A Promoter Recognition Mechanism Common to Yeast Mitochondrial and Phage T7 RNA Polymerases

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Yeast mitochondrial (YMt) and phage T7 RNA polymerases (RNAPs) are two divergent representatives of a large family of single subunit RNAPs that are also found in the mitochondria and chloroplasts of higher eukaryotes, mammalian nuclei, and many other bacteriophage. YMt and phage T7 promoters differ greatly in sequence and length, and the YMt RNAP uses an accessory factor for initiation, whereas T7 RNAP does not. We obtain evidence here that, despite these apparent differences, both the YMt and T7 RNAPs utilize a similar promoter recognition loop to bind their respective promoters. Mutations in this element in YMt RNAP specifically disrupt mitochondrial promoter utilization, and experiments with site-specifically tethered chemical nucleases indicate that this element binds the mitochondrial promoter almost identically to how the promoter recognition loop from the phage RNAP binds its promoter. Sequence comparisons reveal that the other members of the single subunit RNAP family display loops of variable sequence and size at a position corresponding to the YMt and T7 RNAP promoter recognition loops. We speculate that these elements may be involved in promoter recognition in most or all of these enzymes and that this element’s structure allows it to accommodate significant sequence and length variation to provide a mechanism for rapid evolution of new promoter specificities in this RNAP family.

S. cerevisiae YMt and phage T7 RNAPs are both members of the single subunit RNAP family, but their promoter sequences are very different. The T7 promoter is a 21-nucleotide (nt) duplex that includes 4 nt downstream of the transcription start site and 17 nt upstream of +1 (1). The promoters of the closely related T3 phage or the more distantly related Sp6 phage(2) are identical in size to the T7 promoter but differ in sequence. In contrast, the YMt promoter is only 8 nt in length and is therefore similar in size to chloroplast promoters or the mitochondrial promoters of plants and other fungi, which are typically 8–10 nt in length although highly disparate in sequence (3, 4) (Fig. 1).

The mechanisms of promoter recognition by the YMt and T7 RNAP also appear to be distinct. T7 RNAP, like many of the phage polymerases, binds, melts, and initiates transcription as single subunit enzyme without any accessory transcription factor (5). In contrast, the YMt RNAP requires a specific accessory factor (Mtf1 (yeast mitochondrial transcription factor)) to initiate transcription from its promoter (6). This seeming disparity may, however, obscure underlying mechanistic similarity. For example, it was originally suggested (6) that Mtf1 might be homologous and analogous to prokaryotic RNAP σ-factor, which would mean that Mtf1 contributes directly to promoter recognition, a mechanism distinct from that seen with T7 RNAP, where the polymerase contains all of the promoter recognition elements. However, subsequent work showed that YMt RNAP can initiate transcription without Mtf1 if the promoter is supercoiled or contains a heteroduplex (“bubble”) around the transcription start site to facilitate melting (7). This implies that the YMt RNAP itself contains the elements required for specific binding to the promoter sequence, whereas Mtf1 is needed only for promoter melting.

These observations raise a number of questions. First, what structures in YMt RNAP are involved in recognizing the YMt promoter? Second, given the differences in YMt and T7 promoter sequence and length, is it possible that both of these RNAPs use similar or corresponding elements in their structures for promoter recognition? Finally, given the extreme divergence in promoter structure observed within the single subunit RNAP family, may all or many of these polymerases nevertheless utilize a similar promoter recognition mechanism?

T7 RNAP recognizes its promoter using two distinct elements. The −3 to −11 region of its promoter is recognized primarily by the promoter specificity loop, an extended β-hairpin that emerges from the polymerase C-terminal domain, and the −12 to −17 region of the promoter is recognized by elements in the N-terminal domain of the polymerase (8–10). Mitochondrial and chloroplast RNAPs are unlikely to have a structural element that is homologous or functionally analogous to the T7 RNAP N-terminal domain, since the N-terminal domains of these enzymes are highly divergent (11), and the promoter sequences recognized directly by the mitochondrial and chloroplast RNAPs do not extend upstream of −9. However, it has been suggested that the YMt RNAP has a promoter recognition loop like that seen in T7 RNAP (12), and the recently described structure of N4 RNAP exhibits a promoter loop similar to that of T7 RNAP (13), although this RNAP uses a hairpin promoter (Fig. 1) that is very different in structure from the T7 promoter.

To determine if a nonphage RNAP that is distantly related to T7 RNAP also contains an element that is structurally and func-
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| T7 phage | TATACGAGCCTACTATAGGAGGA |
| T3 phage | AATTACCCCTACTAGGAGGA |
| SP6 phage | ATTATAGGAGCCTACTATAGGAGGA |
| S. cerevisiae | ATATAGAT |
| Neurospora | TGTATATAGAT |
| Maize (Plastid) | TATAGATAT |
| Tobacco (Plastid) | CTTTTTATAT |
| Plant Mt. Consensus | YATAGATAT |
| Plant Plastid Consensus | YATANAGA |
| Xenopus | ACRTTAGA |
| Human | TGGGGGGGTGGCTTTAGTCGCAACCAAGATAAAA |
| Mouse | ACTAAAAATATATCTATATTTGAGTTTGGAAATC |

FIGURE 1. Single subunit RNA polymerase promoters are highly divergent. Representative promoter sequences for the indicated single subunit RNAPs illustrate the length and sequence divergence seen in these promoters. The transcription start (+1) sites are in boldface type and underlined. For the two mammalian mitochondrial promoters, the underlined region corresponds to the element bound by the mitochondrial transcription factor A, whereas the boldface sequence is the region bound by the Mt RNAP (27). The N4 phage promoter has a hairpin structure.

experimentally analogous to the T7 promoter recognition loop, we carried out cysteine-scanning mutagenesis of the region of the YMt RNAP that is predicted to encompass this putative recognition loop. We found that such mutations disrupt promoter utilization without affecting promoter-independent transcriptional activity. By tethering chemical nucleases to these mutants, we were able to position this putative promoter recognition loop in the Y Mt initiation complex (IC) and to define the architecture of the complex. Assessment of the effects of the Y Mt RNAP mutations on transcription of a set of mutant promoters identifies a likely amino acid-base pair interaction and corroborates the loop placement suggested by the tethered nuclease studies. In all, our results indicate that the Y Mt RNAP IC architecture is similar to that of T7 RNAP and that both polymerases have nearly identically positioned loops that play similar roles in recognizing their respective promoters.

EXPERIMENTAL PROCEDURES

Enzyme and Template Preparations—Single cysteine-substituted mutants of Y Mt RNAP were constructed with the Stratagene PCR site-directed mutagenesis kit following manufacturer’s instructions. Expression and purification of mutant polymerases and MtT1 transcription factor were carried out as described (14). Purified proteins were stored at −20 °C in buffer containing 10 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 1 mM EDTA, and 50% glycerol. Pol (dI-dC) was from Sigma. A duplex template containing a consensus 14S rRNA Y Mt promoter sequence was prepared by annealing a synthetic 63-base oligonucleotide (Operon Biotechnologies) with the (template strand) sequence AAGCTTATATATATATATATATATAGTATATAAAGAATAGTTTTATACACTATATATAAATAG (where the −1 to −8 bases are in boldface type, and the +1 base is in boldface and underlined) to its reverse complement (template strand). To create a “bubble” promoter template in which the region around the transcription start site is premelted, the −4 to +2 sequence of the nontemplate strand was changed to GCAGCT to generate a heteroduplex in this region. Twelve different fully duplex promoter variants based on this template were generated by introduction of single base substitutions between −1 and −8, as specified under Results. For chemical nuclease experiments the 5′-end of the template (T)-strand was labeled with [γ-32P]ATP (4000 Ci/mmol; ICN) with T4 polynucleotide kinase (InviBrion) as described (15).

Transcription Assays—In vitro transcription reactions were carried out at room temperature for 15 min in 10 μl in transcription buffer (50 mM Tris-Cl (pH 8.0), 10 mM NaCl, 20 mM MgCl2, 10 mM dithiothreitol) containing a 0.5 mM concentration of each NTP (Trilink) and 0.1 mM duplex template or 0.01 μg/ml of poly(dI-dC). RNA synthesis was initiated by the addition of 0.3 mM Y Mt RNAP and 0.6 mM MtT1. Transcripts were labeled by inclusion of 1% (v/v) 3000 Ci/mM 10 mCi/ml [α-32P]ATP from ICN (promoter template) or [α-32P]GTP (poly(dI-dC) template). Reaction samples were quenched by the addition of an equal volume of stop buffer (95% formamide, 20 mM EDTA, 0.01% xylene cyanol). Transcription products were resolved by electrophoresis in 20% (w/v) polyacrylamide gels (19% acrylamide, 1% bisacrylamide, 7 M urea) in TBE buffer and analyzed with an Amersham Biosciences PhosphorImager.

DNA Cleavage with Iron (S)-1-(p-bromoacetamidobenzyl) EDTA (Fe-BABE)-conjugated RNAPs—Fe-BABE (Dojindo Laboratories) was conjugated to cysteine-substituted Y Mt RNAPs as described (16). ICs halted at +0, +3, or +7 were formed at room temperature in transcription buffer containing labeled bubble or duplex 14S rRNA template at 0.01 μM and Fe-BABE-conjugated Y Mt RNAP at 0.03 μM. After a 10-min incubation with varying NTP mixes to form complexes halted at different positions, cleavage was initiated by the addition of sodium ascorbate and H2O2 as described (16). Cleavage reactions were quenched after 5 s by the addition of one reaction volume of stop buffer (95% formamide, 20 mM EDTA, 0.1% xylene cyanol). Cleavage products were analyzed by electrophoresis in denaturing 15% polyacrylamide gels (14.2% acrylamide, 0.8% bisacrylamide, and 7 M urea) and visualized on an Amersham Biosciences PhosphorImager. Cleavage positions were mapped by reference to a Maxam-Gilbert G + A ladder prepared as described (15).

RESULTS

All Single Subunit RNAPs Exhibit a Variable 26 ± 3-Amino Acid Element, Corresponding to the T7 Promoter Recognition Loop, Embedded between Two Well Conserved Blocks of Residues—Hundreds of single subunit RNA polymerase sequences are currently deposited in the data banks. Fig. 2 shows an alignment that includes the T7 and S. cerevisiae Mt RNAPs and a representative sampling of other phage RNAPs; mitochondrial enzymes from plants, fungi, protozoa, and animals; and a chloroplast enzyme. This alignment is centered on the region corresponding to the T7 RNAP promoter recognition loop. The sequences flanking this element are well conserved in this family and include the G, H, and I blocks of conserved elements previously identified in a comprehensive analysis of sequence conservation patterns in the single subunit RNAP family (11). However, within the region corresponding to the T7 promoter recognition loop, conservation breaks down, although all of these RNAPs exhibit a 23–29-residue element of variable sequence between the conserved G/H and I blocks. The consensus secondary structure prediction, based solely on the
aligned sequences of all identifiable members of the single subunit RNAPs in the data bases, is that this element forms a \( \beta \)-hairpin, as it is actually observed to do in T7 RNAP (Fig. 2). This pattern of conservation raises the possibility that this element is involved in promoter recognition in many of these enzymes, with the observed variations in its sequence and length providing a mechanism for recognition of distinct promoter sequences.

Mutations in the Putative YMt RNAP Promoter Recognition Loop Disrupt Promoter-dependent but Not Promoter-independent Transcription—To test this hypothesis in the specific case of YMt RNAP, we carried out cysteine scanning of residues 1125–1136 and 1140 of the \( S. \) cerevisiae enzyme. These residues were selected because homology modeling of the YMt RNAP based on the structure of a T7 RNAP IC containing a 3-mer RNA (IC3) identified them as most likely to be involved in Mt transcription.
promoter recognition. All of the mutant enzymes were well expressed in soluble form in *Escherichia coli* and, when tested for promoter-independent transcription on poly(dI-dC), displayed activity similar to that of the WT enzyme (Fig. 3). However, when tested on the YMt 14S rRNA promoter, mutants K1127C, Q1129C, Q1135C, and T1136C were ~10-fold less active than the WT enzyme, and mutants K1128C, T1132C, and L1134C were 2–5-fold less active. The identification of these residues as important for promoter recognition is in excellent agreement with the model of the YMt RNAP IC that we built based on the T7 IC3. Within the stretch of 13 residues that we mutated, this model places only 4 amino acids within hydrogen bonding distance (≤3.8 Å) of the -1 to -8 nucleotides of the promoter, corresponding to the segment of the yeast Mt promoter recognized by the *S. cerevisiae* Mt RNAP. These 4 residues are Lys-1127, Gln-1129, Gln-1135, and Thr-1136, exactly the residues identified by our mutational analysis as being most critical for Mt promoter utilization.

**A Double Mutant Cycle Indicates That YMt RNAP Gln-1129 and Thr-1136 Are Involved in Recognizing the -7 Base Pair of the Mt Promoter**—To try to identify specific side chain-base pair interactions between the YMt RNAP and its promoter, we tested our library of loop mutants with a panel of 12 Mt promoters mutated at individual base pairs. In such a double mutant cycle, potentially interacting groups are identified as those whose combined mutations do not additively disrupt the interaction between the two molecules in an intermolecular complex (17–19). The 12 mutant promoters tested were -1T→A, -1T→G, -2G→A, -3A→G, -3A→C, -3A→T, -4A→G, -5T→A, -6A→G, -6A→C, -7T→G, and -7T→C. These mutants were selected because a previous study of the YMt promoter found that these substitutions reduced transcription initiation by 5-fold or more (20), indicating that these bases are important for promoter recognition. Nevertheless, all of these mutant promoters displayed at least some (≥2%) residual activity, so that it was still possible to quantitatively compare the WT and mutant RNAP activities on these templates. With most of these promoters, the reductions in transcriptional activity were similar for the WT and mutant polymerases (data not shown). However, the substitutions at the -7-position of the promoter exhibited a distinct pattern: with the WT enzyme and most of the mutant RNAPs, these substitutions reduced initiation by ~20-fold, but with the T1136C and Q1129C RNAPs, substitutions at -7 had little to no effect on transcriptional activity (Fig. 4, A and B). This suggests that the T1136C and Q1129C mutations disrupt interactions with the -7 base pair, so that substitutions at this promoter position have no further significant effect on the activity of these two mutants. Consistent with this possibility, we observe that in the structural model of the YMt RNAP IC, Gln-1129 and Thr-1136 are both within 4 Å of the -7 base pair (Fig. 4C). Moreover, in this model, Gln-1129 superimposes on T7 RNAP Arg-746, which recognizes the guanosine base of the -7 G:C base pair of the T7 promoter (8).

**Chemical Nucleases Tethered to the Putative Promoter Recognition Loop Reveal Loop Position and IC Architecture**—To further define the position of the putative recognition loop in the YMt IC, we tethered the sulphydryl-reactive chemical

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**FIGURE 3.** Mutations in the putative YMt RNAP promoter recognition loop disrupt promoter-dependent but not promoter-independent transcription. A, transcription by the indicated YMt RNAPs on either poly(dI-dC) or on a template containing the 14S rRNA Mt promoter. B, relative transcription (incorporation) rates of the indicated RNAPs on poly(dI-dC). C, relative rates of run-off transcript synthesis from a duplex template containing the 14S rRNA Mt promoter. Error bars, ± S.E. for *n* = 4.
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nuclease Fe-BABE (21) to either the E1125C or E1131C mutant RNAPs. These mutants were selected because neither mutation appeared to affect promoter utilization (Fig. 3) and because they are at opposite ends of the putative promoter recognition loop. Residue 1131 is at the distal end of this loop, at a point predicted by the model to be close to the upstream DNA. Specifically, a nuclease tethered to 1131 is predicted to cleave the T-strand around -8 in an IC with a 3-mer RNA. Residue 1125 is at the proximal end of the loop and is predicted to be close to the downstream DNA. A nuclease tethered to residue 1125 allows us to test the model’s prediction that the DNA downstream of the transcription start site is sharply bent back toward the upstream DNA, as seen in the T7 IC (22–25). Such an architecture would predict that a nuclease at 1125 would cleave the T-strand around +6 in an IC3.

Fig. 5 shows the results of T-strand cleavage by WT, E1125C, and E1131C polymerases derivatized with Fe-BABE. The WT enzyme exhibits T-strand cleavage around -2/-3 in a preinitiation complex (Fig. 5A, lane 3) or in an IC reaction allowing synthesis of a 3-mer RNA (lane 4). The addition of nucleotides allowing synthesis of a 7-mer causes the center of cleavage to shift downstream to +1 (lane 5). The Fe-BABE-derivatized E1125C enzyme shows the same pattern, but in addition a new cleavage site appears centered at +6 in the IC3 (lane 7) and at +10 in the IC7 (lane 8). With the E1131C enzyme, a strong cleavage at -8 is seen in IC3 (lane 10; weaker cutting at the same site is seen in the pre-IC in lane 9), but in the IC7, cutting extends between -8 and -3, and two new sets of cleavages appear centered around +11/12 and +21/22 (lane 11). These cleavage patterns in the 3-mer IC are in perfect agreement with the predictions from the model based on the structure of a T7 IC3, since the nuclease at 1125 and 1131 are seen to cut at +6 and -8, respectively. The movement of the 1125 cleavage site from +6 to +10 upon RNA extension from 4 to 7 nt can be interpreted as reflecting simple translocation of the downstream DNA through the polymerase, but the other changes suggest more complex structural changes during initiation. Downstream cutting by the nuclease at 1125, for example, is not observed in the pre-IC (lane 7), suggesting that the downstream DNA may not bend toward the upstream DNA in the pre-IC. The changes seen, particularly with the 1131 nuclease, upon extension of the RNA from 3 to 7 nt, are likely to reflect structural changes required to accommodate the growth of the RNA during initiation and/or to allow promoter release and formation of the elongation complex. Understanding these changes will require more extensive experimentation, since they are likely to differ from the well-characterized structural changes seen during T7 RNAP initiation (see “Discussion”).

The experiments shown in Fig. 5A were done with a promoter containing a heteroduplex (bubble) between -4 and +2. Such “premelting” increases RNAP affinity for the promoter (7) to enhance saturation and signal strength from the nucleases, especially in the pre-IC. To make sure that our results were not being affected by use of a promoter with this artificial structure, we also looked at cleavage patterns using a normal, fully duplex promoter. As seen in Fig. 5B, the cleavage patterns on this promoter in an IC3 were like those seen on the bubble promoter. We conclude that the cleavage patterns in the IC3 are not being altered by use of the bubble promoter and that the early IC architectures of the YMt RNAP and T7 RNAP are similar, with the putative recognition loop of the YMt RNAP being similarly positioned and fulfilling a similar function as the T7 RNAP recognition loop.
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FIGURE 5. Nucleases tethered to the putative promoter recognition loop cut the promoter as predicted from the T7 RNAP based model. A, cleavage of the (5'-labeled) T-strand of a “bubble” (unpaired from −4 to +1) Mt promoter by the indicated YMt RNAPs derivatized with the chemical nuclease Fe-BABE, which attaches to surface-exposed sulfhydryls. Lane 1, G + A ladder; lane 2, no RNAP control; lanes 3, 6, and 9, reactions with no NTPs (pre-IC); lanes 4, 7, and 10, reactions with ATP and 3'-dUTP, allowing RNA extension to +3; lanes 5, 8, and 11, reactions with ATP, UTP, and 3'-dGTP, allowing RNA extension to +7, B, as in A but for reactions allowing RNA extension to +3 and using a fully duplex promoter. C, solvent-exposed surface model of the YMt RNAP IC3 with the T-strand in blue and the nontemplate strand in gray. Residue 1125 is highlighted in pink, and the T-strand segment cut in the IC3 by the Fe-BABE attached to 1125 is in yellow. D, RNAP model as in C but rotated by −60° around the indicated axis. Residue 1131 is in pink, and the T-strand segment cut by the Fe-BABE attached to 1131 is in yellow.

DISCUSSION

Our observations reveal that the sequence in YMt RNAP that aligns with the T7 RNAP promoter recognition loop plays a role in recognizing the Mt promoter. Although the T7 and YMt RNAPs exhibit less than 30% overall identity (26), a homology model of the YMt RNAP built on the basis of the T7 RNAP early IC accurately places the YMt RNAP promoter recognition loop on the Mt promoter. In fact, this homology model is unexpectedly precise in its predictive power. First, the model places residues Lys-1127, Gln-1129, Gln-1135, and Thr-1136 closest to the promoter, and mutation of exactly these residues is found to have the strongest effects on promoter utilization. Second, the model places residues Gln-1129 and Thr-1136 near the −7 base pair and superimposes Gln-1129 on a T7 RNAP residue that recognizes the −7 base of the T7 promoter, and we find that mutation of Gln-1129 or Thr-1136 eliminates discrimination of the Mt promoter −7 base. Last, the model predicts cleavage at +6 and −8 in an IC3 by nucleases tethered, respectively, to residues 1125 and 1131, and this is exactly what is observed. The nuclease-cut sites also indicate that the early ICs of both the T7 and yeast enzyme have a similar architecture, with the DNA downstream of the transcription bubble similarly bent toward the upstream DNA.

Phylogenetic analyses divide the single subunit RNAP family into three main branches: the phage genome encoded, the mitochondrial plasmid encoded, and the nuclear genome encoded, with the last including mitochondrial, chloroplast, and nuclear enzymes (11). Conservation of the promoter recognition loop function in the T7 phage RNAP and the YMt RNAP therefore encompasses two of these three major branches. Since all members of the single subunit RNAP family display a variable 26 ± 3-residue element with a predicted β-hairpin structure, which aligns with the T7 promoter recognition loop, we speculate that most, if not all, members of this family use this element to recognize the promoter sequence immediately upstream of the transcription start site. Evolution of new promoter specificities in these RNAPs could then occur rapidly (and may be reflected in the sequence variation of the loop itself), since the loop would extend out from the polymerase and make a limited number of contacts with the rest of the protein, so that variation in its sequence is relatively unconstrained by intramolecular interactions.

A conserved promoter recognition mechanism and similar IC architecture for most single subunit RNAPs would appear inconsistent with the extent of the upstream DNA recognized by the transcription apparatus of different members of this RNAP superfamily. The YMt promoter, for example, extends only to −8, whereas the T7 promoter extends to −17, and the human Mt promoter requires sequences that extend to −28 (Fig. 1). Structural and footprinting studies, however, indicate that the core RNAP structure that is present in all the single subunit RNAPs covers and recognizes a similarly sized region around the transcription start site for all of these enzymes. For example, sequences upstream of −10 in the mammalian Mt promoter are recognized and bound by mitochondrial transcription factor A, an HMG-box protein required for initiation of mammalian mitochondrial transcription but not for initiation at YMt promoters. The mammalian Mt RNAP alone recognizes and protects the promoter from DNase I only to about −10 (27), similar to the recognition and protection provided by YMt RNAP on its promoter (7). Similarly, although T7 RNAP protects its promoter up to −17 or −20 from DNase I or MPE-Fe2+ footprinting (28−30), its IC crystal structure reveals that recognition and protection of the region upstream of −11 involves the functionally and structurally nonconserved N-terminal domain, whereas the conserved polymerase domain and recognition loop contact promoter elements downstream of this point (8). Other members of this RNAP family may fit this
pattern, with recognition of promoter elements more than ~10 bp upstream of the transcription start site carried out by accessory factors or domains that are distinct in different members of the family.

Such a pattern suggests a point of necessary divergence in the mechanism of transcription initiation and promoter release by these polymerases. For T7 RNAP, a combination of DNA scrunching and conformational movements of the N-terminal domain accommodates increasing amounts of RNA and DNA in the IC during initial transcription and prior to promoter release (15, 25, 31, 32). To release the promoter then requires movements of the N-terminal domain relative to the promoter recognition loop, which together form a contiguous promoter recognition surface. If the recognition loop and N-terminal domain are locked together via an engineered disulfide bond, T7 RNAP cannot disengage its promoter (15). Given that the YMt RNAP N-terminal domain is structurally and functionally distinct from that of T7 RNAP, it is likely that the conformational mechanisms of initial transcription and promoter release differ significantly between these enzymes. One indication of this is that the cleavage patterns of an Fe-BABE tethered to YMt RNAP loop residue 1131 change significantly upon RNA extension from 3 to 7 nt (Fig. 5), whereas the cleavage patterns of nucleases tethered to the T7 RNAP recognition loop remain unaltered as the RNA is extended from 2 to 8 nt in length (16), consistent with the crystallographic observation that loop-T7 promoter contacts in an IC with a 7- or 8-base RNA are identical to those in a pre-IC (25). More extensive experimentation, however, will be required to determine the details of how the variations in the structures of the N-terminal regions of these polymerases alter the conformational mechanisms of initial transcription and promoter release.

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