 RNA polymerase, when mapped using trypsin, hydroxylamine, 2-nitro-5-thiocyanobenzonic acid, and cyanogen bromide. The RNA 3'-end linking region is between Thr636 and Met666 (Fig. 6). This is a part of the O-helix, which has been considered to include active site residues. A mutation of Tyr639 to Phe previously resulted in incorporation of dNTP as well as rNTP, suggesting that it is close to the C terminus, between Ala724 and Met750 (Fig. 6), as determined from trypsin, hydroxylamine, 2-nitro-5-thiocyanobenzonic acid, and cyanogen bromide. The RNA 3'-end linking region is close to the C terminus, between Ala724 and Met750 (Fig. 6), because the N-terminal 20-kDa fragment has been thought to be associated with RNA binding and processivity of the elongation complex. Exogenous RNA binding was previously abolished in the C-terminal 80-kDa fragment (18). E148A mutant was defective in RNA oligomer binding (25). Also, based on the photocross-linking results with the photoreactive group linked to the 5'-end of nascent RNA, Sastry and Ross (26) proposed that regions between 144 and 168 and between 1 and 93 interact with emerging RNA and form a solvent-accessible RNA binding channel. Based on these previous results, Cheetham and Steitz (9) drew an RNA path toward the thumb domain in the N-terminal region in their model based on the crystal structure of initiation complex.

According to our results, however, elongating RNA travels from the active site region toward a C terminus proximal region rather than an N terminus proximal region (Fig. 6). Elongating and exogenous RNAs appear to bind different regions of the polymerase. In the cross-linking experiments by Sastry and Ross (26), the ternary complex was arrested with only 5–8-mer transcripts, probably had not escaped from abortive initiation cycling, and the linker used was rather long.

In our model, RNA exits in the tunnel through the finger region in the elongation complex (Fig. 6). Thus, the RNA-exiting region is different from the DNA-entering region. The RNA-exiting region (724–750) partially overlaps with the promoter-specificity loop (742–773). This may reflect structural continuation of template DNA strand in initiation and elongation. More interestingly, these two regions form a bent channel in the structure of the initiation complex (9). Recently, DNA in the initiation complex was found to be bent 40–60 degrees around the transcription start site (27), whereas the intrinsic bend of the promoter was much smaller (28). The bending formed to facilitate DNA melting for initiation would be maintained during the elongation stage.

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J. Biol. Chem. 2001, 276:4080-4084.
doi: 10.1074/jbc.M008616200 originally published online October 30, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M008616200

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