

Crucial Role of the RNA:DNA Hybrid in the Processivity of Transcription

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Summary

We present an approach for studying the role of complementary nucleic acid interactions in transcription elongation by *E. coli* RNA polymerase (RNAP). The method involves in vitro reconstitution of a catalytically active elongation complex (EC) by the addition of RNAP to a single-strand DNA oligonucleotide containing the preannealed RNA primer, followed by incorporation of the complementary nontemplate DNA oligonucleotide. In all parameters tested, the reconstituted complex is indistinguishable from normal EC obtained by promoter-specific initiation. Using RNA primers of different lengths, which were fully or partially complementary to the DNA, we determined the minimal transcript length and the degree of its template pairing that is required to stabilize protein/nucleic acid interactions in EC to the high level characteristic of normal transcription. Our data show that a hybrid at least 9 nt long, formed between the template DNA and 3'-proximal RNA transcript, is necessary for the high processivity of EC during RNA chain elongation.

Introduction

The elongation complex (EC) of RNA polymerase (RNAP) represents a principal intermediate in transcription and serves as a target for the action of a variety of regulatory factors (reviewed by Chan and Landick, 1994; Landick, 1997; Uptain et al., 1997). Once assembled on a promoter, EC becomes highly processive and can traverse long distances on the template without dissociation. After encountering an obstacle on the DNA or after spontaneous arrest, EC may remain intact for a long period of time (Krummel and Chamberlin, 1992; Nudler et al., 1995; Komissarova and Kashlev, 1997a). However, when RNAP transcribes through special signals or interacts with termination factors, EC may quickly dissociate.

The remarkable processivity of EC and its regulation in such a broad range of conditions represent a poorly understood biological mechanism that modulates gene expression (Uptain et al., 1997). The current view of the structure of EC contends that during RNA synthesis the enzyme interacts with the transcript and with the double-strand DNA containing a single-strand transcriptional bubble. The template DNA strand in the bubble hybridizes with the 3'-proximal RNA (Gamper and Hearst,

1982; Yager and von Hippel, 1987; Chan and Landick, 1994). Although this nucleic acid region is a primary target for important processes, such as pausing, arrest, termination, and antitermination, its actual structure remains unknown (reviewed by Landick, 1997).

Two models have been proposed to describe the architecture of this part of the complex. Based on theoretical assumptions and on the analysis of a heterogeneous population of ECs, Yager and von Hippel (1987) proposed that an ~12 nucleotide (nt) RNA:DNA hybrid in the normal A form of a double helix is maintained near the 3' end of the transcript, and its length remains unchanged while RNAP progresses along the template. The stability of this helical structure was postulated to determine the processivity of EC, such that a weak hybrid would provoke dissociation of the complex. More recently, an alternative model argued that the hybrid does not exceed 2–3 nt and does not play a significant role in the stabilization of EC (Rice et al., 1991; Chamberlin, 1994; Uptain et al., 1997). Instead, a major role in retention of the RNA was assigned to the single-strand RNA-binding site located in RNAP 2–3 nt from the 3' end of the transcript. The reconciling point of view suggested that the extended RNA/DNA pairing and a putative single-strand RNA-binding site, located upstream of the hybrid, are important for holding together all of the elements in EC (Landick, 1997; Nudler et al., 1997).

Although the crystal structure of EC is not yet available, a growing body of evidence points to the presence of extended pairing between the transcript and template in the complex. It was recently reported that ribonucleotide triphosphate analogs, incorporated into the RNA at variable distances from the 3' end, can be cross-linked to the template DNA strand at a distance of not more than 7 nt from the 3' end (Nudler et al., 1997). This result suggests that the RNA and DNA strands first move in parallel and then branch out, around 7 nt from the catalytic center, which can be interpreted as the presence of a 7 nt hybrid. The strong protection of the template DNA from chemical and enzymatic agents, specific for the single-strand DNA 9–12 nt upstream from the 3' end of the RNA, also supports the idea of the extended hybrid (Metzger et al., 1989; Zaychikov et al., 1995). However, the transcript in EC was shown to be susceptible to different ribonucleases specific for single-strand RNA 3–7 nt from the 3' end (Rice et al., 1991). This result argues that both the 3'-proximal RNA and the template strand opposite from this RNA may at least temporarily adopt a single-strand state. The presence of a specific single-strand RNA-binding site apart from the DNA:RNA hybrid was proposed from the results of RNA/protein cross-linking experiments (Hanna and Meares, 1983; Nudler et al., 1997) and from the fact that RNAP can bind RNA in the absence of DNA to form an equimolar binary complex (Altmann et al., 1994). The vast majority of the experiments circled around the problem of the hybrid's existence without questioning its role in the processivity of RNAP. A clue to understanding this bias lies in the method of halted transcription that was most often used to address RNAP processivity. In this method,

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RNAP was loaded to the DNA by using promoter-specific initiation, followed by controlled walking with the enzyme away from the promoter to the site where the analysis was being performed (Metzger et al., 1989; Kashlev et al., 1993; Nudler et al., 1994; Wang et al., 1995). Although this approach demonstrated a remarkable manipulative power, it has never allowed isolation of ECs containing transcripts shorter than 9–12 nt, which is crucial for studying the role of the RNA:DNA hybrid. This limitation was due to a rapid dissociation of the short RNA species from RNAP in a process called abortive initiation (Johnston and McClure, 1977).

The alternative experimental method presented here expands on a completely different approach that was pioneered by Daube and von Hippel (1992, 1994). This method uses short synthetic DNA and RNA oligonucleotides and the immobilized enzyme to build an *in vitro* authentic ternary complex of RNAP that bypasses promoter-specific initiation and enzymatic synthesis of the transcript. By introducing systematic variations in both the length of the synthetic RNA added to the reconstitution and the degree of its pairing to the template, we demonstrate that an extended RNA:DNA hybrid at the 3' end of the transcript is indispensable for tight binding of RNAP to the DNA and transcript during the processive synthesis of RNA.

Results

Reconstitution of EC from Synthetic RNA and DNA Oligonucleotides

A single-strand 30 nt synthetic DNA oligonucleotide was used as a minimal template DNA strand (TDS³⁰) for EC assembly (Figure 1A). TDS³⁰ contained the sequence of 10 nt upstream and 20 nt downstream from the start site of transcription from the A1 promoter of bacteriophage T₇. The properties of transcription through this sequence were characterized previously (Nudler et al., 1994; Zaychikov et al., 1995; Komissarova and Kashlev, 1997a). The 9 nt 5'-labeled oligoribonucleotide (RNA⁹), coding for the first 9 nt of the initially transcribing region of this promoter, was annealed to TDS³⁰ (see Figure 1A), and the holoenzyme of histidine-tagged RNAP (Kashlev et al., 1993) was added to this hybrid to form the protein/nucleic acid complex. An aliquot of the reaction was then incubated with a complementary 30 nt nontemplate DNA strand (NDS³⁰). EC⁹ (the numerical index indicates the length of the RNA in the complex), containing either one or two DNA strands, was purified by adsorption on Ni²⁺-NTA agarose beads followed by washing the beads with transcription buffer to remove unbound DNA and RNA oligonucleotides. To determine whether the reconstituted complexes were catalytically active, we tested the ability of the immobilized enzyme to extend RNA⁹ to 11 nt by incubating EC⁹ with ATP and GTP. RNA⁹ was quantitatively elongated on both the single- and double-strand DNA (Figure 1B, lanes 1, 2, 7, and 8). The elongation was strictly template-dependent, and EC⁹ did not incorporate noncomplementary nucleotides in the RNA (data not shown). Moreover, EC⁹ showed the unlimited polymerization activity and synthesized the 20 nt runoff RNA after addition of all four NTPs (data not shown).

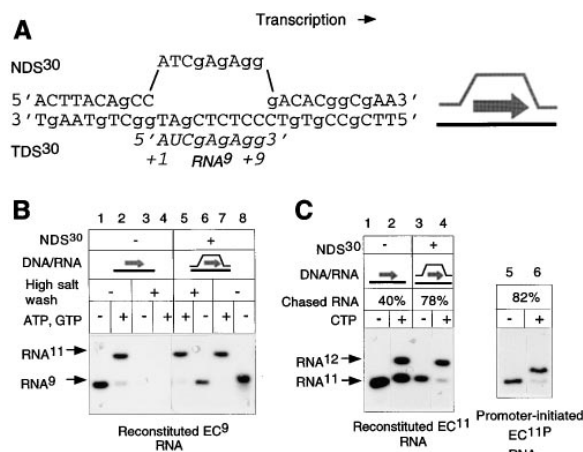


Figure 1. Assembly of Catalytically Active ECs from Short Synthetic Oligonucleotides

(A) The sequences of RNA/DNA oligonucleotides employed for the assembly of EC⁹ and their schematic representation used in the figures. The black and gray lines at the scheme represent the template and nontemplate DNA oligonucleotides, respectively. The thick gray arrow denotes RNA.

(B) Active and stable EC can be assembled from RNAP and synthetic oligonucleotides. EC⁹, containing only the coding strand or both DNA strands, was reconstituted, and its catalytic activity and stability were tested as described in Experimental Procedures.

(C) The catalytic activity of RNAP increased after incorporation of the noncoding strand into ECs. The RNA in reconstituted EC⁹, containing only the coding strand or both DNA strands, was elongated to 11 nt. The reconstituted EC¹¹ and normal promoter-specific EC^{11P} were then incubated at 24°C with 0.01 μM CTP for 5 min in TB.

These results indicated that, during the assembly, the 3' end of RNA⁹ was properly aligned with the template and was positioned in the catalytic center of RNAP.

A noncoding DNA strand was recently demonstrated as having a role in the structural stability of EC (Nudler et al., 1997). To determine whether the addition of NDS³⁰ could increase the stability of EC⁹, we incubated the complex containing either one or two DNA strands with high-salt buffer (1 M KCl), followed by washing the beads with low-salt buffer (40 mM KCl) to remove the dissociated RNA. EC⁹ containing only one DNA strand completely dissociated in the high salt (Figure 1B, lanes 3 and 4), whereas incorporation of NDS³⁰ stabilized EC⁹, which remained intact and catalytically active after high-salt treatment (lanes 5 and 6). In 1 M KCl, half-life of EC⁹ on the double-strand DNA was approximately 60 min, which was close to the stability of the majority of ECs containing the longer RNAs and obtained by either promoter-specific initiation or by using the recently developed technique of template switching (Arndt and Chamberlin, 1990; Nudler et al., 1996).

A direct comparison of the catalytic activity of EC⁹, reconstituted on the double-strand DNA with that of its promoter-specific equivalent, was impossible, because ECs containing less than 11 nt in the RNA (EC^{11P}) could not be obtained from the A1 promoter. To solve this problem, we first incubated EC⁹ with ATP and GTP to elongate RNA⁹ to 11 nt. This step was followed by quantitative comparison of the elongation competence of EC¹¹ with that of its promoter-specific counterpart containing

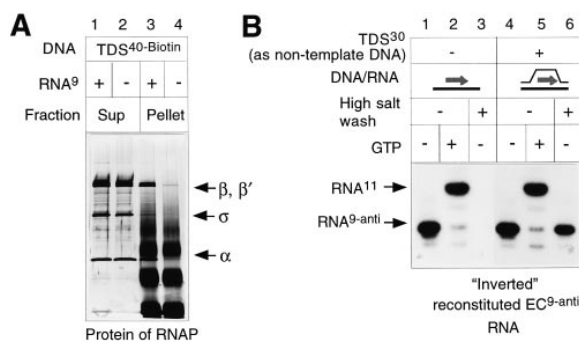


Figure 2. Protein Content of Assembled EC. Reconstitution of Inverted EC^{9-anti} by Template Strand Switching

(A) Dissociation of the σ subunit after binding of RNAP to the RNA:DNA hybrid. RNAP was incubated with TDS⁴⁰-Biotin or with the RNA⁹:TDS⁴⁰-Biotin hybrid as usual except that concentration of the oligonucleotides was two times higher. Avidin-agarose beads (Leinco Technologies) were added to immobilize the 3' biotinylated TDS⁴⁰-Biotin template. After 1 hr of incubation at 24°C, the supernatants (lanes 1 and 2) were removed, and the pellets (lanes 3 and 4) were washed twice with 1 ml of TB. Both fractions were analyzed on 6%–20% gradient SDS-PAGE. The positions on the gel of β , β' , α , and σ subunits are marked.

(B) The template strand switching did not affect the ability to assemble catalytically active and stable EC. The 5'-labeled RNA^{9-anti} oligonucleotide (antisense to the normal RNA⁹ primer) was annealed to NDS³⁰, and reconstitution/immobilization was performed. TDS³⁰ played the role of the nontemplate DNA strand in EC^{9-anti}. The stability (lanes 3 and 6) and activity (lanes 2 and 5) of EC^{9-anti} containing either one or two DNA strands were determined as described in Experimental Procedures.

the same sequence of the RNA. The ECs were incubated with a very low concentration of CTP (0.01 μ M) for the different periods of time. The quantitative analysis of the fraction of chased RNAs showed that the time course of elongation by reconstituted EC¹¹ was almost identical to that by regular EC^{11P}. To illustrate this fact, we selected an intermediate time point (5 min) in which only a fraction of the RNA was extended to 12 nt (78% and 82% of the chased RNA, respectively; lanes 4 and 6 of Figure 1C). In contrast, the activity of EC¹¹ lacking the second strand was much lower (40% of the chased RNA, lane 2). This result implied that our reconstitution method led to successful formation of the authentic EC with an activity very similar to that of the promoter-specific complex, despite the fact that the two approaches for EC formation were completely different. This result also indicated that the noncoding DNA strand has an important role in controlling the rate of RNA polymerization during normal elongation.

Formation of processive EC during transcription initiation at promoters is known to involve dissociation of the σ subunit, which normally occurs when the transcript reaches the length of 9–12 nt (Carpousis and Gralla, 1980; Grachev and Zaychikov, 1980; Hansen and McClure, 1980). Since the purified holoenzyme of RNAP used for the reconstitution contained the σ subunit, it was important to investigate whether the EC assembly also induced the release of the σ subunit. We used the alternative technique to purify reconstituted EC⁹ (Figure 2A). The modified template oligonucleotide (TDS⁴⁰-Biotin), carrying 10 extra bases and the biotin group at the 3'

end, was used to form EC⁹ followed by binding via the DNA to the avidin-agarose beads. The presence of the σ subunit in the template-bound RNAP was analyzed by SDS-polyacrylamide gel electrophoresis after washing the beads containing the immobilized template in the presence or absence of preannealed RNA⁹. RNAP molecules that were bound to the RNA:DNA hybrid contained no σ subunit (lane 3), whereas most of the free subunit was localized in the supernatant, together with an excess of the holoenzyme not participating in the reconstitution (lane 1). Using TDS⁴⁰-Biotin alone did not cause any retention of RNAP in the pellet (lane 4). The quantitation of the protein gel showed that 90% of EC lost σ subunit during the assembly. In a separate experiment, highly purified core enzyme of RNA polymerase was used for the reconstitution of active EC, which confirmed the absence of σ subunit in the assembled complexes. We therefore concluded that the assembly of EC causes release of the σ subunit that is triggered by the interaction of RNAP with the DNA:RNA hybrid.

Since the reconstitution utilized the sequence of the initially transcribing region of the promoter, which is normally used to originate EC in vivo, it was crucial to demonstrate whether the technique could be applied to reconstitute EC by using a sequence that has never been involved in promoter-directed assembly of the elongation machinery. To demonstrate this, we performed the reconstitution of an inverted EC, using NDS³⁰ as a template strand and the 5'-labeled antisense RNA primer (RNA^{9-anti}), which was complementary to regular RNA⁹. EC^{9-anti} was as active and stable as its antipode EC⁹ (Figure 2B, lanes 1, 2, 4, and 5) despite the fact that both complexes faced opposite directions of the DNA and formed totally distinct sets of contacts with the nucleic acids. Incubation with the high-salt buffer (lanes 3 and 6) showed that the stability of EC^{9-anti} also substantially increased in the presence of the noncoding strand (TDS³⁰). Together with the successful reconstitution of EC containing one more set of completely unrelated DNA and RNA sequences (data not shown), this result confirmed the ability of the method to create functional EC in a variety of sequence contexts.

Structural Analysis of Reconstituted EC

To ensure that reconstitution caused the appearance of a nucleic acid structure similar to that found in normal EC (see the Introduction for details), we monitored in EC⁹ the formation of the RNA:DNA and DNA:DNA hybrids and all junctions between them during the reconstitution. We observed the KMnO₄-induced modification of single-strand thymidine residues in 5'-labeled TDS³⁰, performed at each step during the assembly either in the solid phase with the immobilized RNAP (Figure 3A, lanes 1, 3, and 5) or in solution with the nucleic acids alone (lanes 2, 4, and 6). As we expected, after addition of RNA⁹ to TDS³⁰ in the absence of the protein, all three thymidines in the region complementary to the RNA primer (T₁₁, T₁₅, and T₁₇) became completely protected from the modification, indicating the formation of the stable RNA:DNA hybrid in solution (lane 4). NDS³⁰ completely displaced the RNA from the hybrid to form the 30 nt DNA duplex, as shown by the uniform loss of

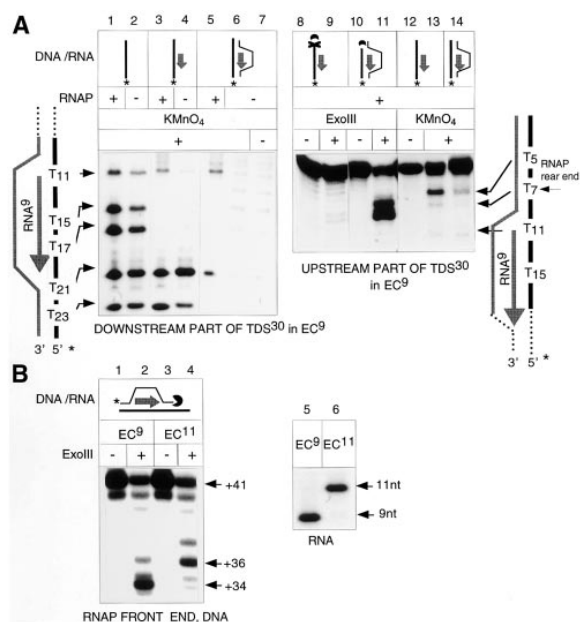


Figure 3. Formation of the Transcriptional Bubble in EC⁹ and Translocation of the Complex along DNA

(A) Lanes 1–7 and 12–14: potassium permanganate footprinting of 5'-labeled TDS³⁰ oligonucleotide at different steps of the assembly of EC⁹ was performed as described in Experimental Procedures. The control samples (lanes 2, 4, and 6) contained no RNAP and were not washed after Ni²⁺-NTA agarose was added. Lanes 8–11: footprinting of TDS³⁰ with ExoIII. The schemes represent the sequence of TDS³⁰ containing the reactive thymidine residues, as counted from its 3' end. Position of RNAP rear end is marked. (B) Lanes 1–4: ExoIII footprinting of the front end of RNAP in EC⁹ reconstituted by using TDS¹¹ and NDS⁴¹ DNA oligonucleotides and in EC¹¹ obtained from the EC⁹. Lanes 5 and 6: the RNA in the ECs.

reactivity to permanganate for all thymidines in TDS³⁰ (lanes 6 and 7). However, in the complex with RNAP, the displacement did not occur, revealing stabilization of the hybrid by the protein (lane 5). Instead, part of TDS³⁰ downstream from the annealed RNA⁹ formed the DNA duplex with NDS³⁰, as shown by complete protection from permanganate for T₂₃ (compare lanes 3 and 5). The T₂₁ residue, located next to the 3' end of RNA⁹ at the junction between the RNA:DNA and DNA:DNA hybrids, remained partially unprotected, indicating the formation of the normal leading edge of a transcriptional bubble within RNAP. The T₁₁ residue, which in the absence of the enzyme was paired to the 5'-terminal adenosine in RNA⁹ (lane 4), showed an increase in accessibility to permanganate (lane 3), suggesting that the 5' end of the primer was partially melted off the DNA after interacting with the protein. The sensitivity to permanganate exhibited by the thymidines in the RNA:DNA hybrid was almost identical in the EC containing both single- and double-strand DNA and in the protein-free hybrid (compare the modification of T₁₅ and T₁₇ in lanes 3 and 5 with that in lane 4). Thus, RNAP did not cause serious distortion of the RNA:DNA hybrid upon binding to it. This pattern of protection of the template DNA strand from permanganate was similar to that observed in normal ECs obtained by halting promoter-initiated transcription (Zaychikov et al., 1995; Komissarova and Kashlev,

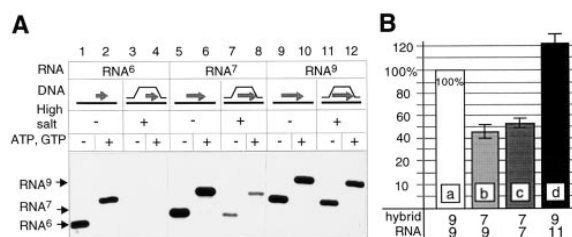


Figure 4. Extended RNA:DNA Hybrid Is Required for EC Stability

(A) Determination of the minimal length of RNA primer required for stabilization of EC. ECs containing 6, 7, or 9 nt 5'-labeled RNA primers were assembled on TDS³⁰ in the presence or absence of NDS³⁰, and their stability and catalytic activity were tested as described in Experimental Procedures.

(B) Stability of ECs with different lengths of the RNA:DNA hybrid. Bars represent the relative stability of the double-strand ECs normalized to the stability of EC⁹. The composition of the hybrids was as follows: (a) RNA⁹:TDS³⁰; (b) RNA⁹:TDS^{30CG}; (c) RNA⁷:TDS³⁰; (d) RNA¹¹:TDS^{30CG}. RNA¹¹ was obtained from RNA⁹ (b) in the presence of ATP and GTP. NDS³⁰ (a and c) or NDS^{30CG} (b and d) were used as nontemplate strands. Numbers in the first row below the graph represent the length of each RNA:DNA hybrid; the total lengths of RNA are shown in the second row.

1997a, 1997b). The incorporation of NDS³⁰ caused the appearance of a similar DNA duplex upstream from the annealed RNA⁹, as revealed by a significant decrease in the reactivity to permanganate for the T₅ and T₇ residues in TDS³⁰ (Figure 3A, lanes 12–14). The treatment of EC⁹ with exonuclease III (ExoIII), which is known to selectively degrade the double-strand DNA (Roger and Weiss, 1980), revealed that incorporation of the noncoding strand caused extensive degradation of the upstream part of TDS³⁰ (compare lanes 9 and 11). This result confirmed the formation of the DNA duplex at the rear end of RNAP. Importantly, the rear-end ExoIII footprint of RNAP in EC⁹ (lane 11) was located at the same distance (13–15 nt upstream from the 3' end of the RNA) as that in the majority of normal ECs obtained by promoter-initiated transcription (Wang et al., 1995; Komissarova and Kashlev, 1997a, 1997b). These results indicate that two complementary DNA strands in RNAP join to form the duplexes at both ends of the RNA primer, thus completing the formation of a structure closely resembling a normal transcriptional bubble.

We next determined whether the position of the front end of RNAP in reconstituted EC⁹ was the same as in the promoter-specific complexes. In many such complexes probed in vitro, RNAP was shown to protect from ExoIII degradation 16–18 nt of the duplex DNA next to the 3' end of the transcript (Nudler et al., 1994; Wang et al., 1995; Komissarova and Kashlev, 1997a). In EC⁹, which contained only 11 nt in the downstream DNA, this part of the template was also completely protected by the protein (data not shown). To make this DNA extruded from RNAP and available for degradation by ExoIII, we assembled EC⁹ on the longer pair of complementary oligonucleotides, containing 11 extra bases at the front end of the complex (TDS⁴¹ and NDS⁴¹). We found that the front end of RNAP in EC⁹ (Figure 3B, lane 2) was located 15–17 nt from the 3' end of the RNA. Incubation of EC⁹ with ATP and GTP made this footprint shift 2 nt

Table 1. Stability of ECs Assembled with Various RNA and DNA Oligonucleotides

#	EC	Composition of RNA:DNA hybrid	Sequence of RNA:DNA hybrid Transcription →	Formation of EC on ssDNA	Stability of EC on dsDNA
1	EC ⁹	RNA-9 TDS-30	5' <i>AUCGAGAGG</i> 3' TGAATGTCGGTAGCTCTCCCTGTGCCGCTT	Y	+
2	EC ^{9anti}	RNA-9-anti NDS-30	<i>CCUCUCGAU</i> AAGCGGCACAGGGAGAGCTACCGACATTCA	Y	+
3	EC ⁷	RNA-7 TDS-30	<i>CGAGAGG</i> TGAATGTCGGTAGCTCTCCCTGTGCCGCTT	Y	+/-
4	EC ⁶	RNA-6 TDS-30	<i>GAGAGG</i> TGAATGTCGGTAGCTCTCCCTGTGCCGCTT	Y	-
5	EC ⁴⁺⁵	RNA-4+5 TDS-30	<i>UGAUC</i> <i>GAGG</i> TGAATGTCGGTAGCTCTCCCTGTGCCGCTT	No	ND
6	EC ^{9CG}	RNA-9 TDS-30CG	<i>AU</i> <i>CGAGAGG</i> TGAATGTCGGCGGCTCTCCCTGTGCCGCTT	Y	+/-
7	EC ^{11CG}	RNA-11 TDS-30CG	<i>AU</i> <i>CGAGAGGGA</i> TGAATGTCGGCGGCTCTCCCTGTGCCGCTT	Y	+
8	EC ^{9CG/GC}	RNA-7+2 TDS-30CG	<i>GCCGAGAGG</i> TGAATGTCGGCGGCTCTCCCTGTGCCGCTT	Y	+
9	EC ^{9-IMP}	RNA-9-IMP TDS-30	<i>AUCIAIAII</i> TGAATGTCGGTAGCTCTCCCTGTGCCGCTT	Y	+/-
10	EC ^{9-DNA}	DNA-9 TDS-30	<i>dATCGAGAGG</i> TGAATGTCGGTAGCTCTCCCTGTGCCGCTT	Y	+/-
11	EC ¹⁰	RNA-10 TDS-30	<i>AUCGAGAGGG</i> TGAATGTCGGTAGCTCTCCCTGTGCCGCTT	Y	+

The RNA primer sequences are shown in italics. The substitutions in the RNA or DNA nucleotides that affected complementary interactions in ECs are underlined. EC^{11CG} contained the normal RNA⁹ primer elongated by RNAP to 11 nt. EC^{9-DNA} had the same sequence as RNA⁹ but was composed of DNA. EC¹⁰ was obtained from EC⁹ by incubation with GTP. The nontemplate strands used were complementary to the template strands. The fifth column shows whether the RNA primers supported reconstitution of EC on the single-strand DNA (Y/No). The sixth column indicates the stability of EC in 1 M KCl achieved after incorporation of the noncoding DNA strand. EC had approximately the same stability as EC⁹ (+). Stability of EC dropped at least 2-fold compared to EC⁹ (+/-). EC was completely salt-sensitive (-). ND, not determined.

downstream (lane 4), which indicated that the synchronous movement of RNAP along the DNA occurred in the assembled complex upon addition of 2 nt to the end of the RNA (lanes 5 and 6). Extending the RNA to 11 nt in EC⁹ allowed a comparison of the RNAP front-end footprint with that in normal EC^{11P} obtained from the A1 promoter, which was shown to occupy exactly the same position on the DNA (Komissarova and Kashlev, 1997a). We therefore concluded that the reconstitution successfully reproduced the normal EC in all tested structural and functional parameters.

Extended RNA:DNA Hybrid Is Required for Stabilization of EC on Double-Strand DNA

The possibility of using synthetic oligonucleotides to reconstitute EC offered an easy approach for addressing the fundamental problem of whether the length of the transcript and the extent of its pairing to the template contribute to the high processivity of RNAP. Figure 4 and Table 1 summarize the results of systematically varying the size of the reconstituted RNA primer or the length/strength of its pairing to the DNA. We measured the stability of ECs containing the labeled primers progressively truncated from the 5' end (Figure 4A). The 6–9 nt primers (RNA⁶, RNA⁷, and RNA⁹) all caused formation of the catalytically active ECs on TDS³⁰ alone with

similar efficiency (lanes 1, 2, 5, 6, 9, and 10). RNA shorter than 6 nt did not support the reconstitution, due to the inability of this primer to form a stable hybrid with the template (data not shown). In contrast to EC⁹, which acquired resistance to high salt on the double-strand DNA (lanes 11 and 12), EC⁶ remained essentially unstable (lanes 3 and 4), whereas EC⁷ was stabilized only moderately (lanes 7 and 8). These results argued that at least 9 nt in the transcript were required for the formation of a stable EC. However, the data do not exclude the possibility that the size of the RNA per se rather than the length of its pairing to the DNA could be responsible for the observed differences in stability. To test this possibility, we changed the sequence of TDS³⁰ by introducing the mismatched CpG residues in two template positions that normally form a hybrid with the two 5'-terminal residues of RNA⁹ (TDS^{30CG}). This replacement reduced the length of the RNA:DNA pairing to 7 nt without affecting both the length and sequence of the RNA in the complex. Although the resulting complex (EC^{9CG}) was formed on the single-strand DNA with an efficiency similar to that of EC⁹, the incorporation of the noncoding strand (NDS^{30CG}) did not stabilize it to the level estimated for normal EC⁹ (Figures 4Ba and 4Bb). Instead, the stability of EC^{9CG} was lower and almost equal to that of the complex containing the shorter RNA⁷ (Figures 4Bb and 4Bc), which indicated that the presence of two unpaired

residues in RNAP upstream from the 7 nt RNA:DNA hybrid did not improve the stability of the complex. However, the stability of EC^{9CG} increased to the normal level after incubating RNAP with ATP and GTP, which moved the complex 2 nt downstream and adjusted the length of the hybrid to 9 nt (Figure 4Bd). This result strongly argued that persistence of the RNA:DNA hybrid at a distance more than 7 nt from the 3' end of the transcript was necessary for the formation of a stable EC. Table 1 summarizes the above results and those of similar experiments in which different parameters of the RNA:DNA hybrid were varied. In particular, restoration of the 9 nt hybrid in EC^{9CG} by replacing two 5'-terminal residues in RNA⁹ with the GpC sequence improved the stability of the complex (Table 1, lane 8). This result indicated that the difference in the hybrid length rather than the alteration introduced in the TDS³⁰ sequence was responsible for the decrease in the stability of EC^{9CG}. The failure of RNAP to form a fully stable complex on the double-strand DNA with the completely paired RNA⁹-IMP in which all of the guanines were substituted by inosines revealed that the strength of the RNA:DNA hybrid was also important for switching RNAP to the tight interaction with the template (lane 9). Similarly, EC^{9-DNA} (containing the DNA analog of RNA⁹) had low stability (lane 10), showing that the nucleic acid-binding site in RNAP that contacted the RNA near the 3' end had a much higher affinity to the RNA:DNA hybrid than to the DNA:DNA duplex. The extension of the 9 nt RNA:DNA hybrid to 10 nt by incubation of EC⁹ with GTP did not improve its stability (data not shown), indicating that 9 nt in the hybrid represents the upper limit required for the full stabilization of EC (lane 11).

Role of the Noncoding DNA Strand in the Stability of EC

The significant difference in the high-salt sensitivity of EC⁹ on the single- and double-strand DNA indicated that the noncoding strand brought the additional component, distinct from the RNA:DNA hybrid, that was necessary for the stabilization of EC. To identify which part of the second strand caused this effect, we assembled the upstream and downstream segments of NDS³⁰ separately into EC⁹, using the conditions developed for the full-length oligonucleotide. We monitored the assembly by KMnO₄ treatment and ExoIII footprinting of 5'-labeled TDS³⁰, as described in Figures 3A and 3B. Both NDS^{11dn} and NDS^{15dn} occupied the proper positions in EC⁹, as revealed by the complete protection from permanganate for T₂₃, located in TDS³⁰ downstream from the 3' end of RNA⁹ (Figure 5A, lanes 3 and 4). When the 15 nt oligonucleotide complementary to the upstream part of the template (NDS^{15up}) was added to RNAP alone or in combination with NDS^{15dn}, the upstream part of TDS³⁰ was extensively degraded by ExoIII, confirming the formation of the DNA duplex upstream from the RNA:DNA hybrid in EC⁹ (lanes 9 and 13). NDS^{15dn} and NDS^{11dn} but not NDS^{15up} increased the stability of EC⁹ to the same level as that of the full-length noncoding strand (Figure 5B, lanes 3–6 and 7 and 8, respectively). This result showed that formation of the DNA duplex downstream of the RNA:DNA hybrid was primarily responsible for

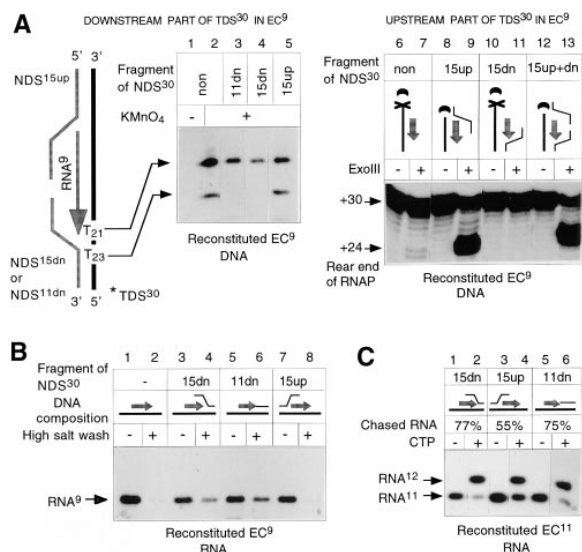


Figure 5. Role of Upstream and Downstream Regions of the Noncoding DNA Strand in the Stability and Activity of EC

(A) Incorporation of the downstream (NDS^{15dn} or NDS^{11dn}) and upstream (NDS^{15up}) regions of the noncoding DNA strand into EC⁹ as detected by KMnO₄ (left) and ExoIII (right) footprinting. The reactive T₂₁ and T₂₃, located next to the 3' end of the RNA, are shown in the scheme.

(B and C) Stability (B) and catalytic activity (C) of EC⁹ containing different segments of NDS³⁰ were determined as described in Experimental Procedures. Bands in lanes 2, 4, 6, and 8 of (B) represent salt-resistant RNA⁹ fractions; upper bands in lanes 2, 4, and 6 of (C) represent chased RNA fractions of the appropriate ECs.

the high stability of EC⁹, which was consistent with the results reported previously (Nudler et al., 1996). The formation of the DNA duplex at the front end of RNAP was sufficient to increase the rate of RNA polymerization in reconstituted EC¹¹ to the level specific for the normal process of elongation (Figure 5C, lanes 1–6). Thus, the placement of the DNA:DNA hybrid next to the 3' end of the RNA was necessary for both the high stability and activity of EC. This result indicates that the core structure of the nucleic acids responsible for all distinct properties of RNAP in transcription elongation, such as the high stability and activity of EC, is composed of the sequential arrangement of two duplexes: the 9 nt RNA:DNA hybrid followed by the DNA duplex.

Discussion

A New In Vitro Method for Promoter-Independent Assembly of EC

We have presented a method for in vitro reconstruction of functionally active EC from the RNAP holoenzyme, a pair of complementary single-strand DNA oligonucleotides, and a short RNA primer (Figure 6). Two principal findings underlie this technique: the ability of RNAP to form a complex with the short RNA primer annealed to the single-strand DNA oligonucleotide (steps 1 and 2) and the ability of this complex to incorporate the complementary DNA strand, causing the formation of the normal transcriptional bubble (step 4, maturation). Although ECs containing only the template DNA strand

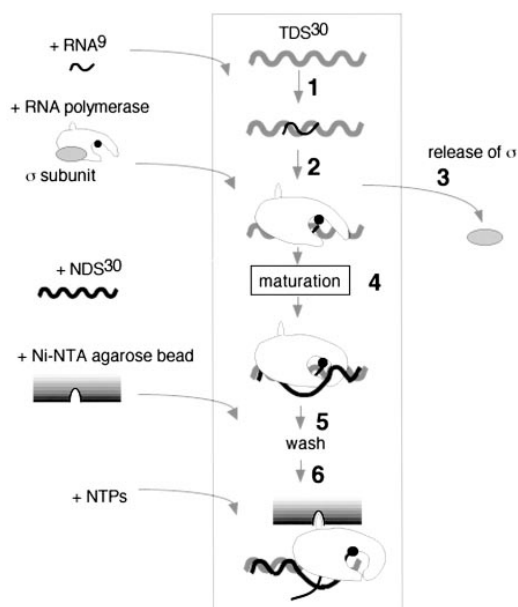


Figure 6. Steps in Promoter-Independent Reconstitution of Authentic EC

Addition of the RNA primer to the template oligonucleotide causes formation of the RNA:DNA duplex in solution (step 1). The holoenzyme of histidine-tagged RNAP, shown by the hand-like shape grabbing the DNA with the jaws and carrying the α subunit in it, forms an unstable complex (step 2) with the hybrid, and the α subunit is released from the enzyme (step 3). The addition of the second DNA strand leads to the formation of normal EC (step 4, maturation). The stabilization is represented as closure of the RNAP jaws around the nucleic acids to emphasize the switch to tight interactions with DNA induced in RNAP by the noncoding strand. The immobilization of the assembled EC on the Ni^{2+} -NTA agarose beads (step 5) is followed by washing the beads with TB. This step represents purification of the assembled complex, which makes it available for the controlled walking along the DNA (step 6).

were relatively unstable, they could be isolated in the low ionic strength conditions in a form fully competent for elongation. Incorporation of the noncoding DNA strand substantially increased the stability and catalytic activity of the complexes to the levels characteristic of normal transcription. Structural analysis of the reconstitution pathway by several footprinting techniques showed that all tested properties of the reconstituted ECs were very similar to those of normal complexes obtained on the same sequence by conventional methods of promoter-initiated transcription (Komissarova and Kashlev, 1997a, 1997b). Using histidine-tagged RNAP bound to Ni^{2+} -NTA agarose beads allowed one-step purification of the reconstituted complexes (step 5) and facilitated their subsequent biochemical analysis.

Reconstitution of the authentic structure of EC without using both catalytic and translocating activities of RNAP makes the present approach an excellent substitute for the conventional methods of *in vitro* analysis of the transcriptional cycle and structural studies of transcription (for a review see Uptain et al., 1997). In combination with the demonstrated ability to perform the assembly in several sequence contexts, followed by movement of the resulting EC away from the original site by the addition of the NTPs (step 6), the technique avoids many

complications associated with the conventional methods of analysis of the transcriptional cycle, such as the low efficiency of initiation and requirement for walking with RNAP a long distance from the promoter to reach a site of interest (Krummel and Chamberlin, 1992; Nudler et al., 1994, 1995; Wang et al., 1995). This technique is also fully applicable to the study of EC architecture by offering a simple way to introduce a wide variety of reporter or cross-linkable groups into different parts of the synthetic RNA and DNA oligonucleotides.

Extended RNA:DNA Hybrid and Processivity of RNAP

Determination of the structure and composition of the RNA required for establishing the tight interaction of RNAP with DNA contributes to the longstanding discussion on the role of the transcript length and the strength of its pairing with the template in the processivity of transcription (Yager and von Hippel, 1987; Chamberlin, 1994; Landick, 1997; Uptain et al., 1997). Here, we demonstrate the requirement of an extended hybrid for formation of the processive EC by measuring the stability of complexes containing two complementary DNA strands and the RNA primers of different lengths, which were completely or partially paired with the template. Our results showed that at least 9 nt of the 3'-proximal RNA, fully hybridized with the DNA, is necessary for the high stability of EC. Shorter RNAs were unable to fully stabilize the complex even when these primers were generated by removing the weakly paired ApU dinucleotide sequence from the 5' end. Shrinking of the hybrid rather than shortening of the RNA mainly contributed to the low stability of EC containing the 6–7 nt RNAs. We based this conclusion on our observation that the EC carrying the 9 nt RNA, in which the number of nucleotides in the hybrid was reduced to 7, was as stable as the complex containing the 7 nt completely paired RNA. Restoration of the original 9 nt hybrid in the mismatched complex by allowing RNAP to elongate the 9 nt primer to 11 nt increased the stability of this complex to the normal level. This result confirmed that the 9 nt RNA:DNA hybrid, rather than the overall length of the RNA per se, was critical for the stabilization of EC.

However, the possibility remained that RNAP containing more than 9–11 nt in the RNA may utilize an additional stabilization mechanism. In that respect, the analysis of the complex containing 20 nt transcript, obtained on a longer template by moving EC⁹ away from the site of assembly, showed that its stability did not improve (I. S., unpublished data). Moreover, the stability of reconstituted EC⁹ was similar to the stability of the majority of promoter-specific ECs containing the 20–100 nt RNAs (Arndt and Chamberlin, 1990). These results argued that holding a longer than 9 nt transcript does not provide an additional strength for the anchoring of RNAP to the DNA.

Since our study is limited by the analysis of EC assembled in a unique template position, it is possible that the requirements for stability may vary from one sequence to another. Moreover, the stabilization mechanism may be different in the complexes obtained by promoter-specific initiation. We addressed both these

issues in a separate study and showed that the long transcripts in a number of ECs obtained from promoter could be truncated to 9–12 nt using the combined treatment with high doses of ribonucleases and greB (N. K. and M. K., unpublished data). The stability of the majority of the truncated ECs carrying 9 nt transcripts was as high as the stability of the reconstituted EC⁹. This fact indicates that the 9 nt rule remains unchanged throughout elongation and suggests that the promoter-specific ECs do not explore an additional stabilization mechanism. For the exception of the inosine-substituted EC⁹, which showed reduced stability, we did not investigate whether the primary sequence of the 9 nt RNA and strength of its pairing to the DNA could affect stability of EC. The experiments addressing this question are currently underway.

All of our observations argue against the earlier proposed model that occupation of a putative single-strand RNA-binding site at a distance of about 3–12 nt, or further upstream from the 3' end of the transcript, improves the processivity of RNAP in elongation (Chamberlin, 1994; Nudler et al., 1997). Rather, our data suggest that all critical interactions between the protein and the transcript in EC occur within a relatively short and very restrictive distance from the growing tip of the RNA. Thus, the minimal RNA-binding site in RNAP is organized to accommodate the stable RNA:DNA hybrid rather than either a single-strand RNA or a combination of both. The significant decrease in the stability of EC after replacement of all guanosines by inosines in the 9 nt hybrid, as well as the inability of the DNA:DNA hybrid with the identical sequence to stabilize EC, indicated that the strength and/or general architecture of the hybrid are also crucial for tight binding to this site in RNAP.

It is well known that RNAP is unable to form ECs at promoters unless the length of the RNA is 9–12 nt (Carpousis and Gralla, 1980; Grachev and Zaychikov, 1980; Hansen and McClure, 1980). In this view, the major rearrangements during the EC assembly and the events that occur during the escape of RNAP from the promoter may have much in common. Both processes are likely to involve formation of the extended RNA:DNA hybrid as a key feature triggering the immediate switch in the enzyme to the processive elongation mode. It is likely that the departure of RNAP from the initiation site is mediated by the appearance of the extended hybrid near the active center of the enzyme, which provides RNAP with a good substrate for strong binding and causes the formation of processive EC.

Distinct Roles for RNA:DNA and DNA:DNA Hybrids in EC

Previous studies have shown that the noncoding DNA strand forming a DNA duplex downstream of the 3' end of the transcript plays a major role in the high stability of EC. This duplex was proposed to promote the formation of a sliding clamp in the protein, which wraps around the DNA and topologically links RNAP to the template (Komissarova and Kashlev, 1997a; Landick, 1997; Nudler et al., 1997). As we showed here, for this stabilization to occur the presence of the extended RNA:DNA hybrid upstream from the DNA duplex is required. This finding

raises the question of whether the mechanisms by which these two spatially arranged duplexes stabilize EC are related. As we demonstrated, even the 6–7 nt RNA:DNA duplex, formed on the single-strand DNA, provided enough information about both the specific binding to RNAP and the correct template-dependent priming for the RNA synthesis. Although the front-end DNA duplex made this preexisting interaction stronger, this result argues that the most important contacts in RNAP are centered on the RNA:DNA hybrid rather than further downstream. The DNA duplex at the front end of the complex may improve the EC stability either directly, by providing the surface for clamping the DNA by the protein, as suggested earlier, or indirectly, by increasing the enzyme's affinity to the RNA:DNA hybrid where the actual clamping may take place. This view is supported by our preliminary data showing that the 3'-proximal RNA of the hybrid in the assembled EC is more accessible to the ribonucleases if the front-end DNA duplex is not established in the complex (M. K., unpublished data).

Experimental Procedures

DNA and RNA Oligonucleotides and Transcription Reaction

The sequences of synthetic RNAs and DNA oligonucleotides are shown in Figure 1 and Table 1. The other DNA sequences (5'→3') are listed below. TDS⁴¹ was identical to TDS³⁰ but carried an additional 11 nt sequence (TGGGATGGCTA) at the 5' end.

TDS^{40-Biotin} was the same as TDS³⁰ except it carried an additional 10 nt sequence (ATCCTATAGG) and the biotin group at the 3' end. NDS^{30GC} was identical to NDS³⁰ except the GC sequence was introduced in the corresponding positions to restore its complementarity to TDS^{30GC}. NDS⁴¹ was the same as NDS³⁰ except it carried an additional 11 nt sequence (TAGCCATCCCA) at the 3' end. NDS^{11dn}: GACA CGGCGAA. NDS^{15dn}: GAGGGACACGGCGAA. NDS^{15up}: ACTTACAGC CGCATA. All oligonucleotides were obtained from Oligos Etc., Inc. (Wilsonville, OR).

Formation of ECs on Synthetic Templates

The standard single reaction (SR), in which the properties of ECs were subsequently tested, contained 10 μ l of ECs in transcription buffer (TB: 20 mM Tris-HCl [pH 7.9], 40 mM KCl, and 5 mM MgCl₂) immobilized on Ni²⁺-NTA agarose beads (Qiagen). ECs for an SR were obtained in a reaction combining 3 μ l of each of the following components dissolved in TB: the RNA oligonucleotide (1.33 μ M), coding DNA strand (0.67 μ M), noncoding DNA strand or its fragments (13.3 μ M), and 0.2 μ l of RNAP (4 μ M). The order of the assembly reaction was as follows. (1) The RNA oligonucleotide was mixed with the coding strand, incubated for 5 min at 45°C, and cooled to room temperature in 2°C/2 min decrements. (2) His-tagged RNAP was combined with the annealed oligonucleotide and allowed to bind for 10 min at 24°C. (3) The noncoding strand was incorporated into EC for 10 min at 37°C. For reactions containing EC on single-strand DNA, an equal volume of TB was added instead. (4) The sample was transferred to a tube containing the Ni²⁺-NTA agarose pellet prewashed with TB, mixed immediately, and agitated for 5 min to bind EC to the solid phase. (5) The Ni²⁺-NTA agarose pellet was then washed four times with 1 ml of ice-cold TB, and the final reaction volume was adjusted to 10 μ l with TB. To obtain enough material for 5–20 SRs, the volumes of individual components in the EC assembly were scaled up proportionately. RNA oligonucleotides were labeled at the 5' end before EC assembly by T4 polynucleotide kinase (New England Biolabs) in a reaction containing 1 μ M of [γ -³²P]ATP (7000 Ci/mmol, ICN Biomedicals). For the footprinting experiments, T4 polynucleotide kinase was used to label 5' ends of the DNA oligonucleotides before assembly. The assembled complexes were walked in the presence of 50 μ M of appropriate NTPs for 5 min at 24°C and then washed with TB.

Catalytic Activity of RNAP and Test for Stability of ECs

For quantitative analysis of RNAP catalytic activity in the experiments of Figures 1C and 5C, at least a 10-fold excess of CTP concentration over the concentration of immobilized ECs was used while measuring the kinetics of RNA extension from 10 to 11 nt. For this purpose, in each SR the EC was diluted 80 times, the volume was adjusted to 10 μ l with TB, and 0.01 μ M CTP was added for 5 min at 24°C. The reaction was stopped by adding 10 μ l of 2 \times gel loading buffer (7 M urea, 100 mM EDTA, 0.025% bromphenol blue, and xylene cyanol). To compare the catalytic activity of the assembled and normal ECs containing the same RNA sequence, the normal EC^{11P} with 5'-labeled RNA was obtained using initiation of transcription from the T₇A1 promoter as described elsewhere (Komissarova and Kashlev, 1997a). EC^{11P} was elongated with 0.01 μ M CTP for 5 min at 24°C. In this and further experiments, the nucleic acid products were analyzed on 20% denaturing urea polyacrylamide gels. To obtain quantitative results, the gels were scanned with a phosphorimager (Molecular Dynamics).

In other experiments, the ECs were incubated with 50 μ M of appropriate NTPs for 5 min at 24°C to test the catalytic activity.

To examine the stability of ECs, the Ni²⁺-NTA agarose pellets containing bound ECs at various steps of assembly were incubated for 20 min in TB containing 1 M KCl at 37°C. Then, the pellets were washed three times with 1 ml of ice-cold TB and loaded on PAGE either directly or after incubation with 50 μ M of appropriate NTPs for 5 min at 24°C.

Potassium Permanganate and ExoIII Footprinting

For each SR, the immobilized complexes were treated with 1 μ l of 6 mM KMnO₄ for 5 min at 16°C. The reaction was stopped by adding 1 μ l of β -mercaptoethanol. The complexes were eluted from Ni²⁺-NTA agarose by 200 μ l of elution buffer (50 mM EDTA, 0.3 M NaOAc, and 25 μ g/ml yeast tRNA) for 30 min at 24°C. DNA in the supernatants was precipitated by ethanol and cleaved with 10% piperidine as described elsewhere (Komissarova and Kashlev, 1997a). In ExoIII footprinting experiments, ECs formed on the template strand were immobilized on Ni²⁺-NTA agarose before incorporation of the non-template strand, the concentration of which was 2.5 times higher than in the standard reconstitution conditions; the incubation was carried out at 24°C for 30 min. For each SR, 10 μ l of ECs was incubated with 100 U of ExoIII (New England Biolabs) for 10 min at 24°C. The reaction was stopped by adding 10 μ l of 2 \times gel loading buffer.

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