Dissociable Rpb4-Rpb7 Subassembly of RNA Polymerase II Binds to Single-strand Nucleic Acid and Mediates a Post-recruitment Step in Transcription Initiation*

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The Rpb4 and Rpb7 subunits of yeast RNA polymerase II form a heterodimeric complex essential for promoter-directed transcription initiation in a reconstituted system. Results of template competition experiments indicate that the Rpb4-Rpb7 complex is not required for stable recruitment of polymerase to active preinitiation complexes, suggesting that Rpb4-Rpb7 mediates an essential step subsequent to promoter binding. Sequence and structure-based alignments revealed a possible OB-fold single-strand nucleic acid-binding motif in Rpb7. Purified Rpb4-Rpb7 complex exhibited both single-strand DNA- and RNA-binding activities, and a small deletion in the putative OB-fold nucleic acid-binding surface of Rpb7 abolished binding activity without affecting the stability of the Rpb4-Rpb7 complex or its ability to associate with polymerase. The same mutation destroyed the transcription activity of the Rpb4-Rpb7 complex. A separate deletion elsewhere in the OB-fold motif of Rpb7 also blocked transcription but did not affect nucleic acid binding, suggesting that the OB-fold of Rpb7 mediates both DNA-protein and protein-protein interactions required for productive initiation.

Cellular RNA polymerases contain a core set of subunits exemplified by the α, β, and β′ subunits of the eubacterial enzyme. Eukaryotic nuclear RNA polymerases have an additional 8–9 accessory subunits that are shared, unique, or similar in the three classes of polymerase (I, II, and III) (1). RNA polymerase II, which is mainly responsible for transcribing protein coding genes, consists of twelve subunits, named Rpb1–Rpb12, from largest to smallest (2). The Rpb1, Rpb2, Rpb3, and Rpb11 subunits are the functional and structural homologues of bacterial core subunits. Along with accessory Rpb subunits, RNA polymerase II uses additional proteins called general transcription factors (GTFs)† to initiate transcription in a promoter-dependent fashion (reviewed in Refs. 3 and 4). The accessory Rpbs and GTFs seem to play a role analogous to that of the σ subunits of prokaryotic RNA polymerases, which enable the core catalytic enzyme (α2ββ′) to bind specifically to start site domains, initiate transcription efficiently, and respond to regulatory inputs.

Several characteristics of a heterodimeric complex of Saccharomyces cerevisiae Rpb4 and Rpb7 bear resemblance to prokaryotic σ factors; the Rpb4-Rpb7 complex is absolutely required for accurate initiation but dispensable for RNA chain elongation (5), and the complex can associate reversibly with core (10-subunit) RNA polymerase II (5). Moreover, the Rpb4 and Rpb7 subunits are less abundant than other polymerase subunits in vivo (6) and appear to be critical for cellular adaptations to stress, a hallmark of many σ factors (6–9). Finally, overexpression of Rpb7 in wild-type yeast induces cell filamentation, suggesting that Rpb7 may promote gene-specific transcription (10).

Rpb7 is essential for cell viability in yeast (11) but Rpb4 is not (12). Some of the stress-sensitive phenotypes linked to an rpb4 null mutation are suppressed by overproduction of Rpb7 (9). Biochemical evidence suggests that the yeast Rpb4 subunit may facilitate association of Rpb7 with core polymerase (5). Taken together, these findings suggest that the Rpb7 subunit is key to the transcriptional activity of the Rpb4-Rpb7 complex. Here we show that Rpb7 contains a putative OB-fold domain that seems structurally and topologically related to a subfamily of domains typically found in proteins involved in ssRNA and ssDNA binding. Biochemical studies with wild-type Rpb4-Rpb7 complex and variants with targeted mutations in this domain reveal a possible link between nucleic acid-binding activity and an essential step in promoter utilization that occurs after formation of a stable preinitiation complex.

EXPERIMENTAL PROCEDURES

Baculovirus Expression of Yeast Rpb4 and Rpb7—Open reading frames (ORFs) were amplified by polymerase chain reaction from yeast genomic DNA using primers containing an NdeI restriction site at the 5′ end and a BamHI restriction site at the 3′ end of each ORF. The RPB4 gene was subcloned into the pET15b bacterial expression plasmid (Novagen), appending a hexahistidine tag and a thrombin cleavage site to the N terminus of Rpb4. The DNA insert encoding His-tagged Rpb4 was excised with XhoI and BamHI and ligated between the XhoI and BglII sites of the baculovirus transfer vector PacAB 4 (Pharmingen) to create PacAB 4/RPB4. The RPB7 gene was cloned into PacAB 4/RPB4

† The abbreviations used are: GTFs, general transcription factors; ss, single-strand; ORF(s), open reading frame(s); pol II Δ4/7; RNA polymerase II lacking Rpb4 and Rpb7 subunits; TF, transcription factor; TBP, TATA-binding protein; D, deletion.

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between the Sma1 and BamHI sites to create PacAB 4/RPB4/7. To create recombinant baculoviruses, PacAB 4/RPB4/7 was cotransfected into Sf9 cells using calcium phosphate precipitation with 0.5 μg of baculovirus Gold™ digested with Bsu 36I (New England BioLabs). Recombinant virus containing the RPB4 and RPB7 genes, identified by blue/white plaque screening, was purified twice and then amplified for large-scale protein expression.

To prepare individual Rpb4 and Rpb7 recombinant baculoviruses, RPB4 and RPB7 genes were cloned into the baculovirus transfer vector PVL 1392 (PharMingen). The RPB7 ORF was first cloned between the Ndel and BamHI sites in PET11a (Novagen), and RPB4 was cloned into pT7-7. Each construct was digested with XbaI and NotI and the resulting ORF-containing fragments were subcloned between the XbaI and BamHI sites of PVL 1392.

In-frame deletions in RPB7 in the PVL 1392 construct were prepared with the QuikChange site-directed mutagenesis system (Stratagene). After confirming each deletion by sequencing, RPB7 alleles were subcloned between the EcoRI and BamHI sites of pFastBac 1 (Invitrogen). Clones were transformed into DH10Bac competent cells (Life Technologies, Inc.) to generate recombinant baculoviruses. Rpb7 variant proteins were coexpressed with Rpb4 in Sf9 cells by coinfection with recombinant baculoviruses.

20 plates of Sf9 cells (150 × 20 mm; Nunc), each containing roughly 2 × 10⁸ cells, were infected with 250 μl of high titer virus stock. The optimized infection for protein expression was achieved by SDS polyacrylamide gel electrophoresis. Infected cells were scraped from plates and collected by centrifugation at 800 × g for 20 min. Cell pellets were washed in 25 ml of 50 mM Hepes (pH 7.5), 150 mM NaCl to remove any remaining medium and then resuspended in Buffer A (50 mM Hepes (pH 7.5), 750 mM NaCl, 10% glycerol, 5 mM imidazole, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine). Resuspended cells were flash frozen and stored at −80 °C.

Purification of Rpb4-Rpb7 Complexes—Infected cells were thawed on ice and broken by sonication (3 times for 1 min). Subsequent steps were performed at 4 °C. The sonicate was clarified by centrifugation at 100,000 × g for 45 min in a Beckman Ti70 rotor. The supernatant was loaded onto a 2-ml His-Bind resin column (Novagen) and washed with Buffer A until the eluate contained less than 10 μg of protein per ml (Bio-Rad protein assay). The His-Bind column was then washed with 10 column volumes of cleavage buffer. The Rpb4-Rpb7 complex lacking the hexahistidine tag (Bio-Rad protein assay). The His-Bind column was then washed with 10 column volumes of cleavage buffer. The Rpb4-Rpb7 complex lacking the hexahistidine tag was then eluted by raising the NaCl and imidazole concentrations to 50 and 500 mM, respectively (unresolved uncleaved His-tagged Rpb4-Rpb7 complex remained on the column and was recovered by elution with 500 mM imidazole). The cleaved (untagged) complex was diluted to 100 mM NaCl and purified further on a Mono Q column (Amersham Pharmacia Biotech) developed with a linear gradient (10 ml) of potassium acetate (pH 7.5) from 100 mM to 1 M at 0.5 ml/min. Fractions containing the Rpb4-Rpb7 complex (identified by SDS polyacrylamide gel electrophoresis) were pooled, dialyzed for 12 h against 50 mM Hepes (pH 7.6), 400 mM potassium acetate, 10% glycerol, 1 mM dithiothreitol, and concentrated in a Centricon 30 device for 12 h against 50 mM Hepes (pH 7.6), 400 mM potassium acetate, 10% glycerol. Bound and free probes were resolved by electrophoresis in TBE. Radiolabeled transcripts were processed for denaturing gel electrophoresis and detected by autoradiography as described (21).

RESULTS

A Predicted Nucleic Acid-binding Domain in Rpb7—The sequence of the archaea Rpb7 homologue is similar to that of the ribosomal S1 domain from E. coli. The S1 protein exemplifies the OB-fold (22), a structure found in many other proteins with single-stranded nucleic acid-binding activity (23, 24) (Fig. 1a).

The OB-fold motif consists of a 5-stranded β-sheet that is coiled to form a closed β-barrel and an α-helix that connects the third and fourth strands (Fig. 1, b and c). Some general observations about the interaction of OB-fold proteins with single-stranded nucleic acid can be gleaned from the two known structures of OB-fold/nucleic acid complexes. First, the β-strands 1–3 (Fig. 1b, green) form a scaffold across which nucleic acid is spliced, making hydrogen bond and stacking contacts. Second, the loop between the first and second strands (L12; Fig. 1b, red) makes key hydrogen bonds with the phosphate backbone. Third, the loop that connects the fourth and fifth β-strands (L45; Fig. 1b, yellow) clamps down on the nucleic acid strand, making hydrogen bond and stacking interactions.

Our study had the following three goals: to purify a transcriptionally active recombinant Rpb4-Rpb7 complex, to test its nucleic acid-binding activity, and to explore its role in transcription initiation. To generate recombinant Rpb4-Rpb7 complex, subunits were first expressed individually or together in E. coli. Although both subunits accumulated to high levels in cells, they proved to be insoluble. However, a soluble complex was recovered from insect cells coinfected with recombinant baculoviruses expressing Rpb4 and Rpb7 or from cells infected with a single recombinant baculovirus encoding both proteins. Purification of the complex from insect cell lysates was facilitated by adding a histidine tag and thrombin cleavage site to the N terminus of Rpb4. The complex was immobilized on a metal affinity column and subsequently released (after extensive washing to remove unbound contaminants) by digestion of Rpb4 with thrombin. Following subsequent anion exchange chromatography, the purified complex was judged to be at least 95% homogeneous by SDS polyacrylamide gel electrophoresis and Coomassie Blue staining (Fig. 2), in a yield of 200 μg per liter of Sf9 cells.

We designed mutant Rpb4-Rpb7 complexes containing small in-frame deletions in the predicted OB-fold of Rpb7 using information from both sequence alignments and detailed structural knowledge of OB-fold interactions with nucleic acids (Fig. 1a).

We constructed three Rpb7 mutants predicted to be compromised for ssDNA-binding (Fig. 1c). The first deletion, Rpb7ΔD1, was predicted to disrupt β-strand 1 and part of the L12 loop. The second deletion, Rpb7ΔD2, was predicted to disrupt β-strand 3, which harbors an aromatic residue (Phe in this case) critical for stacking interactions in all known structures. The third deletion, Rpb7ΔD3, was predicted to disrupt the L45 loop, which contains stacking interactions in one structure but is less well conserved among...
the known OB-fold proteins.

The three Rpb7 variants were coexpressed with histidine-tagged Rpb4 (Fig. 2). The structural integrity of mutant proteins was tested by monitoring the ability of each mutant to dimerize with Rpb4. The Rpb7D1 mutant was expressed but failed to copurify with Rpb4, suggesting severe mis-folding. This mutant was not analyzed further. By contrast, both the Rpb7D2 and Rpb7D3 variants formed a stable complex with Rpb4, as judged by copurification with histidine-tagged Rpb4. Moreover, the resulting mutant Rpb4-Rpb7 complexes were able to interact normally with Pol IIΔ4/7 (5) as judged by comigration with the polymerase on a size-exclusion column (data not shown) and by competition experiments with wild-type Rpb4-Rpb7 complex (see below).

Nucleic Acid Binding by Rpb4-Rpb7 Complexes—To test purified wild-type Rpb4-Rpb7 for single-strand nucleic acid-binding activity, we performed gel mobility shift assays with a radiolabeled ssDNA oligonucleotide probe. As predicted from its OB-fold motif, the Rpb4-Rpb7 complex bound to ssDNA in a saturable and reversible manner (Fig. 3) with an apparent dissociation constant of 0.7 μM (determined by half-maximal binding at equilibrium with protein in large molar excess). Gel mobility shift assays with an RNA oligonucleotide probe revealed similar binding to ssRNA with an apparent dissociation constant of 1.2 μM (data not shown; see Fig. 4).

We were unable to measure the binding activity of wild-type Rpb7 in isolation, because the protein was insoluble in the absence of Rpb4 (the same held true for N- and C-terminal Rpb7 deletion variants and for several internal deletion variants, as well). We therefore tested the Rpb4-Rpb7D2 and Rpb4-

FIG. 1. Panel A, sequence alignment of yeast Rpb7, an S1 domain from E. coli, and the rpoE RNA polymerase subunit from Sulfolobus. Black and gray boxes mark amino acid residues conserved in all three or in two of the three proteins, respectively. Panel B, positions of Rpb7 deletions superimposed on a typical OB-fold structure (residues 180–290 of the largest subunit of human replication protein A). The DNA channel in replication protein A lies between the D1 and D3 deletions when visualized in this orientation. Panel C, boxes enclose missing amino acid residues in Rpb7 deletions D1, D2, and D3.
Rpb7D3 complexes on ssRNA and ssDNA to identify region(s) in Rpb7 that mediate nucleic acid binding. The Rpb7D3 mutation deleting the putative L45 loop between the predicted β-strands had no apparent effect on nucleic acid binding (Fig. 4). By contrast, the affinity of the Rpb4-Rpb7D2 complex for RNA (Fig. 4) and DNA (not shown) was at least 10-fold lower than normal. These results suggest that this part of Rpb7 may be principally responsible for the observed nucleic acid binding, as expected from structure predictions.

**Transcription Initiation in Reconstituted System**—Crude cell extract prepared from yeast lacking the *RPB4* gene fails to support promoter-directed transcription, but activity can be restored by adding purified Rpb4-Rpb7 complex resolved from polymerase by ion exchange chromatography in the presence of urea (5). To confirm that the Rpb4-Rpb7 complex is required in transcription reactions reconstituted with purified components (and therefore lacking nonspecific inhibitors present in crude extracts; see Ref. 13), we assayed the activity of baculovirus-expressed Rpb4-Rpb7 complex with the minimal set of yeast GTFs and highly purified pol IIΔ4/7. As expected (5), pol IIΔ4/7 was completely inactive in promoter-directed transcription in this well defined system. Moreover, addition of purified recombinant Rpb4-Rpb7 complex supported transcription by pol IIΔ4/7 at levels achieved by native (12-subunit) polymerase (Fig. 5a, lanes 1–4).

In contrast to wild-type Rpb4-Rpb7 complex, complexes containing the Rpb7D2 or D3 mutants were completely inactive in transcription (Fig. 5a, lanes 6–11). We performed order of addition transcription experiments to test whether the D2 or D3 mutant complexes could compete with wild-type complex for stable binding to polymerase. When pol IIΔ4/7 was preincubated with wild-type Rpb4-Rpb7 complex, subsequent transcription was only slightly reduced by adding excess mutant complexes (Fig. 5b, lanes 1–5). By contrast, when polymerase was preincubated with the D2 or D3 mutant complex first, subsequent transcription was only slightly reduced by adding excess mutant complexes (Fig. 5b, lanes 6–10). Taken together with size-exclusion chromatography behavior (not shown), these results suggest that both of the mutant Rpb4-Rpb7 complexes bind normally to RNA polymerase II and can even displace wild-type Rpb4-Rpb7, as expected for reversible binding to the same site on polymerase.

To test the generality of the requirement for the Rpb4-Rpb7 complex, we tried transcription with several different promoters. The complex was required for initiation on the *TEF1, CYC1*, and *AdML* promoters (Fig. 5c) regardless of DNA template topology (linear or supercoiled). As expected (25), addition of Mediator to the reconstituted system boosted basal transcription by pol IIΔ4/7 in the presence of wild-type Rpb4-Rpb7 complex, but it did not obviate the strict requirement for Rpb4-Rpb7 (not shown).

**Rpb4 and Rpb7 Act after the Formation of a Stable Preinitiation Complex**—Transcription initiation can be resolved biochemically into an ordered series of steps beginning with the assembly of a preinitiation complex and recruitment (“commitment”) of polymerase to the promoter (see Refs. 26–28; reviewed in Refs. 3 and 4). Previous binding studies comparing the affinity of wild-type (12-subunit) polymerase and pol IIΔ4/7 for a TBP/TFIIB complex implicated Rpb4 and/or Rpb7 in the assembly of a preinitiation complex (29). To explore this ques-
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FIG. 5. Transcription activities of recombinant Rpb4-Rpb7 complexes. Panel A, transcription reactions with a CYC1 promoter template contained pol IIΔ4/7 (lanes 1–4) and indicated amounts (ng) of wild-type (WT) or mutant (D2 and D3) Rpb4-Rpb7 complexes. Control reactions lacked Rpb4-Rpb7 complex (lane 1) or pol IIΔ4/7 complex (lane 5). The arrow marks bands formed by labeled RNA transcripts in a denaturing polyacrylamide gel. Panel B, order of addition experiments with wild-type and mutant Rpb4-Rpb7 complexes. Transcription reactions contained 100 ng of pol IIΔ4/7 and either 10 ng of wild-type Rpb4-Rpb7 complex alone (WT 4/7; lane 5) or with 10 ng of mutant (D2 and D3) Rpb4-Rpb7 complex (lanes 1–4 and 6–11) or wild-type polym-erase (lanes 12–14) and indicated amounts (ng) of wild-type (WT) or mutant (D2 and D3) Rpb4-Rpb7 complexes. Control reactions lacked Rpb4-Rpb7 complex (lane 1) or pol IIΔ4/7 complex (lane 5). The arrow marks bands formed by labeled RNA transcripts in a denaturing polyacrylamide gel. Panel C, supercoiled (sc) or linearized (lin) plasmid templates containing three different promoters (TEF1, AdML, and CYC1) were transcribed with pol IIΔ4/7 in the absence or presence of wild-type recombinant Rpb4-Rpb7 complex (10 ng) as indicated. Transcripts are marked as in A. Panel C, supercoiled (sc) or linearized (lin) plasmid templates containing three different promoters (TEF1, AdML, and CYC1) were transcribed with pol IIΔ4/7 in the absence or presence of wild-type recombinant Rpb4-Rpb7 complex (10 ng) as indicated. Transcripts are marked as in A.

Further in the functional context of transcription, we assessed the ability of pol IIΔ4/7 to form a stable preinitiation complex in the absence or presence of Rpb4-Rpb7. Reactions contained two otherwise identical CYC1 promoter templates encoding G-less cassette transcripts of different lengths (20), and saturating amounts of TBP, TFIIB, TFIIIE, and TFIIF, as determined by factor titrations and by DNase I footprinting assays showing full TATA box occupancy by TBP under identical conditions.2 When pol IIΔ4/7 and the GTFs were incubated with both templates simultaneously, subsequent transcription reactions yielded equal amounts of both sets of promoter-directed transcripts (Fig. 6, lanes 1 and 2). However, if pol IIΔ4/7 and GTFs were preincubated with DNA I prior to adding DNA II and NTPs, transcription ensued almost exclusively on DNA I (lane 3). No such template commitment was observed if TBP and TFIIIB were omitted from the preincubation and added with DNA II (data not shown). In contrast to TBP and TFIIIB, Rpb4-Rpb7 complex was not required for template commitment; the degree of commitment to DNA I was identical regardless of whether the Rpb4-Rpb7 complex was present in the preincubation step (compare lanes 3 and 4). These results indicate that polymerase can join a stable, active preinitiation complex in the absence of Rpb4 and Rpb7 and therefore suggest that the essential function of the Rpb4-Rpb7 complex (Fig. 5a) comes after polymerase binds to the promoter.

DISCUSSION

In this study we focused on the nucleic acid-binding activity of the yeast Rpb4-Rpb7 complex and its role in transcription initiation. Our results show that the Rpb4-Rpb7 complex binds fairly strongly to ssDNA and RNA in vitro, as predicted from the sequence and structural homology of the OB-fold motif of Rpb7 to the S1 domain (22, 30). The complex binds RNA and DNA with nearly equal affinity, and a mutation in the putative OB-fold abrogates binding to both substrates, indicating a common site for interaction. Because the OB-fold occurs in both RNA- and DNA-binding proteins, and the two types of interaction seem structurally indistinguishable, the physiological substrate remains to be determined.

The Rpb4-Rpb7D2 complex bearing a deletion in β-strand 3 of the putative OB-fold DNA-binding surface (Fig. 1) was indeed defective in nucleic acid binding and moreover was completely devoid of transcription activity, raising the possibility that nucleic acid binding is key to the function of the Rpb4-Rpb7 complex. However, the Rpb7 D3 deletion disrupting the putative L45 loop (Fig. 1) was also completely inactive in transcription, even though it retained nucleic acid-binding activity. Our sequence alignment may not accurately predict the exact boundaries of the putative OB-fold, and the D3 deletion may lie outside the critical DNA-binding surfaces. Even if the alignment is correct, the L45 loop in Rpb7 may simply be less important for stabilizing nucleic acid interactions compared with other OB-folds. In any case, this portion of Rpb7 evidently has a key role in transcription distinct from DNA binding. Indeed, the ssDNA-binding activity of the Rpb4-Rpb7 complex may be peripheral to transcription activity, and the deleterious effect of the D2 mutation on both activities may be coincidental. Further genetic, biochemical, and structural studies are needed to fully delineate the role of the OB-fold motif of Rpb7 in DNA/RNA-binding and transcription. The C25 subunit of RNA polymerase III appears to be a Rpb7 homologue (31). It will be interesting to see whether this subunit also possesses

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nucleic acid-binding activity and if so, whether it is important for transcription initiation on class III promoters.

Endogenous pol IIΔ47/Δ7 was incapable of promoter utilization in a crude extract unless supplied with purified Rpb4-Rpb7 complex or (alternatively) Gal4-VP16, a potent transcription activator (5). The extract provided GTFs and Mediator, as well as pol IIΔ47/Δ7, and likely contained Rpb7, as well (Rpb7 is essential for yeast viability). Thus, our previous experiments did not rule out a role for Rpb7 in activated transcription. Results reported here confirm that the Rpb4-Rpb7 complex is indeed required for basal transcription in a completely defined reconstituted system. We also find that the complex is required for transcription in the presence of Mediator, indicating an important role for the Rpb4-Rpb7 complex in transcription by RNA polymerase II holoenzyme (16, 25, 32). Further work is needed to explore the role of Rpb7 in activation by enhancer-binding proteins. The defining biological feature of σ factors is their capacity to confer promoter class specificity on polymerase and thereby orchestrate multigene transcription programs for cellular development and responses to stress (reviewed in Ref. 33). The real test to see whether yeast Rpb4-Rpb7 complex plays a similar role will come from whole-genome analysis with suitable mutants (e.g. see Ref. 34).

Reconstructions of yeast RNA polymerase II structures from electron micrographs place the Rpb4-Rpb7 complex in the putative DNA-binding cleft of the polymerase and suggest that the complex favors a closed conformation of the polymerase “arm” domain around the cleft (29). These observations led to the hypothesis that Rpb4-Rpb7 may stabilize polymerase binding by contacting DNA in the cleft and somehow coupling DNA entry to closure of the cleft. It is tempting to speculate that the Rpb7D2 mutation may disrupt the DNA “sensor,” whereas the D3 mutation may disrupt protein-protein interactions that transduce the DNA signal to the polymerase arm domain. A more detailed structural model of polymerase bound to DNA places Rpb4-Rpb7 downstream of the catalytic site in the cleft (35), and because our biochemical findings indicate that Rpb4-Rpb7 complex mediates a step following template commitment, we speculate that it may stabilize the open promoter complex prior to initiation and/or an early transcribing complex prior to promoter escape, perhaps by binding to ssDNA in the transcript “bubble” (in analogy to bacterial σ factors) or to nascent RNA. Sorting this out will require further biochemical studies using cross-linking, permanganate footprinting, and kinetic measurements along the lines used for analyzing mammalian systems (36–40).

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