Functional Transcription Elongation Complexes from Synthetic RNA-DNA Bubble Duplexes

Shirley S. Daube and Peter H. von Hippel

A synthetic RNA-DNA bubble duplex construct intended to mimic the nucleic acid framework of a functional transcription elongation complex was designed and assembled. The construct consisted of a double-stranded DNA duplex of variable length (the template and nontemplate strands) containing an internal noncomplementary DNA "bubble" sequence. The 3′ end of an RNA oligonucleotide that is partially complementary to the template DNA strand was hybridized within the DNA bubble to form an RNA-DNA duplex with a noncomplementary 5′-terminal RNA tail. The addition of either *Escherichia coli* or T7 RNA polymerase to this construct formed a complex that synthesized RNA with good efficiency from the hybridized RNA primer in a template-directed and processive manner, and displayed other features of a normal promoter-initiated transcription elongation complex. Other such constructs can be designed to examine many of the functional and regulatory properties of transcription systems.

DNA-dependent RNA polymerases from different organisms are not identical. However, most RNA polymerases that have been studied synthesize RNA by the same general mechanism. The functional steps through which the polymerase passes in transcribing an RNA molecule occur in three phases: initiation, elongation, and termination. The transcription cycle in which the reaction mechanisms of these phases has been studied in most detail is that of *Escherichia coli* (*1–3*). During initiation, the *E. coli* RNA polymerase functions as a holoenzyme; it contains the multisubunit ($\alpha_2\beta\beta'$) core polymerase together with a promoter specificity (sigma, σ) subunit. At the end of the initiation phase the sigma subunit is released, and the core polymerase undergoes a major conformational change, yielding a stable and highly processive elongation complex that completes synthesis of the nascent transcript without dissociation from the DNA template.

The elongation complex differs from the initiation complex both structurally and functionally; these differences are manifested in part as changes in the affinity of the core polymerase within the complex for specific transcription factors. When the transcribing polymerase reaches a terminator it releases the nascent RNA, dissociates from the template, and relaxes into its solution conformation. In this form it can again bind a sigma subunit, re-form the holoenzyme, and initiate a new round of transcription.

Homogeneous and fully functional elongation complexes that have been "stalled" (halted) at defined template positions and are suitable for study in solution have been difficult to prepare. Three general strategies have been used to form such complexes. (i) All four nucleotide triphosphates (NTP's) are added to preformed, open promoter-holoenzyme complexes, and synthesis is allowed to proceed for a short time. The reaction is then quenched by the addition of EDTA, which removes the required Mg^{2+} cofactor and stops transcription before most of the transcription complexes have reached terminator sites. The end result is a mixture of complexes positioned along the template at different sites (*4, 5*). (ii) If complexes positioned at a specific template site are required, the template DNA downstream of the promoter can be designed or modified to contain a sequence-specific block to the synthesizing RNA polymer. This block may, for example, be a specific psoralen crosslink or a high-affinity binding site complexed with a specific protein (*6, 7*). This strategy does halt the advancing elongation complexes at a defined template position, but further opportunities to study the complex may be limited by the presence of the block or by the conditions (for example, high salt concentrations) required to remove the blocking agent. (iii) Probably the most useful general approach has been to add only three of the four NTP's to the open promoter-holoenzyme complex and allow synthesis to proceed. In this "missing nucleotide" strategy, transcription ceases specifically at the first template site that requires the incorporation of the missing (fourth) NTP (*8*). Although this approach has generally been the method of choice for obtaining populations of transcription complexes specifically stalled at defined positions, nucleotide misincorporation and NTP contamination permit significant readthrough, resulting in positional heterogeneity even with this relatively straightforward technique (*9*).

We have used DNA and RNA oligonucleotides to assemble synthetic nucleic acid constructs that are designed to avoid these difficulties and to produce defined and homogeneous solutions of specifically positioned transcription elongation complexes. These constructs incorporate structural properties characteristic of the elongation complex, and we have designed them to bypass the need for a promoter. The main feature is a preexisting DNA bubble flanked by double-stranded DNA, which permits a defined RNA primer complementary to the template strand to form a specifically positioned RNA-DNA hybrid within the bubble. In principle, it should then be possible to add core RNA polymerase directly to such constructs "in trans," and thus to obtain specifically positioned and functional elongation complexes.

The RNA-DNA bubble duplex construct. The following structural aspects of real elongation complexes were considered in the design of our nucleic acid construct (*3, 10*). (i) The central feature of an elongation (as opposed to an initiation) complex is the presence of a nascent RNA chain at least 12 nucleotide (nt) residues in length, which serves as a primer for further template-directed RNA chain extension. (ii) Although there is at present some controversy over the steady-state length of the RNA-DNA hybrid within the transcription bubble (*11, 12*), a minimum of 3, and perhaps as many as 12, nucleotide residues at the 3′ end of the growing RNA chain are hybridized to the template DNA strand at all times. In these complexes, the nascent RNA that is more than 12 residues upstream from the 3′ OH-terminus is displaced from the DNA or the polymerase, creating in solution a growing RNA tail that may participate in the regulatory aspects of transcript elongation and termination. (iii) The DNA of natural templates undergoing transcription elongation is melted (by the polymerase) to form an unpaired bubble, within which the 3′ terminal residues of the growing RNA chain are hybridized to the template DNA strand with the catalytic active site of the polymerase located at the downstream end of the bubble. The DNA upstream and downstream of the bubble is double-stranded.

Our designed construct (Fig. 1) fulfills these structural requirements. It consists of an RNA strand that is 20 nt in length, with the 12 nt at the 3′ end being fully complementary to the DNA template strand. The remaining 8 nt are not complementary to the template strand, thus forming the be-

The authors are at the Institute of Molecular Biology and in the Department of Chemistry, University of Oregon, Eugene, OR 97403.

ginning of a free (single-stranded) RNA tail. An additional DNA oligonucleotide serves as the nontemplate strand in the construct. This latter DNA strand is only partially complementary to the DNA template strand, forming a permanently unpaired DNA bubble that accommodates the complementary portion of the RNA strand and allows it to anneal to the template strand within the bubble. The components of this construct (that is, the length of the double-stranded segments, the length of the RNA-DNA hybrid, and the size of the noncomplementary DNA bubble) can all be varied by changing the length and sequence of the constituent oligonucleotides (Fig. 1).

The formation of these constructs (Fig. 1) can be monitored by a nondenaturing gel shift assay. Addition of the template DNA strand to the 5′ end-labeled RNA (Fig. 2, lane 1) shifted the position of the ^{32}P-labeled band, an indication of greater molecular size that corresponds to an RNA-DNA (primer-template) complex (Fig. 2, lane 2). The addition of the nontemplate DNA strand to the preformed primer-template complex shifted the ^{32}P-labeled band to a still lower mobility position, reflecting the formation of a stable three-component nucleic acid complex (Fig. 2, lane 3). The addition of the nontemplate DNA strand only to the labeled RNA did not alter the rate of migration of the latter (*13*), as would be expected if only specific hybridization reactions (Fig. 1) can occur.

The specific formation of the expected construct was further confirmed by testing its sensitivity to cleavage by ribonuclease H. Upon the addition of ribonuclease H to the RNA-DNA bubble duplex construct the 12-nt fragment at the 3′ end of the RNA strand was cleaved as expected, while the 8-nt piece of the unpaired RNA tail remained intact.

RNA primer extension by RNA polymerase. When polymerase was added to primed bubble duplex, the ^{32}P-labeled band was discretely shifted to a still lower mobility position on a nondenaturing gel (Fig. 2, lane 4). A transcription assay was applied (*14*) to demonstrate that the added polymerase not only binds, but can also extend, the RNA primer. Either the core *E. coli* RNA polymerase or the holoenzyme (*15*) was added to the nucleic acid construct, together with all four NTP's (but without Mg^{2+}). Synthesis was initiated by the addition of Mg^{2+} and was terminated by EDTA. The products of the transcription reactions were resolved by denaturing gel electrophoresis (*16*). No enzymatic activity was manifested in the absence of Mg^{2+} (Fig. 3, lanes 1 and 2). The addition of Mg^{2+} resulted in extension of the RNA, as indicated by the appearance of longer RNA transcripts (Fig. 3, lane 3).

In order to ensure that the observed reaction represents "single-hit" primer extension, heparin was added with the initiating Mg^{2+} to serve as a trap for any polymerase that may have dissociated from the synthetic elongation complex (*17*). This assay distinguishes between a fully processive mode of synthesis, in which the polymerase binds to the 3′ OH and extends the RNA primer to the end of the template without dissociation, and a less processive mode in which the polymerase dissociates from and rebinds to the primer-template junction many times in completing each round of transcript synthesis. Except for an initial tendency of the polymerase to dissociate after incorporation of one or two nucleotide residues, most of the initiating polymerases do indeed synthesize processively to the end of the template strand (Fig. 3, lane 3). This was demonstrated by the accumulation of RNA products that were 68 to 72 nt in length, with 72 nt corresponding to the expected full-length product (Fig. 1).

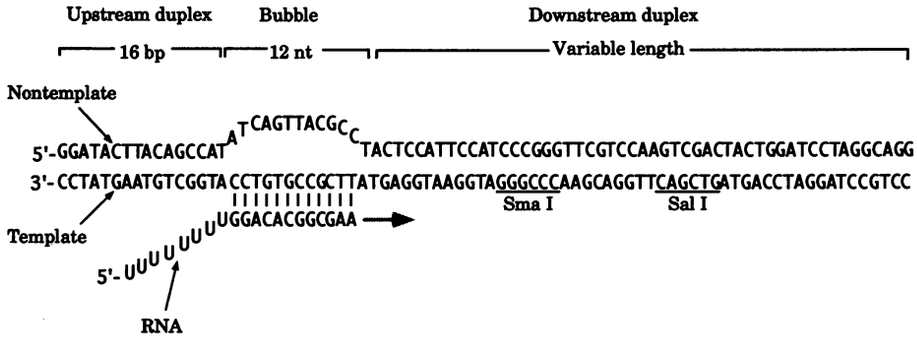

Fig. 1. Design of the synthetic nucleic acid construct. The lengths and sequences of the upstream DNA duplex, the DNA bubble, and the downstream DNA duplex, as well as the RNA primer, are indicated. The sequences of the upstream duplex as well as the template strand of the bubble region are identical to sequences upstream of the A^{20} position following the T7A1 promoter. The sequence of the nontemplate strand in the bubble region was randomly chosen to prevent hybridization with the template strand. The sequence of the downstream duplex was also random and was designed to contain a C-lacking cassette as well as restriction sites. The predicted direction of RNA synthesis is indicated by the stippled arrow. Horizontal lines represent base pairing between the template DNA and the RNA oligonucleotide. The restriction sites Sma I and Sal I within the downstream duplex are underlined. The 20-nt RNA was synthesized on an Applied Biosystems DNA Synthesizer (Model 380B), by the standard phosphoramidite procedure specified for this instrument. The resulting RNA was purified and deprotected (*29*), with slight modifications. The DNA oligonucleotides pictured were obtained (Midland Company, Midland, Texas) in polyacrylamide gel–purified form. Oligonucleotide concentrations were determined by ultraviolet absorbance with the use of a calculated molar extinction coefficient (at 260 nm) of 1.7×10^5 M^{-1} for the RNA 20-nt oligonucleotide (*30*) and 8×10^5 M^{-1} for the two DNA 80-nt oligonucleotides.

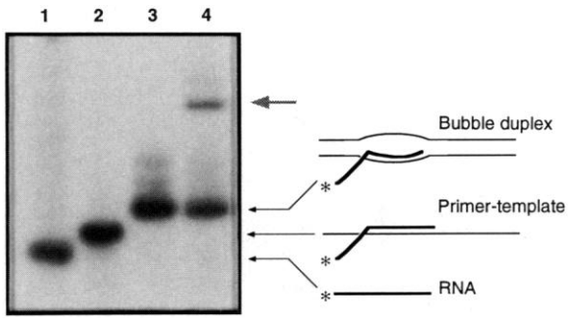

Fig. 2. RNA polymerase binding to a stable nucleic acid construct. Free RNA, the primer-template complex, and the RNA-DNA bubble duplex, in the absence and presence of *E. coli* core RNA polymerase were resolved on a nondenaturing polyacrylamide gel by a band shift assay. To this end, hybridized samples (*31*) were diluted with loading buffer to a final concentration of 8 mM magnesium acetate, 6 percent glycerol, 0.25 percent xylene cylanol, and 0.25 percent bromophenol blue, and placed on a 5 percent nondenaturing polyacrylamide gel (bis:acrylamide ratio of 1:112) in a buffer system containing 8 mM magnesium acetate and 1× TAE buffer [40 mM tris-acetate (pH 8.0) and 2 mM EDTA]. Gels were dried on Whatman 3MM paper and autoradiographed onto x-ray film (Fuji Aif). (Lane 1) Free RNA; (lane 2) RNA hybridized to the template DNA oligonucleotide; (lane 3) RNA hybridized to the template and nontemplate DNA oligonucleotides; (lane 4) bubble duplex incubated with *E. coli* core RNA polymerase as described in (*14*) except that NTP's were omitted from the reaction mixture and Mg^{2+} was added. Nucleic acid structures corresponding to bands with different mobilities are shown along the right side of the gel. The gray arrow marks the shifted position of the bubble duplex by *E. coli* core RNA polymerase. The asterisk (*) represents the ^{32}P-label at the 5′ end of the RNA.

RNA synthesis that is initiated from a promoter is directed by the sequence of the DNA template. To demonstrate that synthesis in our constructs is also template-directed, we initiated reactions that contained only ATP, CTP, and UTP. The longest RNA product obtained in this experiment was 36 residues (Fig. 3, lane 4), corresponding exactly to the first template position at which the next nucleotide required for RNA extension was the omitted GTP (Fig. 1, relevant sequence). As in promoter-initiated elongation complexes, RNA polymerase stalls at this missing nucleotide position and can then resume synthesis (be chased) on the addition of GTP (Fig. 3, lane 5).

In addition to its resistance to heparin (*17*), the RNA polymerase bound to the bubble duplex was also resistant to rifampicin. Thus, no effect on product formation was observed when rifampicin was added to RNA polymerase bound to the bubble duplex prior to the synthesis (*13*). This resistance to heparin and to rifampicin is characteristic of elongation complexes, and is in contrast (i) to the absolute inhibition by rifampicin of de novo synthesis by holoenzyme in transcript initiation from open promoter complexes (*18*), and (ii) to the sensitivity of open promoter complexes to heparin (*19*).

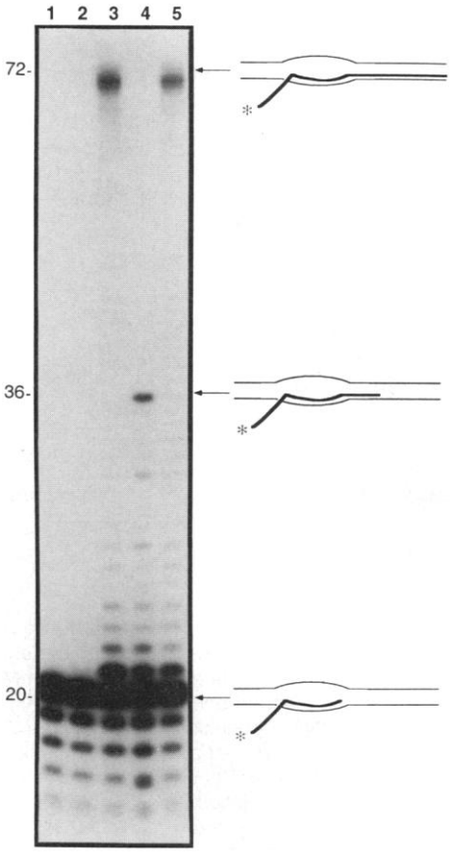

Fig. 3. RNA primer extension by *E. coli* core RNA polymerase. Transcription assays were performed by combining *E. coli* core RNA polymerase and the end-labeled bubble duplex (*14*) in various incubation schemes, and the reaction products were resolved by denaturing polyacrylamide gel electrophoresis (*16*). Stalled complexes were obtained by following the above transcription protocol, except that only ATP, CTP, and UTP (each at a final concentration of 5 μM) were first incubated with the nucleic acid components and the *E. coli* core RNA polymerase at 30°C for 2 minutes. Reactions were then incubated for 2 minutes after the addition of Mg^{2+} and heparin (*14*). A portion was then quenched with 100 mM EDTA, while all four NTP's (at a final concentration of 1 mM) were added to the remaining transcription solution for an additional 2 minutes before the addition of EDTA. (Lane 1) The bubble duplex incubated in transcription buffer in the absence of enzyme and Mg^{2+}; (lane 2) the bubble duplex incubated with *E. coli* core RNA polymerase and all four NTP's, but without Mg^{2+}; (lane 3) identical to lane 2 except that Mg^{2+} (and a heparin trap) were added at time zero; (lane 4) stalled complexes formed by omitting GTP from the reaction mixture; (lane 5) the RNA products obtained by "chasing" the stalled complexes (lane 4) with all four NTP's. The diagrams alongside the gel represent the extent to which the RNA has invaded the downstream duplex. Numbers on the left side of the gel are the length (in nucleotide residues) of the RNA products that have migrated to the corresponding position in the gel.

The above data (Fig. 3) were obtained by addition of core *E. coli* RNA polymerase to the nucleic acid constructs. Qualitatively similar results were obtained with the *E. coli* holoenzyme (*13*), suggesting that the sigma subunit was not required for RNA synthesis from these constructs. These findings indicate that functional elongation complexes were formed after the addition of RNA polymerase directly (in trans) to these RNA-DNA bubble duplex complexes.

An important characteristic of RNA polymerases is the high processivity with which they transcribe RNA on natural templates after the initiation phase of transcription is complete. Only at a terminator does the polymerase dissociate from the DNA template and release the nascent RNA chain. The *E. coli* RNA polymerase also functions processively when extending the RNA primer within our synthetic constructs (Fig. 3). However, the degree of processivity observed seemed to vary somewhat depending on the details of the construct.

We therefore investigated the relation between the length of the downstream duplex and the degree of processivity. Digestion of the downstream DNA at defined restriction sites (Fig. 1) provided constructs that differed only in downstream duplex length. Comparison of the products from transcription of the 52-bp duplex (Fig. 4, lane 2) with those of the 33-bp duplex (Fig. 4, lane 3) and the 16-bp duplex (Fig. 4, lane 4) revealed complexes displaying two classes of processivity. Low processivity was

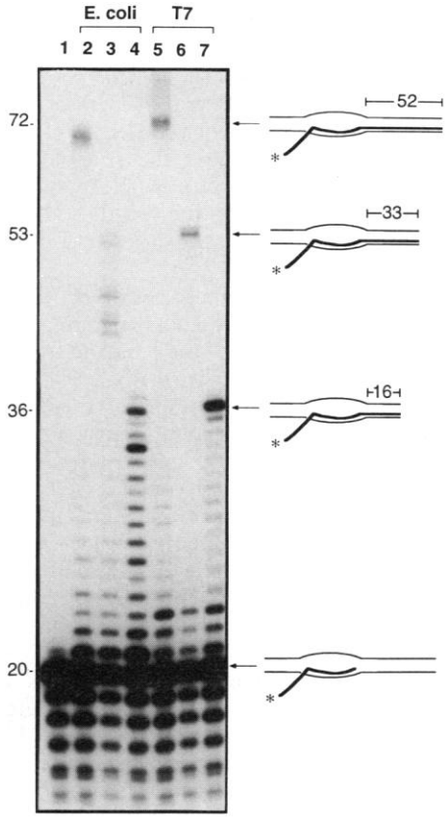

Fig. 4. Processive synthesis by *E. coli* and T7 RNA polymerases. The processivities of *E. coli* (lanes 2 to 4) and T7 (lanes 5 to 7) RNA polymerase are demonstrated with RNA-DNA bubble duplex constructs that have varying lengths of downstream DNA (*32*). Lanes 2 and 5 correspond to synthesis on the original bubble duplex. Lanes 3 and 6 show synthesis on bubble duplexes digested with Sal I, and lanes 4 and 7 show synthesis on bubble duplexes digested with Sma I. Lane 1 is a control to which no Mg^{2+} has been added. Structures of bubble duplexes digested to different extents are indicated to the right of the gel; the length of the downstream duplex is indicated. The corresponding number on the left side of the gel represents the length (in nucleotide residues) of the full-length RNA product. Transcription with T7 RNA polymerase was performed by first incubating 200 units of the enzyme with the appropriate nucleic acid (30 to 50 nM) construct at 37°C. The rest of the transcription protocol with T7 polymerase was identical to that used for *E. coli* RNA polymerase, except that the salt concentration (30 mM potassium glutamate) was lower. Control transcription reactions with *E. coli* core RNA polymerase at this low salt concentration resulted in patterns similar to those obtained at high salt, an indication that the properties of the two enzymes transcribing within these transcription complexes can legitimately be compared.

exhibited by the polymerase for incorporation of the first few nucleotides on all three constructs. Synthesis of downstream sequences showed low processivity for the 16-bp duplex, but became highly processive as the length of the downstream duplex increased (see below).

When these experiments were repeated with T7 RNA polymerase (Fig. 4, lanes 5 to 7), the T7 RNA polymerase showed processive synthesis with these constructs at all downstream duplex lengths (Fig. 4, lanes 2, 3, and 4 with lanes 5, 6, and 7, respectively). In addition to validating the generality of our findings, this result demonstrated that the T7 polymerase is less demanding than the *E. coli* enzyme in its structural requirements for processive synthesis.

Transcription on the primer-template construct (Fig. 5, lane 2) was less processive than that with the complete RNA-DNA bubble duplex construct (Fig. 5, lane 3); almost no full-length products were obtained with the primer-template construct in the presence of a heparin trap. This result shows that interactions between the nontemplate DNA strand and the *E. coli* polymerase are essential for the highly processive synthesis seen with promoter-initiated elongation complexes.

Comparison of initiation and elongation modes of RNA transcription. We have used a combination of RNA and DNA oligonucleotides to put together a nucleic acid structure that we have termed the RNA-DNA bubble duplex. This synthetic construct can work as a template and substrate for both the *E. coli* and the T7 RNA polymerases. Although technical considerations provided the initial impetus for this synthetic approach to elongation, it is clear that many mechanistic problems can be considered in this way, including questions that cannot be addressed by studying elongation complexes formed by conventional means.

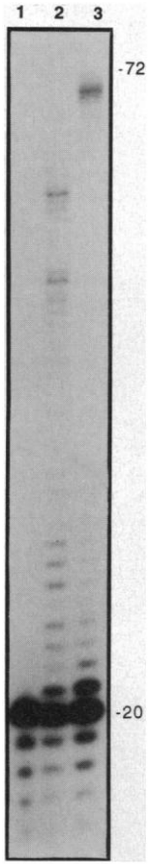

Fig. 5. Transcription on primer-template and RNA-DNA bubble duplex. *E. coli* core RNA polymerase was incubated with either the primer-template (lane 2) or the RNA-DNA bubble duplex (lane 3) construct, and allowed to extend the ^{32}P-labeled RNA primer by the addition of Mg^{2+} and heparin (except in lane 1, which is a control). The numbers indicate the length of the original RNA primer (20 nt) and the expected full-length product (72 nt).

Nonphysiological DNA structures, including poly[d(AT)] (*20, 21*) and poly(rC)-tailed templates (*22*), have been used in special situations to initiate transcription without passing through a promoter. In an approach that may be somewhat closer to ours, Martin *et al.* (*23*) used synthetic DNA oligonucleotides to study transcript initiation by T7 RNA polymerase. However, the direct formation of a functional elongation complex with synthetic oligonucleotides has not, to our knowledge, been previously demonstrated.

Perhaps the most basic concept that is challenged by the use of the RNA-DNA bubble duplex construct is that transcription must pass through the promoter-polymerase complex initiation phase to result in the formation of a stable and functional elongation complex. The successful extension of an RNA primer by T7 and *E. coli* RNA polymerase in the absence of promoter sequences suggests that the nucleic acid framework itself suffices to induce the RNA polymerase to undergo the conformational rearrangements required for the formation of a stable and processive elongation complex.

The resistance of *E. coli* RNA polymerase to rifampicin when bound to the RNA-DNA bubble duplex supports the view that our artificial construct closely resembles an elongation complex in this respect. We have shown that rifampicin does inhibit RNA synthesis when the polymerase is incubated with the drug before being added to the RNA-DNA bubble duplex (*13*). Our data are consistent with the view that polymerase uses the same binding site for the nascent RNA and for rifampicin. Such order-of-addition experiments could not have been performed with "stalled" (promoter-initiated) elongation complexes formed in the conventional manner.

The central observation (Figs. 3 to 5) that the RNA primer within the bubble duplex construct can be extended processively is consistent with the proposal that structures characterized by many of the features of natural elongation complexes can be successfully formed by the addition of core polymerase in trans to synthetic constructs of this type. Whether a fully functional elongation complex, subject to physiological regulation by template and nascent RNA sequence as well as by added protein factors, has indeed been formed remains to be determined.

An observation made in our study that requires further investigation is that although a molar excess of RNA polymerase to nucleic acid construct was maintained throughout our experiments, the primers within the bubble constructs were reproducibly extended with 40 to 50 percent efficiency as determined by quantitation of individual lanes in the gels (*13*). Since direct binding (Fig. 2, lane 4) also showed polymerase binding efficiencies of 40 to 50 percent to the constructs, we can rule out the formal possibility that some polymerase molecules that could bind to the synthetic complexes might not be able to extend the RNA primer, and thus block access of fully active polymerase molecules to these constructs. Instead we have some evidence that excess single-stranded DNA oligonucleotides present in the reaction mixture may have formed complexes with some of the polymerase molecules and prevented them from binding to the rest of the RNA-DNA bubble constructs (*13*). This complication does not affect our demonstration of specific extension of an RNA primer in a processive and template-dependent manner, but should be avoided to increase the subsequent efficient utilization of these constructs.

Structural requirements for processive transcription. One possible interpretation of the correlation between the length of the downstream duplex and the processivity observed (Fig. 4, lanes 2 to 4) is that during the initial steps of RNA primer extension the total DNA bubble size is increased. This size increase can be attributed to the fact that the sequences of the template and nontemplate strands located behind the bubble in its initial stages are not complementary and thus cannot reclose. Only on constructs with long enough downstream duplexes can the complex move into a region in which these strands can rehybridize behind the complex, allowing the transcription bubble to return to its original size. This allows the polymerase to maintain the simultaneous contacts with the double-stranded DNA regions ahead and behind the complex that seem to be required for processive synthesis. This interpretation is consistent with the results of foot-printing studies of stalled and paused elongation complexes (*24–26*) and with the effect of downstream sequences on transcriptional pausing (*27*).

Our synthetic constructs are equally useful for investigating transcription by T7 RNA polymerase. The RNA synthesis catalyzed by the T7 polymerase is processive on all the constructs that we have used (Fig. 4, lanes 5 to 7). This observation is

consistent with the faster rate of transcription catalyzed by T7 polymerase compared to that catalyzed by *E. coli* enzyme, since a higher intrinsic synthesis rate should allow the elongation reaction to compete with polymerase dissociation processes that might be potentiated by unstable nucleic acid structures (such as those encountered by the polymerase near the noncomplementary DNA bubble) (*12*). Alternatively, the high processivity displayed by T7 RNA polymerase, even on short downstream duplexes (Fig. 4, lanes 5 and 6), may be only a reflection of its smaller size (*28*).

REFERENCES AND NOTES

1. P. H. von Hippel, D. G. Bear, W. D. Morgan, J. A. McSwiggen, *Annu. Rev. Biochem.* **53**, 389 (1984).
2. W. R. McClure, *ibid.* **54**, 171 (1985).
3. T. D. Yager and P. H. von Hippel, in Escherichia coli *and* Salmonella typhimurium: *Cellular and Molecular Biology*, F. C. Neidhardt, Ed. (American Society for Microbiology, Washington, DC, 1987), pp. 1241–1275.
4. G. Rhodes and M. J. Chamberlin, *J. Biol. Chem.* **249**, 6675 (1974).
5. K. M. Arndt and M. J. Chamberlin, *J. Mol. Biol.* **213**, 79 (1990).
6. Y.-B. Shi, H. Gamper, B. Van Houter, J. E. Hearst, *ibid.* **199**, 277 (1988).
7. P. A. Pavco and D. A. Steege, *J. Biol. Chem.* **265**, 9960 (1990).
8. J. R. Levin, B. Krummel, M. J. Chamberlin, *J. Mol. Biol.* **196**, 85 (1987).
9. D. A. Erie, T. D. Yager, P. H. von Hippel, *Annu. Rev. Biophys. Biomol. Struct.* **21**, 379 (1992).
10. T. D. Yager and P. H. von Hippel, *Biochemistry* **4**, 1097 (1991).
11. G. A. Rice, C. M. Kane, M. J. Chamberlin, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4245 (1991).
12. P. H. von Hippel and T. D. Yager, *Science* **255**, 809 (1992).
13. S. S. Daube and P. H. von Hippel, unpublished observation.
14. For transcription assays, the desired nucleic acid construct (at a total molar concentration of 30 to 50 nM complex) was incubated at 30°C with *E. coli* core RNA polymerase (100 to 150 nM) in transcription buffer containing 1 mM DTT and (at 1 mM each) ATP, CTP, GTP, and UTP. After 2 minutes, single-round transcription reactions were started by the addition of magnesium glutamate and heparin to a final concentration of 10 mM and 250 μg/ml, respectively. The complete (10 μl) mixture was again incubated for 2 minutes and EDTA was added to a final concentration of 100 mM. Mixed yeast tRNA (Boehringer Mannheim) at 200 μg/ml was added to each reaction as a carrier, and samples were subjected to extraction with phenol and chloroform.
15. Purified *E. coli* core and holoenzyme RNA polymerase were provided by D.-J. Jin (NCI, NIH) and W. Rees (our laboratory), respectively. The source of the T7 RNA polymerase (100 U/μl) was U.S. Biochemicals.
16. After transcription had been terminated, the nucleic acid components of the reaction were precipitated with ethanol and centrifuged. Pellets were dried, resuspended in formamide loading buffer, and the suspension was boiled for 2 minutes. Samples were then chilled rapidly and subjected to denaturing gel electrophoresis on 12 percent polyacrylamide (20:1 acrylamide:bis) gels (0.4 mm) containing 8 M urea (50°C, 1.5 hours, 40 W).
17. Heparin dilution experiments showed that heparin was not an active trap for RNA polymerase under these assay conditions; heparin at the concentrations used can still trap any RNA polymerase in solution but does not compete with the construct for transcribing polymerase [see M. K. Reddy, S. E. Weitzel, P. H. von Hippel, *J. Biol. Chem.* **267**, 14157 (1992)].
18. A. Sippel and G. Hartmann, *Biochim. Biophys. Acta* **157**, 218 (1968).
19. S. R. Pfeffer, S. J. Stahl, M. J. Chamberlin, *J. Biol. Chem.* **252**, 5403 (1977).
20. U. M. Hansen and W. R. McClure, *ibid.* **255**, 9564 (1980).
21. A. R. Wheeler, A.-Y. M. Woody, R. W. Woody, *Biochemistry* **26**, 3322 (1987).
22. T. R. Kadesch and M. J. Chamberlin, *J. Biol. Chem.* **257**, 5286 (1982).
23. C. T. Martin, D. K. Muller, J. E. Coleman, *Biochemistry* **27**, 3966 (1988).
24. M. Kainz and J. Roberts, *Science* **255**, 838 (1992).
25. D. N. Lee and R. Landick, *J. Mol. Biol.*, in press.
26. W. Metzger, P. Schickor, H. Heumann, *EMBO J.* **8**, 2745 (1989).
27. D. N. Lee, L. Phung, J. Stewart, R. Landick, *J. Biol. Chem.* **265**, 15145 (1990).
28. M. J. Chamberlin and J. Ring, *ibid.* **148**, 2235 (1973).
29. K. R. Webster, Y. Shamoo, W. Konigsberg, E. K. Spicer, *BioTechniques* **11**, 658 (1991).
30. M. M. Warshaw and C. R. Cantor, *Biopolymers* **9**, 1079 (1970).
31. For assembly of the nucleic acid constructs, the 5′ terminus of the synthesized RNA primer strand (100 pmol) was labeled with [γ-^{32}P]ATP (New England Nuclear, Dupont) in the presence of T4 polynucleotide kinase (U.S. Biochemicals) (*33*). This 5′-labeled RNA oligonucleotide was hybridized either with the template-strand DNA oligonucleotide, yielding a primer-template construct, or with both the template- and the nontemplate-strand DNA oligonucleotides, yielding an RNA-primed DNA bubble duplex. The oligonucleotides to be hybridized were dissolved in buffer containing 20 mM Hepes, pH 8.0, 20 mM sodium acetate, 10 mM magnesium acetate, and 0.1 mM EDTA, at a total oligonucleotide strand concentration exceeding 1 μM and at a ratio of 1.2:1 for each DNA strand to RNA primer strand so that maximum hybridization of the labeled RNA was attained. The hybridization reaction was allowed to proceed at 70°C for 5 minutes; the samples were slow-cooled to room temperature over a 2-hour period. If DNA constructs only were desired (that is, for restriction enzyme treatment of bubble duplexes), the same procedures were used, except that the labeled RNA was omitted from the hybridization mixture (*32*). To separate nucleic acid components from free (labeled) ATP and Mg^{2+}, the primer-template and primer-bubble duplex mixtures were passed through Bio-Spin 30 columns (Bio-Rad) that had first been equilibrated with transcription buffer containing 20 mM Hepes, pH 8.0, 150 mM potassium glutamate, 1.0 mM EDTA, and acetylated bovine serum albumin at 125 μg/ml (U.S. Biochemicals). Free (labeled) RNA was separated from small molecule contaminants by passing the solution through a Bio-Spin 6 (column) that had been equilibrated with the same buffer. The purity and yield of the eluted nucleic acids were determined on thin-layer chromatography plates (EM Science), which were then quantified on a radioanalytic scanner (AMBIS, San Diego, CA).
32. DNA bubble duplex constructs were cut at specific restriction sites (Fig. 1) after hybridization of the template and nontemplate DNA strands. Portions were incubated overnight at room temperature with Sma I (Boehringer Mannheim), or at 37°C with Sal I (Boehringer Mannheim), each in their optimal buffers. The restriction enzymes were then inactivated by heating the samples for 10 minutes at 70°C. The resultant DNA bubble duplexes were separated from Mg^{2+} on Bio-Spin 30 columns as described above, and hybridized with the end-labeled RNA primer by reheating the solution at 70°C for 5 minutes, and then slowly cooling to room temperature.
33. T. Maniatis, E. F. Fritsch, J. Sambrook, in *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
34. This work is being submitted (by S.S.D.) to the Graduate School of the University of Oregon in partial fulfillment of the requirements for the Ph.D. degree in Chemistry. We thank our laboratory colleagues for useful suggestions; M. Reddy for insights, support, and comments; D. Jin for polymerase; and A. Das, D. Lee, and R. Landick for discussions. Supported in part by USPHS research grants GM-15792 and GM-29158 (to P.H.v.H.), by a grant to the University of Oregon from the Lucille P. Markey Charitable Trust, by the U.S. Department of Education program for Graduate Assistance in Areas of National Need (S.S.D.), and by an American Cancer Society Research Professorship (P.H.v.H.).

3 June 1992; accepted 20 October 1992