Characterization of T7 RNA Polymerase Transcription Complexes Assembled on Nucleic Acid Scaffolds*

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We have used synthetic oligomers of DNA and RNA to assemble nucleic acid scaffolds that, when mixed with T7 RNA polymerase, allow the formation of functional transcription complexes. Manipulation of the scaffold structure allows the contribution of each element in the scaffold to transcription activity to be independently determined. The minimal scaffold that allows efficient extension after challenge with 200 mM NaCl consists of an 8-nt RNA primer hybridized to a DNA template (T strand) that extends 5-10 nt downstream. Constructs in which the RNA-DNA hybrid is less than or greater than 8 bp are less salt-resistant, and the hybrid cannot be extended beyond 12-13 bp. Although the presence of a complementary nontemplate strand downstream of the primer does not affect salt resistance, the presence of DNA upstream decreases resistance. The addition of a 4-nt unpaired “tail” to the 5’ end of the primer increases salt resistance, as does the presence of an unpaired non-template strand in the region that contains the 8-bp hybrid (thereby generating an artificial transcription “bubble”). Scaffold complexes having these features remain active for over 1 week in the absence of salt and exhibit many of the properties of halted elongation complexes, including resistance to salt challenge, a similar trypsin cleavage pattern, and a similar pattern of RNA-RNA polymerase cross-linking.

During the early stages of transcription, T7 RNAP¹ (like all known RNAPs) forms an unstable initiation complex (IC) that synthesizes and releases short abortive initiation products before clearing the promoter and forming a stable elongation complex (EC) (1-9). The transition is accompanied by release of upstream promoter contacts, changes in the size of the footprint of the polymerase on the DNA, increased resistance to challenge with agents such as salt and heparin (which disrupt and inactivate the IC), and changes in accessibility to cleavage by a variety of proteases (4-7). Taken together, these changes suggest that significant alterations in the organization of the complex occur during the transition.

A variety of lines of evidence demonstrate that the transition is complex and may involve multiple steps (6, 8-13). Recent fluorescence probing and nuclease sensitivity experiments indicate that promoter clearance and collapse of the upstream edge of the transcription bubble occur when the RNA-DNA hybrid achieves a length of 8-9 bp (13, 14) but that the length of the hybrid increases to 10 bp before the transcription bubble collapses to yield a hybrid length of 8 bp, as is observed during elongation (Ref. 13 and see below). The final phase (between 10 and 14 nt) appears to involve displacement of the 5’ end of the RNA from the upstream end of the hybrid and its association with an RNA product-binding site (11, 15).

Crystal structures have now been solved for free RNAP, for RNAP complexed with a specific inhibitor of transcription (T7 lysozyme), for a binary promoter-RNAP complex, and for an initiation complex that has transcribed the first three bases in the template strand (16-19). However, no structure has yet been published for an elongation complex or for any intermediate complexes that may form during the transition to an EC.

Biochemical studies have revealed a number of features of T7 RNAP elongation complexes. In a halted EC the enzyme protects a 24-bp region that extends ~19 bp upstream and ~5 bp downstream from the active site from digestion with DNase I or MPE-Fe(II) (4, 20, 21). A somewhat smaller region that extends ~13 bp upstream and ~5 bp downstream is protected from digestion with exonuclease III or λ exonuclease, and this footprint is shifted downstream in the presence of the incoming NTP (which stabilizes the EC in the post-translocation state) (14). The RNA-DNA hybrid is ~8 bp (14, 15, 22) and is enclosed in a transcription bubble of ~9 bp. The association of the displaced nontemplate (NT) strand of the transcription bubble with the RNAP may help to stabilize the complex (11). Fluorescence studies and KMnO₄ sensitivity indicate that the upstream edge of the transcription bubble in the EC is very close (within 1 bp) to the point at which the RNA is displaced from the template and that downstream border is very close (within 1 bp) to the 3’ end of the RNA (in the active site) (14, 22). The latter conclusion is supported by the observation that on templates in which the two DNA strands have been covalently joined by a psoralen cross-link, the RNA may be extended up to the cross-link (23). The 5’ end of the nascent RNA becomes accessible to ribonuclease (emerges to the surface of the complex) ~4-6 nt after its displacement from the template, resulting in a total protected RNA length of 12-14 nt (14, 15).

To gain understanding of the organization and properties of T7 RNAP transcription complexes, we annealed together synthetic oligomers of RNA and DNA to construct nucleic acid “scaffolds” that resemble the structural elements thought to be present in an EC. Incubation of these structures with T7 RNAP resulted in the formation of functional transcription complexes that exhibit many of the properties of halted elongation complexes. The use of such nucleic acid structures has enabled us to characterize the contribution of each of the components in

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¹ The abbreviations used are: RNAP, RNA polymerase; IC, initiation complex; EC, elongation complex; nt, nucleotide; NTCB, 2-nitro-5-thiocyanobenzoic acid; MOPS, 4-morpholinepropanesulfonic acid.

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the scaffold to transcription activity. Similar approaches have been used to examine transcription complexes formed by multi-subunit RNAPs such as \textit{Escherichia coli} RNA polymerase and yeast polymerase II (24, 25).

**EXPERIMENTAL PROCEDURES**

Assembly of Scaffolds and Transcription Conditions—Histidine-tagged T7 RNAP was purified as described.\(^2\) Synthetic DNA oligonucleotides were obtained from Macromolecular Resources (Fort Collins, CO). RNA oligonucleotides were purchased from Dharmacon Research Inc. (Lafayette, CO). Where indicated, RNA and DNA oligomers were labeled at their 5’ ends using \(\gamma\)-\(^{32}\)P-ATP and polynucleotide kinase. The sequences of all oligomers are given in Table I.

To assemble scaffolds, oligonucleotides were taken up in water, mixed together at a concentration of 10 \(\mu\)M each, heated to 70 °C for 5 min, and cooled slowly to room temperature. Except where noted, RNAP-scaffold complexes were formed in a reaction volume of 10 \(\mu\)l containing transcription buffer (20 mM Tris-HCl, pH 7.9, 15 mM MgCl\(_2\), 0.1% Tween 20, 5 mM \(\beta\)-mercaptoethanol), 1 \(\mu\)M scaffold, and an equimolar concentration of RNAP for 10–20 min at room temperature.

**FIG. 1.** Assembly of a functional T7 RNAP scaffold complex. A, scaffold 1 was assembled by annealing together an 8-nt RNA primer (RNA8) labeled at its 5’ end with \(\alpha\)-\(^{32}\)P (asterisk), a 28-nt template strand (TS1), and an 18-nt non-template strand (NT1), resulting in a gap of 2 nt between the 3’ end of the RNA primer and the 5’ end of the downstream NT strand. B, complexes formed by incubation of the scaffold with an equimolar amount of His\(_6\)-tagged T7 RNAP were immobilized on Ni\(^ {2+}\)-agarose beads (15, 26) (step 1). The complexes were washed with transcription buffer (step 1) and incubated with GTP for 2 min (step 2), washed again and incubated with UTP (step 3), and then incubated with CTP (step 4). The aliquots were removed at each step and analyzed by electrophoresis in 20% polyacrylamide gels. C, RNAP-scaffold complexes formed as in A were challenged by a 5-min exposure to increasing concentrations of NaCl, and the ability of the complexes to extend the RNA primer during the subsequent 2-min incubation in the presence of the next template-directed nucleotide was determined as above.
from the start site of transcription (EC14) were formed by annealing together oligos DT6 and DT7 and incubation with RNAP in the presence of GTP, ATP, and UTP as previously described (15).

**Trypsin Cleavage**—Trypsin cleavage was performed in 10-μl reactions containing 20 mM Tris-HCl (pH 7.9), 15 mM MgCl₂, 0.1% Tween 20, and 5 mM β-mercaptoethanol at room temperature for 10 min. The reactions contained 1 μM T7 RNAP or T7 RNAP complexes and trypsin at a 20:1 ratio (w/w, respectively). The reactions were stopped by the addition of protein PAGE loading buffer and resolved in a 4–12% Nu-PAGE gel using a MOPS buffer system (Invitrogen). The identities of the cleavage fragments were confirmed by Edman amino acid sequence analysis.

**RNA-RNAP Cross-linking**—RNAP photocross-linking experiments were carried out using a synthetic RNA oligonucleotide (DT11sU; Dharmacon; Table I) that contained a photoreactive uridine derivative, 4-thio-UMP (sU). The RNA oligonucleotide was annealed to template strand TS1 as described above to give sU-containing scaffold 23. The RNAP-scaffold complexes were labeled by extension of the RNA primer with [α-32P]UTP and irradiated for 10 min on ice with a 6 W UV lamp (Cole-Parmer 9815 series, Chicago, IL) using a 312-nm optical filter. The cross-linked products were precipitated with an equal volume of saturated (NH₄)₂SO₄ and subjected either to NTCB or CNBr cleavage.

**RESULTS**

**Assembly of Functional Scaffold Complexes**—Two approaches involving the use of artificial scaffolds were employed in previous studies of multi-subunit RNAPs. One group of investigators explored the use of minimal scaffold assemblies that consisted of a single-stranded DNA template hybridized to an RNA primer (24). Other investigators explored more complex structures that included duplex DNA upstream and downstream of the primer and a mispaired nontemplate strand in the region of the primer-template hybrid (an artificial transcription bubble) (27, 28). To simplify studies of parameters that affect the function of T7 complexes, we initiated our studies using a scaffold that consists of a 28-nt template strand hybridized to an 8-nt RNA primer and an 18-nt NT strand downstream (Fig. 1, scaffold 1). Such a scaffold had previously been shown to allow the assembly of functional transcription complexes with E. coli RNAP (24, 29). When incubated with histidine-tagged T7 RNAP and the next incoming nucleotide, the 8-nt primer was quantitatively extended to 9 nt (Fig. 1B, steps 1 and 2). Subsequent washing of immobilized complexes and incubation with UTP and then CTP allowed further extension of the primer to 10 and 11 nt (steps 3 and 4). The incorporation of NTPs was template-specific, because primers were extended only in the presence of the appropriate substrate (data not shown).

Unlike binary promoter-RNAP complexes and initiation complexes, which are rapidly inactivated by exposure to 200 mM NaCl, T7 RNAP elongation complexes are resistant to salt challenge (1, 2, 9, 11, 30). As shown in Fig. 1C, transcription complexes assembled on the minimal scaffold are also resistant to challenge with 200 mM NaCl.

**Effects of Removing the NT Strand of the DNA and of Changing the Length of the RNA-DNA Hybrid**—Previous studies have shown that whereas the binding region of the T7 promoter (−17 to −5) must be double-stranded to allow promoter binding, the NT strand of the DNA in the initiation region (−4 to +6) is not required for promoter function and that removal of the NT strand downstream of −4 results in tighter RNAP binding and increased rates of initiation (30–36). In a similar manner, the NT strand downstream from the primer is not required for transcription activity on the minimal scaffold, and complexes assembled on a scaffold that lacks the NT strand (Fig. 2, scaffold 2) were as resistant to salt challenge as complexes that were assembled on a scaffold in which the NT strand was present (scaffold 1).

The length of the RNA-DNA hybrid in a halted T7 RNAP EC has been determined to be 8 bp (14, 15, 22). We determined the effects of changing the length of the RNA-DNA hybrid on the behavior of scaffold complexes as follows. To examine the effects of shortening the primer we utilized a 7-nt RNA primer (RNA7, scaffold 3). To examine the effects of increasing the length of the primer, we extended the 8-nt RNA primer (RNA8) to 9 or 10 nt by the addition of GTP or GTP and UTP (scaffolds 4 and 5). The ability of the polymerase to extend these primers

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**Fig. 2. Effects of removing the NT strand of the DNA and of changes in the length of the RNA-DNA hybrid.** A. scaffolds were assembled using either an 8-nt RNA primer (RNA8; scaffolds 1 and 2) or a 7-nt RNA primer (RNA7; scaffold 3). To form complexes involving scaffolds 4 and 5, the 8-nt RNA primer in scaffold 2 was extended 1 or 2 nt by incubation with GTP alone or with GTP and UTP, respectively. Unincorporated substrates were removed by centrifugation on quick spin columns. A T7 RNAP elongation complex halted 14 nt downstream from the start site (EC14) was assembled by annealing template and NT DNA strands that contain a consensus T7 promoter sequence (DT6 and DT7) and incubation with T7 RNAP in the presence of GTP, ATP, and UTP (100 μM each). B. RNAP-scaffold complexes were challenged by a 30-min exposure to 200 mM NaCl, and the ability of the complexes to extend the RNA primer was determined as for Fig. 1C. The data were quantified by PhosphorImager analysis and are given as fraction of primer extended for each scaffold and for an elongation complex halted at +14 (EC14).
after challenge with 200 mM NaCl was determined as described above. Shortening the RNA-DNA hybrid to 7 bp or extending it to 9 or 10 bp resulted in significant decreases in salt resistance (Fig. 2, scaffold 2 versus scaffolds 3–5).

**Effects of Downstream DNA Configuration on Complex Stability**—The observation in Fig. 2 that the presence of the NT strand is not required to provide salt resistance to T7 RNAP complexes is in contrast to previous findings with *E. coli* RNA polymerase RNAP (24) but not with yeast polymerase II (37). To examine the contribution of the downstream DNA in more detail, we varied the length of the downstream DNA both in the presence and in the absence of a complementary NT strand downstream. B, the ability of T7 RNAP assembled on such scaffolds to extend the primer following a 30-min challenge with 200 mM NaCl was determined as described for Fig. 2B. C, to determine whether transcription complexes remained associated during the course of the reaction, scaffolds 7–9 (1 μM) were mixed either with an equimolar concentration of RNA polymerase (1 μM) or a 20-fold lower concentration of RNAP (0.05 μM). The ability of the polymerase to extend the primer during a subsequent 40-min incubation without NaCl was determined by gel electrophoresis of the products.
activity (cf. scaffold 1 versus scaffold 2 and scaffold 6 versus scaffold 7). To determine the contribution of the template strand to salt resistance, we progressively shortened the length of the downstream strand from 20 nt (scaffold 2) to 2 nt (scaffold 9). A significant loss in salt resistance was observed when this strand was shortened to 5 nt.

The resistance of transcription complexes to salt challenge is usually interpreted as an indication of their stability (i.e. the tendency of the components of the complex to remain together). To determine whether the components of the T7 scaffold complexes remained associated during the course of the reaction or whether the RNAP dissociates from one template and then associates with another, we examined primer extension under conditions in which the RNAP concentration was either equimolar to the scaffold (1/2M each) or 20-fold lower than the scaffold (50 nM) (Fig. 3C). Using scaffold 1 (which is highly resistant to salt challenge), we found that when the concentrations of RNAP and scaffold were equimolar, nearly 100% of the primers were extended, whereas when the concentration of polymerase was only 5% that of the scaffold, only 5% extension was observed. This indicates that there is little turnover of the RNAP after primer extension during the 40-min incubation period on this template. In contrast, when the NT strand protrudes only 2 nt downstream from the primer (scaffold 9), more than 50% of the primers are extended during the reaction, indicating that the polymerase dissociates from this scaffold after extending the primer and may subsequently associate with other scaffolds. Scaffolds in which the NT strand extends 5 or 10 nt downstream from the 3' end of the primer (scaffolds 7 and 8) exhibit an intermediate stability. From these results, we conclude that the minimal length of the downstream template strand that is required to confer maximal stability on the transcription complex is 5–10 nt. This is consistent with the size of the footprint of T7 RNAP in a halted EC (4, 14).

Previous studies have shown that on templates in which the two DNA strands are covalently joined via a psoralen cross-link, T7 RNAP can extend the RNA up to the site of the cross-link (23). In the experiments above in which a complementary NT strand was present (scaffolds 1 and 6), the 3' end of the RNA primer was separated by a gap of 2 nt from the 5' end of the downstream NT strand. To determine what happens when primer extension in a scaffold complex results in an encounter with the NT strand, we constructed templates in which the gap between the 3' end of the primer and the 5' end of the downstream NT strand was reduced from 2 to 1 or 0 nt (Fig. 3, scaffolds 1, 10, and 11, respectively). In the absence of salt, the primer was efficiently extended by 1 nt in all three of these constructs (data not shown). However, in the presence of 200 mM NaCl, primer extension was significantly reduced when there was no gap (scaffold 11).

**Toward the Assembly of a Complete Scaffold: Examining the Effects of Upstream DNA, a Transcription “Bubble,” and a 5' RNA “Tail”**—In halted T7 RNAP elongation complexes, the RNA product is displaced from the template strand of the DNA about 8 bp upstream from the active site, but the 5' end of the product but does not emerge to the surface until an additional

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**Fig. 5.** The RNA-DNA hybrid may only be extended to 12 bp. A, scaffolds were assembled as shown. Template strand TS2 is 2 nt shorter than TS1; when annealed to RNA8 (scaffold 21), this results in a noncomplementary 5' tail of 2 nt on the RNA primer and an RNA-DNA hybrid length of 6 bp (as opposed to a tail of 0 nt and a hybrid length of 8 bp in scaffold 2). Both scaffolds were incubated with RNAP in the presence of all 4 NTPs, and the products were examined by gel electrophoresis. The primer in scaffold 2 was extended to a limit length of 12–13 nt, corresponding to the formation of a 12–13-bp RNA-DNA hybrid. The primer in scaffold 21 was extended to a limit length of 14–15 nt, which also corresponds to a 12–13-bp hybrid. B, RNAP complexes involving scaffold 22 were incubated in the presence of GTP, UTP, and CTP (to give scaffold 22a) or all four NTPs (lower left). RNAP-scaffold 22 complexes were loaded onto Ni²⁺-agarose beads and incubated with GTP, UTP, and CTP, and the integrity of the complexes before and after extension was monitored by retention of the labeled primer and NT strand on the beads (b) or their presence in the wash (w) (lower right).
Fig. 6. Organization of RNAP-scaffold complexes. A, free T7 RNAP, a binary T7 RNAP-promoter complex, EC14, and a T7 RNAP complex assembled with scaffold 16 (see Fig. 4) were digested with trypsin at room temperature for 10 min. Digestion products were resolved in a 4–12% Nu-PAGE gel using a MOPS buffer system (Invitrogen). The peptides were identified by molecular mass and by N-terminal sequence analysis (Ref. 39 and this work). B, a scaffold for RNA-RNAP photocross-linking was prepared by annealing RNA oligonucleotide DT11sU with DNA.
4 nt have been added, presumably because the RNA is involved in protective contacts with the RNAP (14, 15, 38).

To explore the possible contribution of the displaced RNA to complex stability, we modified the 8-nt RNA primer used above (RNA8) to include a noncomplementary tail of 4 nt at its 5’ end (Fig. 4; RNA12) and examined the properties of complexes formed using these two different primers. A number of templates were tested, including those in which the nature of the upstream DNA was varied and those that included a noncomplementary NT strand in the region of the RNA-DNA hybrid (thereby creating an artificial transcription bubble).

With regard to the effects of upstream DNA, we observed that the presence of single-stranded template DNA upstream of the primer resulted in a significant decrease in salt resistance (scaffold 2 versus scaffold 12 and scaffold 16 versus scaffold 17) and that salt resistance decreased even further if the upstream DNA was made double-stranded by the addition of a complementary NT strand (scaffolds 13, 14, 18, 19). In fact, the latter complexes are so unstable that we were unable to detect their assembly (as determined by failure to form labeled complexes formed using these two different primers. A number of templates were tested, including those in which the nature of the upstream DNA was varied and those that included a noncomplementary NT strand in the region of the RNA-DNA hybrid (thereby creating an artificial transcription bubble).

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N-terminal amino acid sequence analysis, we determined that the 88-kDa fragment results from cleavage after Arg96 (data not shown). In the crystal structures of a T7 RNAP-promoter complex and of an IC, the former sites lie in a solvent exposed loop in the N-terminal domain, whereas the latter site forms part of the AT-rich recognition loop that is involved in upstream promoter contacts at –17 (17, 18). As noted below, changes in the accessibility of these sites to trypsin are likely to reflect conformation changes in the enzyme that occur during promoter release and the transition to an EC.

The RNA Cross-links to RNAP in a Similar Manner in Scaffold Complexes and in an EC—As the nascent RNA is displaced from the RNA-DNA hybrid in an EC it becomes associated with an element of the RNAP (the specificity loop) that was earlier involved in promoter recognition (15). Thus, in a halted EC the RNA nucleotide that lies 9 nt upstream from the active site may be cross-linked to a region that includes amino acids 744–750. To determine the nature of RNA-RNAP interactions in a scaffold complex, we synthesized an RNA primer that contains a photocross-linkable uridine derivative, 4-thio-U, 9 nt upstream from the 3′ end and determined the location of the cross-link made to the RNAP in complexes assembled with this RNA (Fig. 6B). Analysis of the products that result from digestion of the cross-linked complex with NTB and CNBr reveals that the base at –9 in the scaffold complex forms a cross-link with the specificity loop in the interval from residues 723 to 750, as is observed in the EC (15). The same results were obtained with a complete scaffold that contains a transcription bubble (data not shown).

**DISCUSSION**

In this work, we used synthetic oligomers of DNA and RNA to assemble nucleic acid scaffolds that allow the formation of functional T7 RNAP transcription complexes. Manipulation of the scaffolds allowed us to examine the contribution of each of the structural elements to transcription activity and salt resistance. The minimal scaffold that allowed efficient extension after challenge with 200 mM NaCl consists of an 8-nt RNA primer hybridized to a DNA template that extends 5–10 nt downstream. Constructs in which the RNA-DNA hybrid is less than or greater than 8 bp are less salt-resistant, and the hybrid cannot be extended beyond 12–13 bp. Although the presence of a complementary NT strand downstream of the primer does not affect salt resistance, the presence of either duplex or template strand DNA upstream of the hybrid decreases salt resistance. The addition of a 4-nt unpaired tail to the 5′ end of the primer increases salt resistance, as does the presence of an unpaired NT strand in the region that contains the 8-bp hybrid (thereby generating an artificial transcription bubble). The minimal scaffold (scaffold 7), as well as complexes containing upstream and downstream elements, together with the bubble (scaffold 20), exhibit many of the properties of a promoter-initiated elongation complex, including resistance to challenge by salt, trypsin cleavage patterns, and a similar pattern of RNA-RNAP cross-linking. The features of the complete scaffold correspond closely to available information concerning the organization of a halted T7 RNAP EC (14, 15, 22). The RNA-DNA hybrid length is 8 base pairs, there is a 1-nt gap between the 3′ end of the RNA and the displaced template strand, and there is no gap between the displaced RNA at the upstream end of the RNA-DNA hybrid and the trailing edge of the transcription bubble. In Fig. 6A we show that although trypsin cleaves the IC at positions 173 and 180, these sites become less accessible in the EC and that cleavage at Arg96 becomes more prominent. The reason for this switch in cleavage sites is understandable in light of recent cross-linking experiments that probe changes in the structural organization of the transcription complex during the transition from an IC to an EC (40). Whereas the cleavage sites at positions 173 and 180 are in a solvent-exposed loop that is not in contact with nucleic acids in the IC, after isomerization this region becomes associated with the template and nontemplate strands of the DNA at the upstream edge of the transcription bubble (19, 40). In contrast, Arg96 is in close association with the upstream region of the promoter prior to isomerization, because it forms part of the AT-rich recognition loop that contacts the promoter in the –17 region, but these contacts are released during the transition. Thus, the switch in cleavage sensitivity observed here is the signature of a transcription complex that has cleared the promoter and isomerized to a stable EC. These results are consistent with the results of UV laser cross-linking experiments demonstrating that the interaction between the RNAP and the promoter at –17 is a characteristic of a late IC that is lost upon the transition to an EC (5).

In previous work, the laboratory of Peter von Hippel explored the use of similar “complete” scaffold templates to study elongation by T7 RNAP but observed poor efficiency of primer extension (27, 28). As shown in Fig. 5, this is likely due to the length of the RNA-DNA hybrid length used in the earlier studies (12 bp, as opposed to 8 bp in the current study). We found that an 8-bp hybrid may only be extended to 12–13 bp and that further extension results in dissociation of the complex. Liu and Martin (13) have shown that during initiation the RNA-DNA hybrid may achieve a length of 10–11 bp before the bubble collapses to 8 bp and the complex isomerizes to a stable EC. Structures involving a hybrid of >8 bp may therefore represent an interesting subject for studies of a strained IC that forms just before isomerization to an EC.

The stable scaffold complexes described here remain active after storage of up to 1 week in the absence of salt, suggesting that they may prove useful in structural studies. Importantly, the scaffolds do not involve a T7 promoter sequence and thus allow the characterization of mutant phase RNAPs that are defective in promoter binding and/or the early stages of transcription.

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