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The 8-Nucleotide-long RNA:DNA Hybrid Is a Primary Stability Determinant of the RNA Polymerase II Elongation Complex*

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The sliding clamp model of transcription processivity, based on extensive studies of *Escherichia coli* RNA polymerase, suggests that formation of a stable elongation complex requires two distinct nucleic acid components: an 8–9-nt transcript-template hybrid, and a DNA duplex immediately downstream from the hybrid. Here, we address the minimal composition of the processive elongation complex in the eukaryotes by developing a method for promoter-independent assembly of functional elongation complex of *S. cerevisiae* RNA polymerase II from synthetic DNA and RNA oligonucleotides. We show that only one of the nucleic acid components, the 8-nt RNA:DNA hybrid, is necessary for the formation of a stable elongation complex with RNA polymerase II. The double-strand DNA upstream and downstream of the hybrid does not affect stability of the elongation complex. This finding reveals a significant difference in processivity determinants of RNA polymerase II and *E. coli* RNA polymerase. In addition, using the imperfect RNA:DNA hybrid disturbed by the mismatches in the RNA, we show that nontemplate DNA strand may reduce the elongation complex stability via the reduction of the RNA:DNA hybrid length. The structure of a "minimal stable" elongation complex suggests a key role of the RNA:DNA hybrid in RNA polymerase II processivity.

Characterization of the processivity determinants in yeast RNA polymerase II (Pol II)[1] is crucial for understanding the mechanisms controlling eukaryotic gene expression at the levels of promoter escape, pausing, arrest, and release of the RNA from transcription terminators (1, 2). The polymerase proceeds through the nucleosomal structure of the template and survives a prolonged pausing or arrest in the genes without dissociating from the template. Therefore, the formation of a highly stable elongation complex (EC), in which RNA polymerase is tightly bound to the nascent transcript and template, is absolutely required for the enzyme processivity (3). The mechanism that reconciles the strong stable binding of Pol II to the DNA with the high speed of forward translocation is unknown.

Elongation of a promoter-initiated transcript occurs in the absence of general initiation factors, which dissociate from the enzyme during promoter escape (4). Processive elongation by eukaryotic Pol II can also be achieved using purified polymerase in a promoter- and factor-independent transcription system (5). Therefore, it is likely that the basic processivity function belongs to the core Pol II enzyme. Although it was shown that substantial changes in the nucleic acid array accompany the switch to a processive RNA synthesis (6), the role of DNA and RNA in the Pol II EC stability remains speculative (7). The elucidation of this role has been hampered by the extreme complexity of the native eukaryotic EC, which contains multiple transcription elongation factors (8). Differentiation between the effect of elongation factors on EC stability and activity and the role of the Pol II core enzyme interaction with RNA and DNA requires a simple, "minimal" *in vitro* system. Here, we develop a novel technique for obtaining Pol II ECs, which bypasses the need for protein factors or for introduction of mismatches in DNA to promote transcription initiation. This approach involves a direct assembly of intermediates in the elongation pathway using purified hexahistidine-tagged core Pol II enzyme and synthetic RNA and DNA oligonucleotides. We demonstrate that the resulting complex has structural and functional properties that resemble those of the promoter-initiated ECs in all structural parameters that we have tested. The method allows us to assess the impact of nucleic acids components by introducing various changes to any site of the EC nucleic acid array through the sequence, length, and pairing affinity, which cannot be done using a promoter-initiated system or "artificial transcription bubble templates" (9, 10).

Below, we apply the assembly approach for the analyses of the minimal nucleic acid composition required for formation of a stable EC by the Pol II core enzyme. The interactions of two DNA strands and RNA within the region of the transcription bubble and their role in the maintenance of a stable EC were analyzed in complexes assembled with synthetic RNAs and nontemplate DNA strands fully or partially complementary to the template DNA.

EXPERIMENTAL PROCEDURES

Yeast Strains and Transformation—Protease-deficient strain BJ5464 (11) was cultured on YPD plates at 30 °C. The *rpb3* ts strain Z251 (a gift from Dr. R. Young) was cultured on YPD plates at 25 °C. Plasmid DNA was transformed into yeast cells by electroporation (12).

DNA and RNA Oligonucleotides—All oligos were obtained from Oligos Etc., Inc. (Wilsonville, OR). Sequences of oligonucleotides used in transcription reactions are summarized in Fig. 1*B*. Both RNA and DNA oligonucleotides were labeled at the 5′-end by phosphorylation with T4 polynucleotide kinase (New England Biolabs) in the presence of 1.5 μM [γ-^{32}P]ATP (7000 mCi/mmol, ICN Biomedicals). RNA primers were annealed to DNA as described (13).

Rpb3 Subcloning and Introduction of the Affinity Tag Sequence—The *Rpb3* promoter region and open reading frame were amplified by polymerase chain reaction from BJ5464 genomic DNA and subcloned into the NUVEC16 shuttle vector (a gift from Dr. J. Strathern). Double-strand oligonucleotides encoding tag sequence were subsequently in-

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[1] The abbreviations used are: Pol II, RNA polymerase II; EC, elongation complex; nt, nucleotide(s); TB, transcription buffer; NDS, nontemplate DNA strand; TDS, template DNA strand.

serted between the promoter and open reading frame regions. The sequences of primers and tags are available upon request. Integration plasmid was constructed on the basis of the YIp5 vector. The resulting plasmid was linearized with *Hpa*I in the middle of the *Rpb3* coding region for targeting the integration into the *Rpb3* locus of the genome.

Protein Purification—Glutathione *S*-transferase-BirA fusion protein expression was induced in strain C14-pDW364 by isopropyl-1-thio-β-D-galactopyranoside, and glutathione *S*-transferase-BirA was purified by affinity chromatography (14). Wild-type Pol II purification from strain BJ5464 grown to stationary phase in YPD was done according to Ref. 15.

Pol II with the affinity tag was purified from a BJ5464 derivative carrying a stable insertion of tagged *Rpb3* gene. All purification procedures were done at 4 °C. All buffers contained protease inhibitors (15). The yeast culture (6 liters) was grown to saturation in YPD; cells (110 g) were harvested and resuspended in 50 ml of 3× lysis buffer (1× lysis buffer: 150 mM Tris acetate (pH 7.9), 50 mM potassium acetate, 1 mM EDTA, 2 mM dithiothreitol, 20% glycerol). BirA and biotin were added to 10 μg/ml and 0.1 mM, respectively, and cells were disrupted in a BeadBeater apparatus (BioSpec Products). The cell lysate was prepared as described (15). The diluted lysate was incubated for 1.5 h with 100 ml of Bio-Rex 70 (Bio-Rad) resin, equilibrated with Buffer A(100) (Buffer A: 20 mM HEPES (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, and 10% glycerol; the number in parentheses indicates the potassium acetate concentration in mM). The resin was washed with 500 ml of Buffer A(100) and then with 500 ml of Buffer A(300); the Pol II-containing fraction was eluted with 400 ml of Buffer A(700). The eluate was adjusted to 500 mM potassium acetate with Buffer A(0) and loaded at 0.5 ml/min onto a 3-ml column packed with SoftLink Soft Release monomeric avidin resin (Promega) equilibrated with Buffer A(500). The column was washed with 30 ml of Buffer A(500), and the bound proteins were eluted with 10 ml of Buffer A(500) containing 5 mM biotin. The eluate was diluted 5-fold with Buffer B(0) (Buffer B: 20 mM Tris acetate (pH 7.9), 1 mM EDTA; the number in parentheses indicates the potassium acetate concentration in mM) and loaded at 0.5 ml/min onto a MonoQ HR5/5 column equilibrated with Buffer B(100). A 15 ml of a linear gradient of potassium acetate from 100 to 2000 mM in Buffer B was applied to the column. Fractions of 0.3 ml were collected, and Pol II-containing fractions were pooled. The enzyme was stored either at 4 °C immobilized on Ni^{2+}-NTA agarose (Qiagen) or at −20 °C in Buffer B(100) containing 50% glycerol.

Electrophoresis of Proteins and Western Blot Analysis—Proteins were resolved on precast 8–16% gradient Tris-glycine polyacrylamide gels (Novex) and detected by silver staining with a Silver Stain Plus kit (Bio-Rad). Western blot analysis was performed by a semidry electrophoretic transfer of proteins as described (12), and the membrane was probed with horseradish peroxidase-streptavidin conjugate (Amersham Pharmacia Biotech) with a subsequent detection using ECL reagent (Amersham Pharmacia Biotech).

Promoter-independent Initiation and Assembly of ECs on Synthetic Templates—All transcription reactions were performed in low salt transcription buffer (TB) (20 mM Tris-HCl (pH 7.9), 40 mM KCl, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol) at 25 °C. Transcription on a dC-tailed template (TDS46-NDS31 duplex) was initiated by a 5-min preincubation of polymerase with the template (1.33 μM), followed by the addition of labeled ApUpC (2 μM) and 50 μM each of ATP and GTP, and it was allowed to proceed for 10 min.

ECs were assembled according to the protocol developed for bacterial RNA polymerase (13). Pol II (approximately 0.01 μM) was incubated with 0.133 μM RNA:DNA hybrid for 10 min, followed, where indicated, by the addition of 2.6 μM nontemplate DNA and incubation at 37 °C for 10 min. ECs were immobilized on Ni^{2+}-NTA agarose (16) and washed four times with 1 ml of ice-cold TB. Where specified, the high salt wash was performed: the EC was incubated for 15 min in 1 ml of TB with 1 M KCl and then washed twice with 1 ml of TB. The transcription and subsequent EC wash were done in TB with 40 mM KCl, which allowed the formation of ECs, which are unstable under high salt conditions. The concentration of the NTPs used for transcript elongation was 10 μM, unless indicated otherwise. Where indicated, transcripts were labeled by incorporation of 40 μCi of [α-^{32}P]NTP (3000 mCi/mmol; NEN Life Science Products). Analysis of the DNA and RNA content of ECs was performed by denaturing electrophoresis in 20% polyacrylamide gels.

ExoIII, Potassium Permanganate, and RNase Footprinting—ExoIII footprinting was performed on ECs containing labeled DNA (template or nontemplate strand) that were immobilized and isolated as described above. ExoIII (100 units, New England Biolabs) was added to the 10-μl reaction, and the digestion was allowed to proceed for 5 min.

$KMnO_4$ footprinting of immobilized ECs was performed as described previously (17). When EC20 was analyzed, RNA9 was extended to 20 nt by incubation of the initial complex with 500 μM each of ATP, GTP, and CTP for 10 s or 10 min before the addition of 10 mM $KMnO_4$. The reactions were stopped after 5 s by the addition of β-mercaptoethanol. The modified DNA was cleaved with piperidine at 90 °C for 15 min.

RNase T1 (Roche Molecular Biochemicals) treatment was performed for 10 min with 300–1000 units/ml of RNase. ECs were washed with 1 ml of TB to remove excess ribonuclease. The reactions were stopped by the addition of phenol (18).

Tests for EC Stability and Catalytic Activity—For quantitation of stability the ECs were assembled and purified from the excess oligonucleotides by TB wash. Then, equal amounts of each EC were incubated in TB or 1 M KCl TB for 15 min and washed with low salt TB. One-half of the washed EC was incubated with ATP and GTP to demonstrate that RNA in the EC is transcriptionally engaged. The samples were loaded onto gels, and the gels were scanned with a phosphorimager (Molecular Dynamics). The stability was calculated as the ratio of the transcriptionally active EC remained in the high salt-washed sample to the transcriptionally active EC in the low salt-washed sample.

The catalytic activity assay was done by isolation of EC and transcript extension with a 1 μM concentration of the next NTP. Catalytic activity was calculated as the ratio of the extended transcript quantity to the total transcript quantity, as determined by phosphorimager scanning.

RESULTS

Affinity Purification of His-tagged Biotinylated Pol II—The affinity purification of Pol II was achieved by adding a combinatorial affinity tag to the amino terminus of the Pol II Rpb3 subunit. The amino acid sequence of the tag (MGSHHHHHH-SNSGLNDIFEAQ**K**IEWHEDTGSSE; the last Glu residue corresponds to the fourth amino acid residue of wild-type Rpb3) includes a hexahistidine stretch (underlined) and a biotin acceptor peptide (the biotinylated lysine is shown in boldface) for the *Escherichia coli* biotin holoenzyme synthetase (14). A low copy number plasmid, carrying the tagged gene under the control of its own promoter, completely rescued the *rpb3* temperature-sensitive phenotype of strain Z251 (19) (data not shown). Tagged Pol II was biotinylated *in vitro* and purified in three chromatographic steps (Fig. 1*A*). The electrophoretic mobility of the Rpb3 subunit with its affinity tag was decreased compared with that of the wild-type Rpb3 (Fig. 1*A, lanes 5* and *6*). The Rpb3 subunit of the purified Pol II was specifically recognized by horseradish peroxidase-streptavidin conjugate (*lanes 7* and *8*), and because of the His tag, it retained the ability to bind to Ni^{2+}-NTA agarose beads (see below).

The comparison of wild-type and tagged Pol II activities was made using the standard transcription elongation assay on an oligo-dC-tailed template (5). The double-strand oligonucleotide with a protruding 3′ single-strand oligo-dC region, made of template and nontemplate DNA strands (TDS46 and NDS31, respectively; numerical index indicates the length of the oligonucleotide; for the sequences, see Fig. 1*B, scheme I*), was preincubated with Pol II. Transcription was initiated with a cognate labeled trinucleotide ApUpC. The substrates CTP and UTP were omitted from the transcription reaction to obtain a defined 11-nt RNA. We found that the wild-type and tagged polymerases transcribed the template with equal efficiency (Fig. 1*C,* compare *lanes 1* and *2* with *lanes 3* and *4*). Both Pol II enzymes showed activity comparable to that of bacterial RNA polymerase on the same template (Fig. 1*C, lanes 5* and *6*).

Transcription of the oligo-dC-tailed templates often results in the formation of ECs with an undisplaced transcript (20), making this method unsuitable for the analysis of nucleic acid architecture of EC. Therefore, we developed an alternative method of obtaining of Pol II EC in the absence of promoter sequence and transcription factors. It was shown (13) that bacterial RNA polymerase binds the RNA annealed to single-strand template DNA, and the nontemplate DNA strand sub-

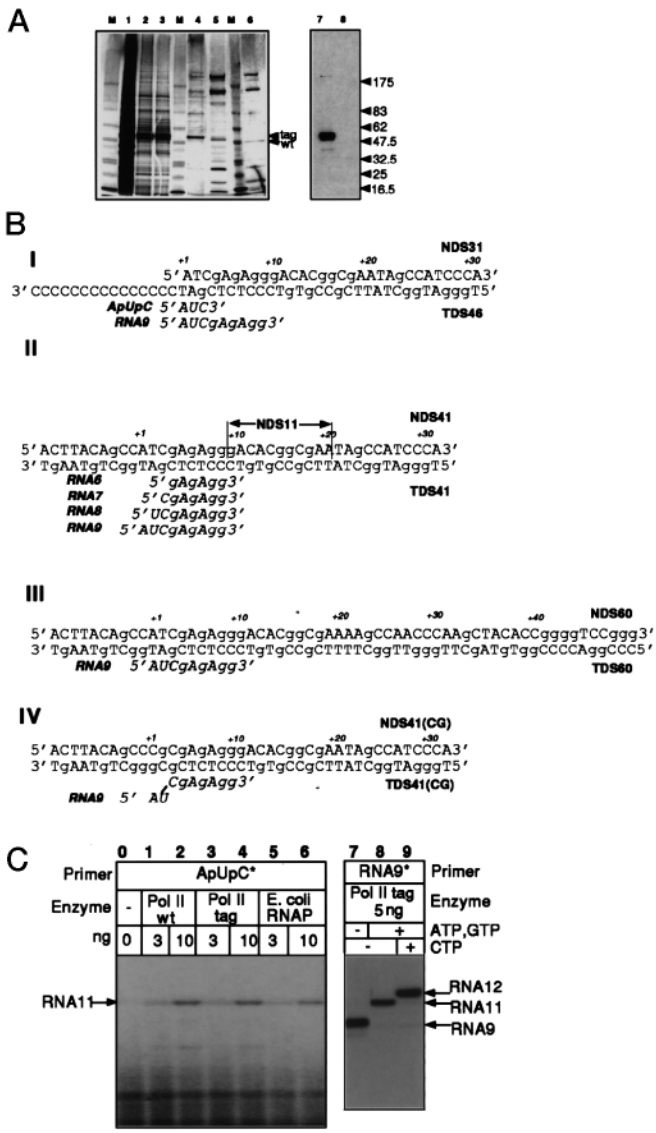

FIG. 1. **Affinity purification and activity testing of Pol II with tagged biotinylated Rpb3 subunit.** *A,* consecutive purification steps of Pol II. The gel was loaded with 10 μl of each of crude cell lysate (*lane 1*), Bio-Rex 70 eluate before (*lane 2*) and after (*lane 3*) passing through avidin column, avidin column eluate (*lane 4*), combined fractions 32–34 of Mono Q column eluate (*lane 5*), and wild-type Pol II (*lane 6*). *Lanes 7* and *8* show an immunoblot of Pol II with tagged biotinylated Rpb3 subunit (*lane 7*) and of wild-type Pol II (*lane 8*) probed with horseradish peroxidase-conjugated streptavidin. Lanes marked *M* represent prestained molecular weight markers. *Arrows* indicate the tagged (*tag*) and wild-type (*wt*) Rpb3 subunits. The molecular mass of markers in kDa is indicated on the *right*. *B,* templates and primers utilized in transcription assays in this work. All sequences are aligned to show complementary pairing. RNA sequences are *italicized*. The numerical indexes indicate the length of the oligonucleotides. Coordinates are indicated above the NDS, with +1 corresponding to the 5′-end of primer RNA9. *C,* tag insertion does not affect Pol II activity. Wild-type Pol II (*lanes 1* and *2*), tagged Pol II (*lanes 3* and *4*), and *E. coli* RNA polymerase (*lanes 5* and *6*) were used to synthesize an 11-nt RNA on a dC-tailed template. *Lane 0* is a negative control without added polymerase. In a parallel experiment (*lanes 7–9*), the EC was assembled on TDS46 with RNA9, followed by the addition of NDS31. The transcription activity of EC9 (*lane 7*) is shown by a sequence-specific extension of RNA (*lanes 8* and *9*). *Asterisks* indicate labeled primers.

sequently enters this complex, completing the formation of a normal transcription bubble. We extended this approach to assemble the Pol II EC on the same TDS46-NDS31 DNA array, which was used for activity testing. The synthetic RNA9 oligonucleotide corresponding to the first 9 nt of the transcribed sequence of the TDS46 (Fig. 1*B, scheme I*) served as a primer in this experiment. His-tagged Pol II was added to TDS46 with a prehybridized labeled RNA9 primer to obtain a Pol II-RNA-DNA complex in solution. This initial complex was then incubated with the complementary nontemplate DNA oligonucleotide (NDS31). EC9 (the numerical index indicates the length of the RNA in the EC), containing the two DNA strands and the RNA, was purified from the excess oligonucleotides by adsorption of the polymerase to Ni^{2+}-NTA agarose beads (Fig. 1*C, lane 7*). Fig. 1*C, lanes 8* and *9,* illustrates the "walking" of the assembled EC9 along the double strand DNA in the presence of subsets of NTPs (16, 21) to form EC11 and EC12. The elongation was quantitative and template specific, because the addition of an inappropriate NTP did not allow the extension of the RNA (data not shown). Thus, the elongation complex assembly with yeast Pol II was successful, and in EC9, the 3′-end of the RNA was located in the active center of the enzyme and in the correct register with the template DNA strand. Because the assembly of EC and the oligo-dC-dependent initiation were done on the same DNA, it can be concluded that the assembly is significantly more efficient than the initiation on the oligo-dC-tailed template (Fig. 1*C,* compare *lanes 4* and *8*).

The efficiency of the assembly was about 10–30% of the core enzyme in the reaction containing 0.04 μM Pol II and 0.013 μM RNA:DNA hybrid, but the yield of the complex dropped to less than 1% at an enzyme concentration below 0.005–0.01 μM (data not shown).

Structural Analyses of Assembled EC—Although functional, the structure of the EC assembled on the dC-tailed template relative to the natural EC was incomplete, because part of the NDS responsible for the formation of the DNA duplex at the upstream edge of Pol II was missing. Next, we assembled the EC by using the same RNA9 primer with two fully complementary 41-nt DNA oligonucleotides (TDS41-NDS41) (see Fig. 1*B, scheme II*). To determine whether the nucleic acid architecture of the assembled EC was characteristic of the ECs originating from the promoter, we analyzed in detail the structure of the assembled complex. The retention of the labeled NDS41 in EC9 after incubation of the EC in 1 M KCl and washing off the dissociated components (Fig. 2*A, lane 1*) showed that the NDS was incorporated into the complex. In the complex, the double strand DNA-specific exonuclease III (ExoIII) digested the parts of both NDS41 and TDS41 that were not covered by Pol II (Fig. 2*A, lanes 2* and *9*). This revealed a successful annealing of the two DNA strands beyond both edges of the enzyme. The rear end and front end ExoIII footprints of Pol II in EC9 were similar to those observed in the promoter-specific ECs (22). Therefore, the assembled EC9 has the proper alignment of the polymerase mainframe on the double strand DNA. The incubation of EC9 with ATP and GTP, which led to a 2-base extension of the 9-nt RNA, caused the appropriate shift of the ExoIII footprint, demonstrating the translocation of the assembled complex along the template (Fig. 2*A, lanes 3* and *8*).

The normal structure of the transcription bubble in EC9 was confirmed by $KMnO_4$ footprinting, which revealed the single-strand thymidines in the NDS. Fig. 2*B* shows that the T_2 thymidine residue (the numerical index indicates the distance of the residue from the 5′-end of RNA9 primer) was sensitive to the modification, whereas T_{21}, located 10 nt downstream from the 3′-end of the primer, was resistant (*lane 1*). This result suggested that two DNA strands were unpaired in the area containing RNA9 and paired at the leading edge of the complex. Fig. 2*B, lane 2,* demonstrates the advancement of the bubble immediately after incubation of EC9 with ATP, GTP, and CTP, producing a 20-nt RNA. In the newly obtained EC20, the sensitivity of the T_2 site to permanganate was now significantly

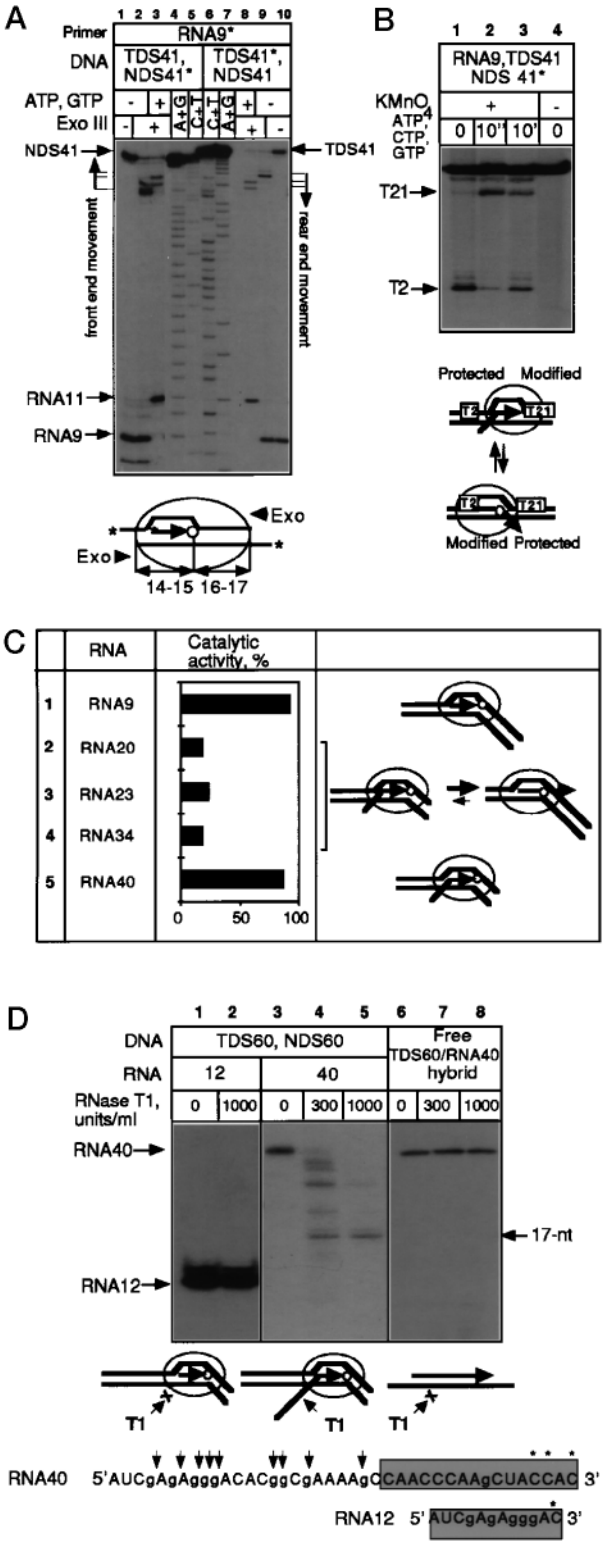

FIG. 2. **Structural analysis of Pol II ECs obtained by promoter-independent assembly.** *A,* detection of the enzyme position on the DNA in EC9 and EC11 by ExoIII footprinting. ECs were assembled on TDS41 with labeled RNA9, followed by the addition of NDS41. EC11 was derived from EC9. The front end footprint of the enzyme was detected with 5′-labeled NDS41 (*lanes 1–5*); the rear end footprint was detected with 5′-labeled TDS41 (*lanes 6–10*). The positions of ExoIII footprints were identified using A+G (*lanes 4* and *7*) and C+T (*lanes 5* and *6*) sequence markers. Distances between the 3′-end of the transcript and borders of the enzyme are shown in the *scheme*. DNA strands are depicted as *solid lines*; RNA is shown by a *gray arrow* directed toward the 3′-end of the transcript. The *asterisks* indicate the positions of labeling. Pol II is shown as an *oval,* with the *dot* indicating the active center. *B,* transcription bubble movement demonstrated by $KMnO_4$ footprinting of the NDS. EC was assembled on TDS41 and RNA9 followed by the addition of labeled NDS41. EC20 was obtained by a 10-s (*lane 2*) or 10-min (*lane 3*) chase with ATP, CTP, and GTP. *Arrows* indicate the intact NDS41 and its 11-nt (*T2*) and 30-nt (*T21*) cleavage products. The *scheme* illustrates the equilibrium between the two positions of transcription bubble. *C,* catalytic activity of Pol II ECs. ECs were assembled on TDS41 (*lanes 1* and *2*) with labeled RNA9 or on TDS60 (*lanes 3–5*) with unlabeled RNA9 (*lanes 3* and *5*) or labeled RNA9 (*line 4*) with the subsequent addition of a corresponding NDS. RNA20 and RNA34 were obtained from RNA9 in one step. RNA23 was obtained by primer elongation in four steps and labeled by incorporation of [α-^{32}P]GTP to position 23 in the presence of 50 μM ATP. RNA40 was obtained in two steps and labeled in positions 37, 38, and 40 with [α-^{32}P]CTP in the presence of 50 μM ATP and UTP. The catalytic activity was determined after a 5-min chase with 1 μM each of ATP and GTP (EC9), CTP (EC23), ATP and UTP (EC34), or GTP (EC40). The proposed shifts in the equilibrium between the two extreme positions of the Pol II active center in the RNA range of 20–34 nt, as detected by a change in the catalytic activity, are shown in the *scheme*. *D,* protection of the transcript from RNase T1 in EC12 and EC40. ECs were assembled on TDS60 and RNA9 primer followed by the addition of NDS60. EC12 (*lanes 1* and *2*) and EC40 (*lanes 3–5*) were obtained in two steps. Free RNA40-TDS60 hybrid (*lanes 6–8*) is a product of dissociation of EC40 obtained on a single-strand template. The RNA sequences and positions of cleavage sites are shown in the *scheme*. The *asterisk* indicates the labeled nucleotide. The *shaded box* denotes the region of the RNA that was protected from RNase digestion in the EC.

reduced, whereas the downstream region containing T_{21} became sensitive. Thus, successful reannealing of the two DNA strands occurred at the rear end of the transcribing Pol II, indicating that the normal architecture of nucleic acids was preserved during translocation of Pol II along the DNA duplex.

It has recently been reported that Pol II ECs halted in the vicinity of promoter have a tendency to fall into an arrested state by sliding backwards to the 5′-end of the 15–35-nt RNA, whereas the irreversible escape from the promoter occurred at a length of approximately 40 nt of the RNA (23). To test whether the arrest occurs in the assembled complex, we performed $KMnO_4$ footprinting of the transcription bubble in EC20 after a 10-min delay to provide sufficient time for the development of the arrested state. In this case, the T_2 signal strengthened, whereas the T_{21} signal weakened (Fig. 2*B, lane 3*), which indicated that the transcription bubble in EC20 moved backward. The elongation arrest was also observed in the catalytic activity test performed with the ECs obtained on a longer DNA (TDS60-NDS60; see Fig. 1*B, scheme III*). The complexes containing 20–34-nt RNA have a low catalytic activity compared with EC9 (Fig. 2*C, lines 2–4*). Importantly, upon reaching the 40-nt RNA length, the Pol II activity increased to the normal level specific for the processive elongation (Fig. 2*C, line 5*).

To test whether the normal RNA displacement pathway was established in the assembled EC, the single strand RNA-specific ribonuclease T1 was used. *Lanes 1* and *2* of Fig. 2*D* show that the transcript in EC12 was completely protected from RNase T1. At the same time, the 5′-terminal 23 nt of the transcript in EC40 were cleaved off (Fig. 2*D, lanes 3–5*), which demonstrated that this region was extruded from the protein and displaced from the hybrid with the template. In the control experiment, the polymerase-free RNA40:TDS60 heteroduplex was completely resistant to RNase T1 (Fig. 2*D, lanes 6–8*). The 3′-proximal part of the truncated RNA remained in the complex after washing and could be extended to the longer products (data not shown). This protection pattern is consistent with the 17–20-nt protected zone in the promoter-specific EC (24). Thus, the assembly results in formation of a functional EC, closely resembling the natural EC in all parameters that we tested.

Stability Determinants of Pol II EC—We analyzed stability determinants of Pol II EC using the treatment with KCl at a high concentration, which is commonly used to detect whether

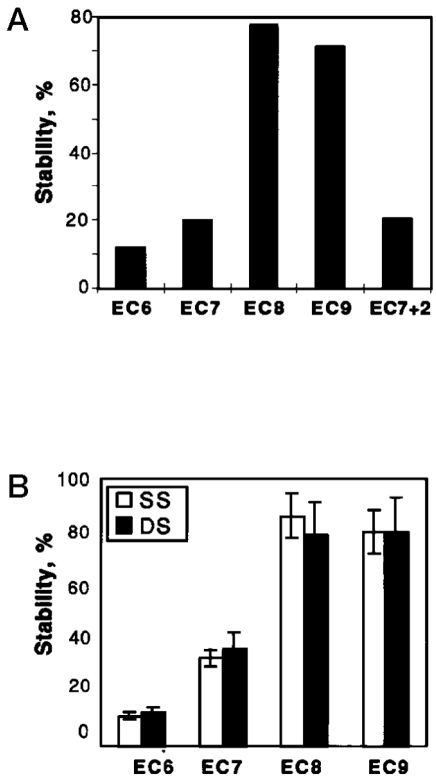

FIG. 3. **Stability determinants of Pol II EC.** *A,* formation of an 8-nt RNA:DNA hybrid is required for EC stabilization. ECs containing a 6-, 7-, 8-, or 9-nt RNA primer were assembled on TDS41 or TDS41(CG) in the presence of NDS41 or NDS41(CG), respectively. EC stability was assayed by determining the percentage of the transcriptionally active EC resistant to 15 min of incubation with 1 M KCl as described under "Experimental Procedures." Data shown are representative of three independent experiments. *B,* NDS does not affect the stability of ECs with 6–9-nt RNA. ECs containing a 6-, 7-, 8-, or 9-nt RNA were assembled on TDS41 in the absence or presence of NDS41 (*SS* and *DS,* respectively). EC stability was tested as described for *A*. Each *bar* represents the mean of three independent experiments; the *error bars* show the S.D.

the switch from ionic to strong hydrophobic interactions has occurred in the enzyme. The resistance of EC to high salt treatment indicates that the complex has escaped the initiation mode and has acquired a conformation necessary for processive transcription (25, 26).

First, to determine the properties of RNA essential for EC stability, the length of the RNA oligonucleotide used for assembly was varied in the range from 6 to 9 nt (see Fig. 1*B, scheme II*). We tested the stability of these ECs by a high salt incubation in the same manner as it was done for the assembled *E. coli* EC (13). EC6 and EC7 were sensitive to a high salt treatment, whereas EC8 and EC9 were resistant (Fig. 3*A*). The same result was obtained in a separate experiment with a set of RNA primers of unrelated to RNA9 sequence (data not shown). These data identified 8 nt as the minimal transcript length that is sufficient for the stabilization of Pol II EC, consistent with the previously reported cessation of the abortive initiation in Pol II ECs (6, 9).

Next, the role of the extent of transcript-template pairing in EC stability was analyzed. We assembled a complex (EC7+2) containing the same 9-nt RNA and modified DNA oligonucleotides with two bases noncomplementary to the 5′-end of the RNA (TDS41(CG) and NDS41(CG); see Fig. 1*B, scheme IV*). The stability of this complex was the same as that of the complex containing 7-nt RNA (EC7) completely paired to the DNA and was significantly less than the stability of EC9 on TDS41 (Fig. 3*A*). This result indicates that the complementary pairing between the transcript and template persists in the Pol II EC at a distance of at least 8–9 nt from the 3′-end of the transcript, and it is the hybrid that is required for the stability function.

Apart from RNA:DNA hybrid, the second element of the nucleic acid array that is crucial for the stabilization of bacterial EC is a double strand DNA downstream from the hybrid (13, 27, 28). The current "sliding clamp" model positions the hydrophobic channel observed in low-resolution crystals of RNA polymerase II over the downstream template region (29). Surprisingly, Fig. 3*B* shows that the stability of the ECs of Pol II assembled with 6–9-nt RNAs and template DNA did not change after incorporation of the nontemplate DNA strand. The EC stability in this case was determined solely by the RNA:DNA hybrid length. Moreover, truncation of the downstream part of the template up to the +12 position did not affect the stability of EC9, either (data not shown). This result is dramatically different from the data obtained for *E. coli* RNA polymerase in the same experimental setting, where ECs with from RNAs of 6–9 nt assembled on single-strand template DNA all had an extremely low stability, which was significantly increased upon the incorporation of the nontemplate DNA strand (13). Hence, the formation of a stable EC by Pol II requires only an 8-nt RNA:DNA hybrid, whereas the double-strand DNA at both ends of the transcription bubble apparently have no effect on the EC stability.

Nontemplate DNA Strand Interacts with RNA:DNA Hybrid and Destabilizes EC with the Imperfect Hybrid—The simplicity of a "stable" eukaryotic EC poses a question about the role of the nontemplate DNA strand in eukaryotic transcription. An interaction of the nontemplate DNA with the upstream part of the RNA:DNA hybrid was revealed in our experiments with the EC containing mismatched RNA. On the double-strand template, EC7+2 containing the 9-nt RNA with two unpaired bases at the 5′-end is significantly less stable than EC9 with the intact 9-nt RNA:DNA hybrid of the same RNA sequence (Fig. 3*A*). This effect turns out to be absolutely dependent on the presence of the nontemplate DNA strand: EC7+2 on a single-strand template has the same high stability as EC9 (Fig. 4*A*). Importantly, the facts that EC7+2 and EC9 are equally stable on the single-strand DNA and that their stability is higher than that of single-strand EC7 (Fig. 3*B*) suggest that the RNA:DNA hybrid binding site in the protein can accommodate the unpaired 5′-end of the RNA without serious loss of the binding affinity. The stabilization of EC7+2 on the single-strand DNA might be achieved by formation of the alternative rA:dG base pair and the positioning of the noncomplementary rU:dC pair into the RNA:DNA heteroduplex, as is schematically shown in Fig. 4*C* (*EC7+2 SS*). The incorporation of the nontemplate DNA strand does not affect the stability of EC9 but results in a significant decrease of the stability of EC7+2 (Fig. 4*A*). This result suggested that the presence of the nontemplate DNA strand in the EC triggers the mechanism that enables Pol II to "sense" the absence of the correct transcript-template pairing at the 5′-end of the 9-nt RNA:DNA hybrid.

The experiment shown in Fig. 4*B* directly demonstrates that the portion of the nontemplate DNA strand complementary to the template strand in the upstream region of the RNA:DNA hybrid is responsible for the difference in the stability of EC9 and EC7+2. The substitution of NDS41(CG), which is fully complementary to the template DNA strand in EC7+2, with NDS41 (see Fig. 1*B, scheme IV,* for the sequences) resulted in a significant increase of the stability of EC7+2. Apparently, the mismatch weakened the competition of this portion of the nontemplate strand with the 5′-end of the 9-nt RNA for hybridization to the template. In this case, an equilibrium between the

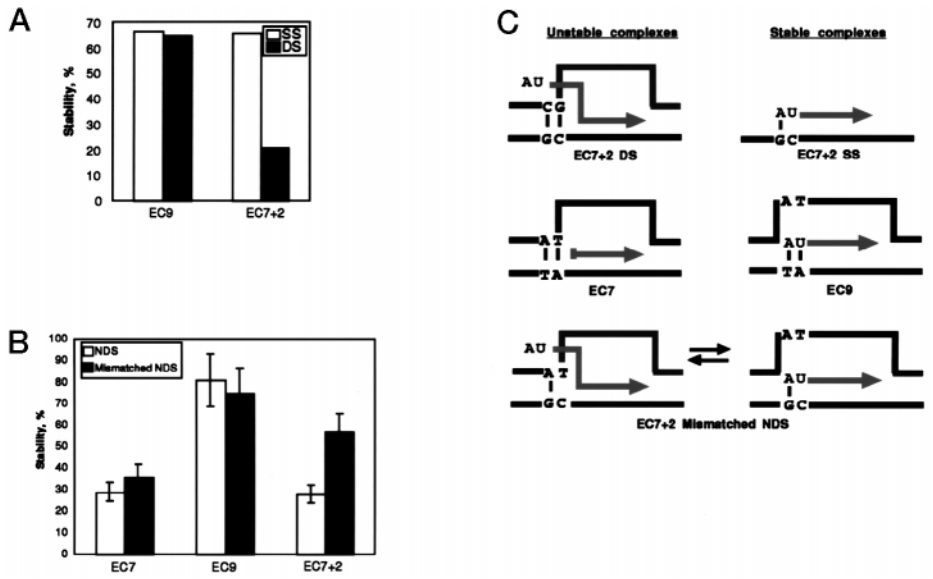

FIG. 4. **Nontemplate DNA strand negatively modulates stability of Pol II EC: fully complementary nontemplate DNA prevents formation of imperfect RNA:DNA hybrid.** *A,* a nontemplate DNA strand is essential for destabilization of the EC with the mismatch in RNA. EC stability was tested as described under "Experimental Procedures." The graph demonstrates the quantitative analysis of this experiment. *B,* the nontemplate DNA strand interacts with the upstream part of the RNA:DNA hybrid. ECs containing a 7- or 9-nt RNA primer were assembled on TDS41 or TDS41(CG) in the presence of NDS41 or NDS41(CG). NDS41 is a "mismatched NDS" for TDS41(CG), and NDS41(CG) is a "mismatched NDS" for TDS41. Each *bar* represents the mean of three independent experiments; the *error bars* show the S.D. *C,* the RNA:DNA architecture of ECs and its dynamics as analyzed in this work (an explanation is given in the text).

formation of RNA:DNA rA:dG and DNA:DNA dA:dG pairs is likely to be established (Fig. 4*C, EC7+2 Mismatched NDS*). It is difficult to predict, however, which conformation is thermodynamically favored: although the dA:dG pair is stronger (30, 31), rA is located at the end of the RNA oligonucleotide, and therefore, the rA:dG interaction in this context is less structurally constrained. The shift in the equilibrium between the two states of the 5′-end of the RNA toward the abnormally paired state determines the higher stability of the complex with mismatches in both the RNA and nontemplate DNA strands (Fig. 4*C,* compare *EC7+2 Mismatched NDS* with *EC9*). This result suggests that the fully complementary nontemplate DNA strand in Pol II EC contacts the 5′-proximal part of the 9-nt RNA:DNA hybrid and can compete with the transcript for pairing to the template (Fig. 4*C, EC7+2 DS*). However, NDS41 does not effectively compete with the base pairs at the 5′-end of the 9-nt RNA in EC9, because the stability of EC9 did not increase upon substitution of NDS41, fully complementary to the template DNA strand in EC9, with NDS41(CG) (Fig. 4*B*). Therefore, the nontemplate strand-mediated signaling mechanism exists in Pol II, which becomes activated when the RNA:DNA hybrid is disturbed at a distance of 7 nt from the 3′-end of the RNA.

DISCUSSION

One of the most difficult problems in conducting *in vitro* analysis of transcription elongation in eukaryotes is the formation and isolation of an EC in the absence of multiple auxiliary factors. The current methods for the promoter-less EC formation using the dC-tailed templates (5, 8, 20) or the artificial transcription bubble constructs (8–10) are either not efficient or yield complexes the structure and activity of which may not be similar to the properties of a native EC. Here, we develop a new method of a promoter- and factor-independent EC formation. This method exploits the ability of Pol II to bind the 3′-end of a short RNA oligonucleotide annealed to a single-strand DNA oligonucleotide serving as a template. This intermediate then accepts the fully complementary nontemplate DNA strand to generate a normal transcription bubble. The assembled complexes quantitatively and sequence-specifically elongate the synthetic RNA primer. Elongation of a transcript results in movement of the polymerase along the template with a coordinate movement of the transcription bubble and displacement of the 5′-end of the RNA from the hybrid with the template. These properties indicate that the structure of the assembled EC closely resembles the structure of the EC obtained by a promoter-specific initiation.

In this work, the assembly method is applied to the analyses of the processivity determinants of Pol II ECs. The comparison of properties of ECs formed by *E. coli* RNA polymerase and *S. cerevisiae* Pol II reveals a substantial difference in the stability requirements of prokaryotic and eukaryotic ECs. EC8 and EC9 formed on a single-strand template by yeast Pol II are stable (Fig. 2*B*), whereas the same ECs of bacterial RNA polymerase are unstable (13).[2] Double-strand DNA downstream from the RNA:DNA hybrid does not increase the stability of the eukaryotic EC as it does for the prokaryotic EC. Thus, an 8-nt RNA:DNA hybrid representing one out of two stability determinants of the bacterial enzyme EC may be considered a main stability determinant of the Pol II EC.

The observed difference in EC stability determinants in prokaryotes and eukaryotes is surprising, considering that multiple experimental results indicated a significant homology in the three-dimensional structures of the two enzymes and in the biochemical activities of their ECs (32–34). Biochemical analyses of transcription performed on *E. coli* RNA polymerase lead to the conclusion that both prokaryotic and eukaryotic enzymes form a sliding clamp around the duplex DNA, which anchors the protein to the template but does not interfere with the lateral motion of the enzyme (Refs. 35 and 36 and references therein).

However, structural analyses of yeast Pol II enzyme (37), as well as a two-dimensional crystal structure of Pol II elongation complex (38), position the hybrid in the active site cleft in the protein structure. Downstream DNA apparently lies on the Pol II surface and is partially surrounded from above and below by the arms of the enzyme (38). Our results are consistent with a speculation based on crystallographic analyses of Pol II enzyme (39) that, at least in the Pol II enzyme, the crucial protein-nucleic acid contact, which is necessary for the processive elongation, is located within the region of the RNA:DNA hybrid, rather than downstream of the hybrid. Based on a significant structural homology of the prokaryotic and eukaryotic RNA polymerases, we believe that the requirement of the downstream DNA duplex by *E. coli* RNA polymerase for the formation of a stable EC may indicate a weaker clamping action of the bacterial enzyme on the RNA:DNA hybrid, which is

[2] N. Komissarova, unpublished observation.

strengthened by the polymerase interaction with the double-strand DNA downstream. It is necessary to emphasize that the interaction with the downstream DNA duplex is likely to take place both in prokaryotic and eukaryotic ECs and has various functional consequences. For example, Pol II pausing near the end of a linear template (40), as well as a positive effect of the downstream DNA duplex on the catalytic activity of EC formed on a single-strand template and containing a 34-nt transcript,[3] indicates that the interaction with the front end DNA duplex may be important for modulating catalytic competence of the EC.

Although the effect of the Pol II interaction with the duplex DNA downstream from the RNA:DNA hybrid on the Pol II EC stability was not detected in our experimental system, the nontemplate DNA strand is evidently closely involved in the negative regulation of the EC stability. The processivity control by the nontemplate strand, summarized in Fig. 4*C,* occurs via modulation of the RNA:DNA hybrid length. Apparently, the mismatched portion of the transcript is actively displaced by the nontemplate strand (Fig. 4*C, EC7+2 DS*), making the nucleic acid array in this complex structurally similar to that of the EC with the 7-nt RNA (*EC7 DS*). Introduction of a corresponding mismatch into the nontemplate DNA strand (Fig. 4*C, EC7+2 Mismatched NDS*) abolishes this competition and thereby provides the opportunity for the RNA:DNA hybrid binding site in Pol II to accommodate the mismatched RNA. In this case, EC7+2 may acquire a conformation resembling the stable complex with the 9-nt RNA:DNA hybrid (EC9). Thus, the nontemplate DNA strand interacts with the upstream part of the RNA:DNA hybrid, modulating the EC stability. Yet another example of the positive processivity control by the nontemplate strand is a displacement of nascent RNA in the ECs with transcripts longer than 15–16 nt. In these complexes, the nontemplate DNA strand prevents the formation of an abnormally long RNA:DNA hybrid, which, as shown elsewhere, has a dramatic destabilizing effect on the Pol II EC.[4]

The main prediction of our model is that the length of the RNA:DNA hybrid is a possible target for regulation of the transcription processivity. Our results suggest an active involvement of the nontemplate strand on the upstream edge of the transcription bubble in the induction of transcript release during termination or abortive RNA synthesis (9, 41–44). Various positive and negative transcription elongation factors, which modify Pol II and/or template DNA (reviewed in Ref. 2; see also Refs. 45–47) may directly or indirectly affect the interaction of a nontemplate DNA strand with the RNA:DNA hybrid, promoting stabilization or dissociation of the elongation complex.

Importantly, the conclusions that we have drawn cannot be generalized without a future detailed comparison of the assembled EC with a more "natural" promoter-initiated complex formed in the presence of the general transcription factors. Experiments are under way to address the issues of how the transcription factors and the phosphorylation of Pol II CTD, which occurs during the escape from promoter, would affect the minimal RNA/DNA requirements for the EC stability and the other properties of the assembled complex.

[3] M. L. Kireeva, unpublished observation.
[4] M. L. Kireeva, submitted for publication.

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