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RNA template-directed RNA synthesis by T7 RNA polymerase

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ABSTRACT **In an attempt to synthesize an oligoribonucleotide by run-off transcription by bacteriophage T7 RNA polymerase, a major transcript was produced that was much longer than expected. Analysis of the reaction indicated that the product resulted from initial DNA-directed run-off transcription followed by RNA template-directed RNA synthesis. This reaction occurred because the RNA made from the DNA template displayed self-complementarity at its 3′ end and therefore could form an intra- or intermolecular primed template. In reactions containing only an RNA template, the rate of incorporation of NTPs was quite comparable to DNA-dependent transcription. RNA template-directed RNA synthesis has been found to occur with a great number of oligoribonucleotides, even with primed templates that are only marginally stable. In one instance, we observed a multistep extension reaction converting the oligonucleotide into a final product longer than twice its original length. Presumably, such a process could have generated some of the RNAs found to be efficiently replicated by T7 RNA polymerase.**

The RNA polymerases from bacteriophage T3, T7, and SP6 have been widely used to prepare RNAs for biochemical and biophysical studies (1). A 17-nucleotide 5′ promoter sequence directs efficient initiation at a unique position on the DNA template (2). While the transcription generally initiates with a nucleoside triphosphate (NTP), extension of a mono-, di-, or trinucleotide primer can occur if it is present in high concentration and has a sequence appropriate to initiate at or near the normal start site (3). In the absence of a promoter, bacteriophage polymerases can bind to the end of a DNA fragment and, with much lower efficiency, produce an "end-to-end" transcription product (4).

In most *in vitro* transcription reactions, the expected run-off RNA corresponding precisely to the template sequence is not the only product obtained. A number of abortive initiation products of 2–8 nucleotides are generally produced in substantial amounts (1, 5). In addition, a fraction of the transcripts have one or two additional, non-template-encoded nucleotides of mixed sequence added to their 3′ end. Finally, several unusual products from *in vitro* transcription reactions have been reported. Krupp (6) has shown that with certain DNA templates that contain no promoter, both T7 and SP6 polymerase can either elongate the template to produce hybrid DNA-RNA molecules or produce RNAs that are multimers of the template sequence, presumably by a rolling-circle mechanism. Konarska and Sharp (7) have reported that certain T7 RNA polymerase preparations contain several RNA species that can be efficiently replicated by the enzyme in reactions that produce two complementary strands. The sequences of these replicating RNAs have substantial symmetry, suggesting that they may fold into hairpin or cloverleaf forms during their replication cycle (8).

In this paper, we analyze an unusual reaction product from an *in vitro* transcription reaction and conclude that it is the result of extension of an RNA-primed RNA template. Further analysis leads to the conclusion that T7 RNA polymerase is extremely efficient in this type of reaction.

MATERIALS AND METHODS

RNA Synthesis. Oligomer **1** (5′-pppGGAAUGUCGGUCG-3′) was synthesized by transcription of partially duplex synthetic DNA templates with T7 RNA polymerase (9). Radiolabeled RNA was obtained by incubating 0.2 μM template with T7 RNA polymerase (0.1 mg/ml) together with 1 mM ATP, 1 mM UTP, 1 mM GTP, 0.2 mM CTP, and 350 μCi of [α-^{32}P]CTP (≈3000 Ci/mmol; 1 Ci = 37 GBq) in 50 μl of 40 mM Tris·HCl, pH 8.1 (at 37°C)/10 mM $MgCl_2$/1 mM spermidine/5 mM dithiothreitol/0.01% Triton X-100 for 1 hr at 37°C. Nonradioactive RNA was obtained similarly except that each NTP was 4 mM, $MgCl_2$ concentration was raised to 25 mM, the final volume was 5 ml, and the incubation was for 3 hr at 37°C. Transcripts were purified by electrophoresis in a 1.5-mm-thick 20% polyacrylamide sequencing gel, located by autoradiography or UV shadowing, eluted by crushing in 0.25 M ammonium acetate/10 mM Tris·HCl, pH 8.0/1 mM EDTA, and concentrated by DEAE chromatography and ethanol precipitation. RNA was dissolved in sterile water and stored at −20°C.

Oligomers **1A** (GAAUGUCGGUCG), **3** (GCUAGCAUCC), **4** (GAAUGUGGAUGC), and **5** (CGGUCG) were chemically synthesized on an Applied Biosystems DNA synthesizer using phosphoramidite chemistry (10), deprotected, and purified by gel electrophoresis. Concentrations of oligomers were determined by their absorbance at 260 nm, with extinction coefficients calculated from their base composition and sequence (11).

5′-End-labeled oligonucleotides were obtained by reaction with T4 polynucleotide kinase and [γ-^{32}P]ATP after prior dephosphorylation with bovine alkaline phosphatase if needed. Extension reactions were performed in the same buffer and same NTP concentrations used for transcription of radioactive RNAs except that the DNA template was omitted and replaced by RNA oligonucleotides. At various times aliquots were removed mixed with an equal volume of "stop" mixture containing 7 M urea, dyes, and 50 mM EDTA; heated for 1 min at 98°C; and loaded on a 20% polyacrylamide sequencing gel.

Sequence and Structure Analyses. The 3′-terminal nucleotide of transcripts was identified by RNase digestion of oligonucleotides that had been 3′ end-labeled with [5′-^{32}P]pCp (12), followed by two-dimensional PEI-cellulose thin-layer chromotography to separate the 3′-NMPs (13). RNAs obtained by transcription or elongated by T7 polymerase in the presence of one [α-^{32}P]NTP were completely digested by RNases and analyzed in the same ways. The relative amounts of 3′-NMPs were quantitated with a PhosphorImager (Molecular Dynamics) (14).

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RNA sequence determination was based on phosphorothioate chemistry (15). Transcription was performed in three separate reactions with three nucleoside [α-thio]triphosphates (ATP[αS], UTP[αS], and CTP[αS] generously provided by Fritz Eckstein, Max-Planck-Institut für Experimentelle Medizin Göttingen, Germany). The transcripts were then purified by gel electrophoresis, dephosphorylated, 5′ end-labeled, heated in the presence of iodoethanol to cause partial cleavage at thiophosphate linkages, and analyzed in a sequencing gel together with 5′ end-labeled oligonucleotide which had been subjected to mild alkaline hydrolysis. The products of extension reactions were sequenced in the same way using 5′-^{32}P oligonucleotides as primers.

Melting curves of oligonucleotides were performed in 40 mM Tris·HCl, pH 8.1 (at 37°C)/6 mM $MgCl_2$. Absorbance was recorded at 260 and 280 nm with data points every 20 sec from 2°C to 70°C. The rate of temperature increase was 30°C/hr.

RESULTS

An Unexpected Transcription Product. The partially double-stranded DNA template shown in Fig. 1*A* was designed (5, 9) to produce a 13-nucleotide RNA molecule upon transcription with T7 RNA polymerase. However, a time course of the reaction (Fig. 1*B*) shows that while the product of the expected length, **1**, was produced early in the reaction, its amount did not increase as the incubation continued. Instead, a substantially longer product, **2**, appeared after a short lag and accumulated in very large amounts. Smaller amounts of even longer products and several abortive initiation (or breakdown) products were observed as well.

Initial attempts to determine the sequence of **2** by means of sequence-specific nucleases (16) were only partially successful due to the unusual resistance of **2** to enzymatic digestion. Despite prior denaturation of the sample and the semidenaturing conditions used for nuclease digestion, only the partial sequence 5′-GGXXUGUCGGUCXXCXXXXX-3′ (X, unknown nucleotide) was obtained (data not shown). While this sequence suggests that at least the first 12 nucleotides are encoded by the DNA template in the expected manner, the sequence of eight additional 3′ nucleotides remained largely unknown. The resistance of **2** to nuclease digestion suggested the presence of extensive secondary structure. This expectation was confirmed by the fact that **2** showed a melting curve with a sharp transition at a high temperature (T_m = 79°C in 0.1 M NaCl/10 mM sodium cacodylate, pH 7.0).

The complete sequence of **2** was deduced by the method of Gish and Eckstein (15) based on phosphorothioate chemistry, which is insensitive to RNA secondary structure (Fig. 2*A*). The presence of a 3′-terminal cytidine residue was confirmed by 3′-end analysis (12, 13). Oligonucleotide **2** has the 13-nucleotide sequence predicted by the DNA template, but the 13-mer was extended by an additional 7 residues that are complementary to part of itself. Oligomer **2** is therefore a self-complementary RNA that could form either an intramolecular hairpin or an intermolecular duplex (Fig. 2*B*), thereby accounting for its high T_m and nuclease resistance.

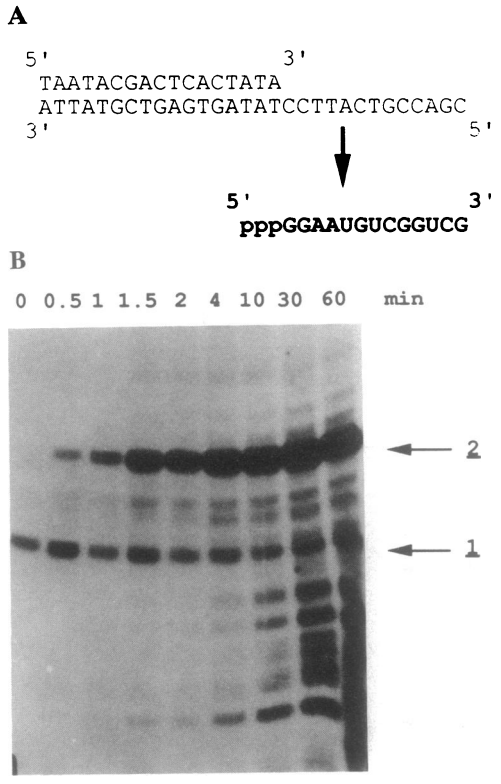

FIG. 1. (*A*) Transcription from synthetic DNA (5) to give the expected product, oligomer **1**. (*B*) Time course of the transcription reaction depicted in *A*.

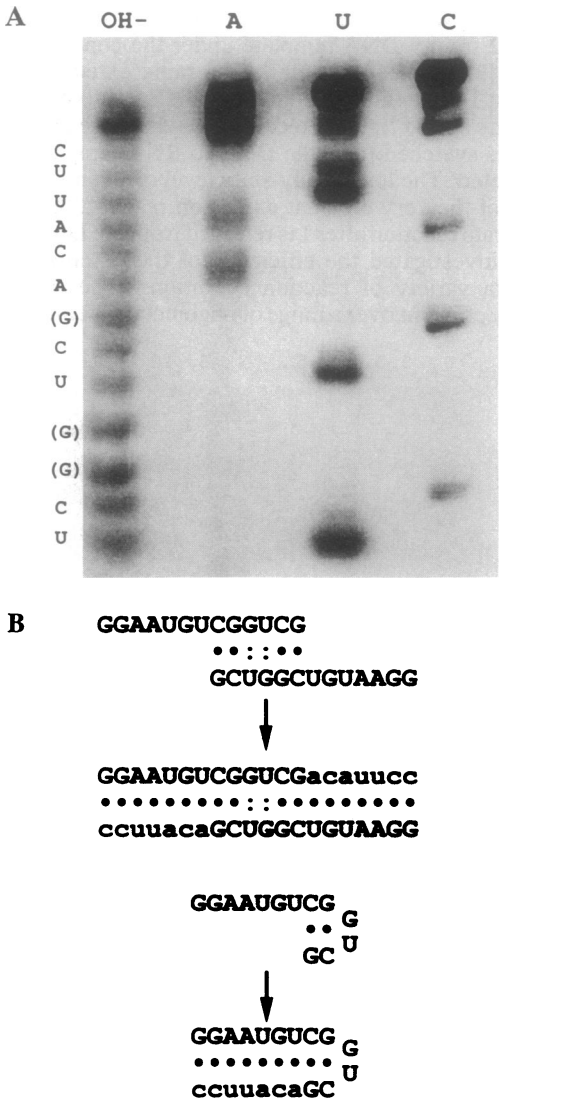

FIG. 2. (*A*) Partial phosphorothioate sequence analysis (15) of oligomer **2**. (*B*) Possible reaction schemes for the conversion of oligomer **1** (uppercase letters) into oligomer **2** through primer extension by T7 RNA polymerase. The extended nucleotides are shown as lowercase letters.

The sequence of **2** suggests a possible mechanism for its formation in the transcription reaction. Oligonucleotide **1** could first be produced from the DNA template in the normal fashion. Then, due to its self-complementary 3′-terminal sequence, **1** could form an intra- or intermolecular primed template and be extended to **2** by T7 polymerase (Fig. 2*B*). If this extension reaction were reasonably efficient, it would account for the low amounts of **1** accumulating in the transcription reaction.

RNA-Directed Primer Extension. We tested whether T7 RNA polymerase could extend RNA in the absence of a DNA template under standard transcription conditions. As shown in Fig. 3*A*, **1** was rapidly extended to an oligomer that comigrated with **2**. To be sure that our preparation of **1** did not contain any DNA, we prepared an oligonucleotide one residue shorter on the 5′ end (**1A**) by chemical synthesis. As shown in Fig. 3*B*, the 12-nucleotide **1A** was extended to an 18-mer termed **2A** as expected. The nucleotide composition of **2** and **2A** prepared by extending **1** and **1A** in the presence of [α-^{32}P]CTP was found to be consistent with their expected sequences. Under the reaction conditions used, the initial rate of the extension reaction in Fig. 3*A* was 114 nmol of nucleotide incorporated per min per mg of enzyme. This rate is considerably greater than the combined rate of synthesis of **1** and **2** from a DNA template under the conditions used in Fig. 1 [27 nmoles/(min·mg)]. The rapid extension of **1** to **2** without DNA template eliminates the need to hypothesize a template strand transfer mechanism where the growing RNA chain is switched from the DNA to RNA template after **1** is completed. The low steady-state level of **1** can simply be the result of the very efficient extension reaction that occurs in a separate reaction after **1** is released from the DNA template.

We investigated the efficiency of the extension reaction under a variety of reaction conditions. The reaction was equally efficient over a range of oligomer concentrations from 1 nM to 5 μM, with >80% of **1** converted to **2** in 10 min. The reaction did not require Triton or spermidine and was insensitive to added NaCl to 0.1 M. Extension was efficient from 25°C to 42°C.

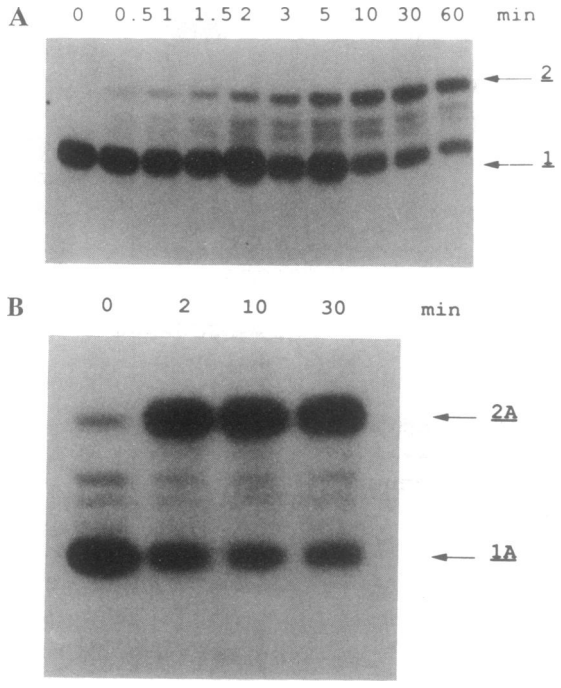

FIG. 3. Time course of the elongation reaction of enzymatically synthesized oligomer **1** (*A*), and of the chemically synthesized oligomer **1A** (*B*). (*A*) Final concentrations of oligomer **1** (5.2 μM) and of T7 RNA polymerase (0.025 mg/ml) were chosen to allow determination of the initial rate. (*B*) Final concentrations of oligomer **1A** (0.6 μM) and enzyme (0.1 mg/ml) are similar to those found in the transcription reaction mixture after a few copies of the DNA template have been completed.

In an attempt to determine whether the template of the extension reaction was an intramolecular hairpin or an intermolecular duplex (Fig. 2*B*), the thermal denaturation of **1** was carried out at several strand concentrations in buffer containing the same free Mg^{2+} concentration present in the extension reactions (6 mM). Since a clear concentration dependence of T_m was observed, a majority of the molecules must form intermolecular duplexes and not hairpins. Of course, this does not necessarily mean that hairpins are not elongated. Even at relatively high concentrations of **1** (5 μM), the T_m is quite low (21°C), so only a few percent of the molecules are in either duplex or hairpin form at 37°C. This suggests that the enzyme must have a high affinity for the primed template. Indeed, since the reaction works equally well at 1 nM **1**, where virtually no duplex forms at 37°C, the enzyme may promote the annealing of the two strands or the formation of the hairpin template.

In an alternative approach to determine whether the extension template was an intermolecular dimer, oligomers **3** and **4** were designed to be complementary to each other for six 3′-terminal residues (Fig. 4*A*). When 5′-^{32}P-labeled **3** was tested for extension, no product was observed. However, when nonradioactive **4** was included in the reaction, the expected 16-nucleotide elongation product was made (Fig. 4*B*). This experiment clearly shows that, in this case, an intermolecular duplex was the substrate of the extension reaction. When the reciprocal experiment was carried out with 5′-^{32}P-labeled **4**, several unanticipated extension products were produced with **4** by itself, greatly complicating the analysis (see below, Fig. 6). When nonradioactive **3** was added to that reaction mixture a small amount of product of a length (16 nucleotides) expected from a bimolecular extension could be detected (data not shown).

Many Different Extension Reactions. The preceding results prompted testing of 18 other nonradioactive RNA fragments for extension using [α-^{32}P]CTP. Although these fragments varied in both length (6–37 nucleotides) and sequence, they were made for other purposes (17, 18) and therefore do not systematically explore the requirements of the reaction. While many of these fragments were produced by *in vitro*

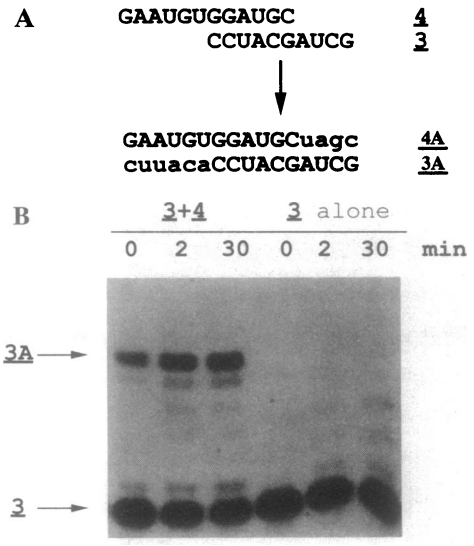

FIG. 4. (*A*) Expected elongation products from a template formed from oligomers **3** and **4**. (*B*) Elongation reaction performed with 5′-^{32}P-labeled oligomer **3** mixed with 43 pmol of oligomer **4** (left lanes) or 39 pmol of oligomer **3** (right lanes).

transcription, no significant amounts of alternative products were observed in any of the transcription reactions. Nevertheless, it is striking that when each of these 18 RNAs was incubated with NTPs and T7 RNA polymerase, 14 gave significant amounts of extension product. A representative gel is shown in Fig. 5*A*. In most cases we have not sequenced the products, but a likely extension product can often be proposed on the basis of the length of the extended product and the sequence of the fragment (Fig. 5*B*). It appears that extension can occur with primed templates that are only poorly complementary over a stretch as short as a few base pairs. It is interesting that oligonucleotides **b–g** have the same 3′ end and consequently can pair in the same way. However unlike oligonucleotides **b–f**, oligonucleotide **g** does not form an extended product. Thus, the sequence requirements for extension are incompletely understood.

In several of the reactions in Fig. 5, multiple elongation products were seen. In most cases, these probably reflect alternative pairing possibilities, but it is clear that in one case (oligonucleotide **4**), some of the products were intermediates in a multistep extension reaction. This is seen in Fig. 6*A* where the time course of the extension of 5′-^{32}P-labeled **4** is followed. During the first few minutes of incubation, **4** was converted to two major products, **4A** and **4B**, and several minor products. As incubation continued, **4A** and **4B** disappeared and two even longer products, **4C** and **4D**, accumulated. This second phase of the reaction did not occur when 1 mM spermidine was present in the extension reaction mixture. Although we have not fully characterized all of the intermediates, the sequences of **4A** and **4B** determined by the phosphorothioate method suggest that they are extension products of two alternative pairing possibilities of **4**. In one, two molecules of **4** form a weak 8-bp helix containing four G·U pairs and two A·G pairs and are extended to produce the 16-nucleotide **4A**. In the other, two molecules of **4** form a 6-bp helix containing two G·G mismatches and are extended to produce the 18-nucleotide **4B**.

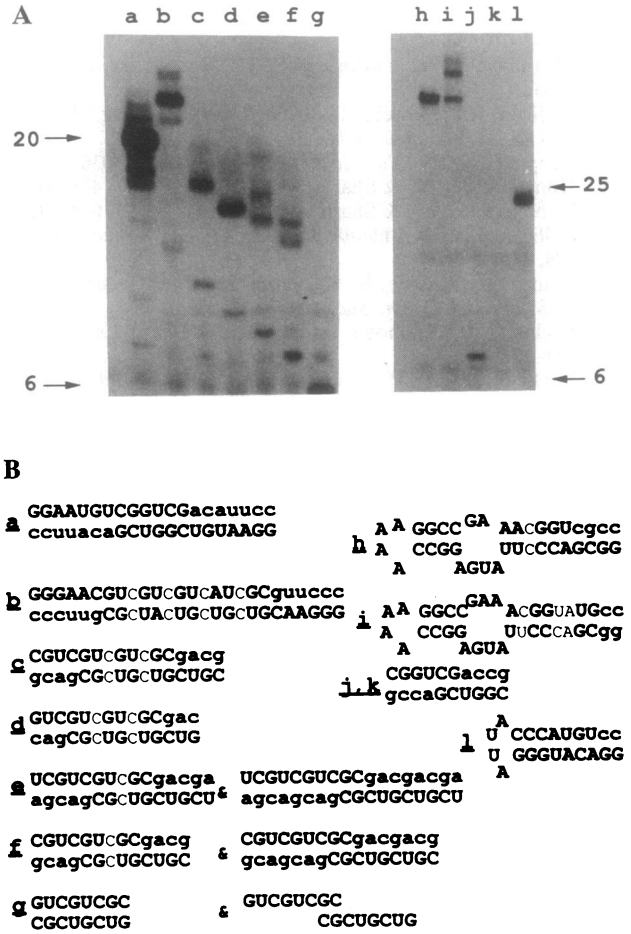

FIG. 5. (*A*) Extension of various nonradioactive oligoribonucleotides. Each reaction mixture contained 1 μM oligonucleotide (10 μM for **k**) and 0.1 mg of T7 RNA polymerase per ml. Incubation was 1 hr at 37°C. Arrows correspond to approximate sizes (lengths in nucleotides) of oligonucleotides inferred from the migration of oligomer **1** and of the dyes. (*B*) Sequences (uppercase letters) of oligonucleotides tested and proposed extensions (lowercase letters) inferred from their estimated length. Uppercase letters not in bold type indicate mismatched pairs formed in the primed template.

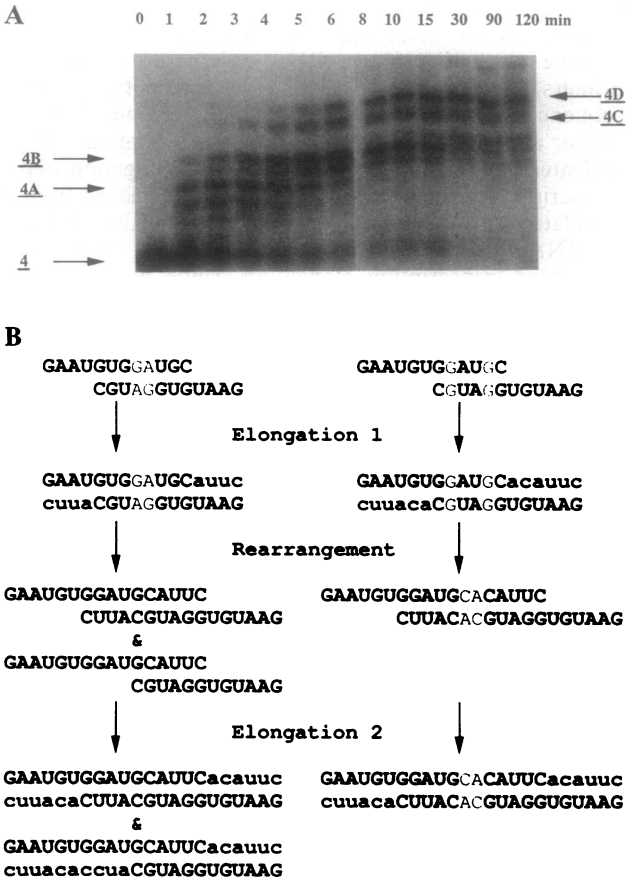

FIG. 6. (*A*) Time course of the elongation reaction of 1 μM oligomer **4** by T7 RNA polymerase. (*B*) Proposed mechanism for the extension reactions compatible with the determined sequence of some of the intermediates.

The further reaction of **4A** and **4B** is less well understood. The major extension products **4C** and **4D** are heterogeneous in sequence, but the majority of the material can be understood to be the result of the dissociation of the **4A** and **4B** helices, followed by rehybridization to form a new primed template that is subsequently extended (Fig. 6*B*). It appears that **4D** is primarily a 24-nucleotide extension product of two **4B** molecules. The 22-nucleotide **4C** is a mixture of sequences containing extension products of two **4A** molecules as well as a **4A** paired to a **4**.

DISCUSSION

It has been known for many years that phage RNA polymerases will extend short RNA oligonucleotides that are complementary to a DNA template in the region of a promoter sequence containing a promoter (3, 19). This reaction, termed priming, can be understood as an alternative to the rate-limiting initiation step with an NTP (usually GTP). As a

result, priming can occur only while the RNA polymerase is on the promoter in the open complex. More recently, it has become clear that phage RNA polymerases can extend primers on DNA templates that do not contain promoter sequences (6). A recent study of the extension of an RNA primer annealed to a synthetic "bubble" template (20, 21) indicates that this reaction resembles the elongation steps in transcription, since the product strand is displaced from the template. It is likely that the extension of an RNA primer on an RNA template described in this paper most closely resembles transcription elongation, since no promoter is needed and the A-form RNA·RNA resembles the A-form DNA·RNA hybrid that forms transiently during transcription.

Our understanding of the sequence and structure requirements of the RNA extension reaction remains incomplete. It is clear that a wide number of sequences can be extended as either intra- or intermolecular primed templates. In a survey of 21 oligonucleotides, 16 of them produced detectable extension products. It appears that at least two normal Watson–Crick base pairs at the primer terminus are needed for any extension to be observed, although the stabilizing effect of upstream non-Watson–Crick pairs is often important. While it is clear that the primed template can be composed of two different RNA molecules, it is likely that intramolecular primed templates can be extended as well. Our observation that T7 RNA polymerase can efficiently extend a very unstable RNA helix suggests that it may actually stabilize the primed template. A careful enzymological study of the extension reaction will be needed to fully understand its sequence and structural requirements.

It is likely that many of the unexpected products that often arise in *in vitro* transcription reactions are the result of the RNA template-directed extension reaction. Indeed, our attention was first drawn to the phenomenon by an exceptional case where the major product of a transcription reaction was much longer than predicted by the DNA template. Based on our extension experiments without DNA present, we conclude that longer transcription products are the result of the 3′ terminus of the normal run-off product forming a short helix with another part of the molecule. The extension reaction could also potentially produce RNA products shorter than the desired RNA in a transcription reaction. This could come about by one of the abortive initiation products hybridizing to a region of the product RNA to form a primed template. Since abortive initiation products are generally G+C-rich and quite abundant, we anticipate that this is a common side reaction. Despite the large number of potentially efficient side reactions based on primer extension, most *in vitro* transcription reactions produce substantial amounts of the correct run-off product and very little of the extension products because of the great preference of T7 RNA polymerase to bind promoter DNA over primed template. Many of the RNAs that produced abundant extension products when incubated with T7 RNA polymerase were not extended in the transcription reactions that produced them.

In one case, T7 RNA polymerase was found to extend a single oligoribonucleotide into a large number of different products, some twice as long as the original fragment. Although the intermediates in the reaction were not all characterized, it appears that the products arose from a combination of extension of alternative pairing possibilities and subsequent rearrangement of the extended products to permit another round of extension. As a result of the two rounds of extension, the longest products had two pseudoaxes of symmetry. It is quite striking that the small RNAs that replicate with T7 RNA polymerase (8) also contain two pseudoaxes of symmetry, suggesting that they arose by two separate extension reactions.

Finally, it is possible that the extension reaction can be exploited to prepare RNAs with unique modifications in predefined positions. An appropriate primed template can be extended by one residue with a modified or radiolabeled NTP, the NTP removed, and the template further extended with the four normal NTPs. This procedure would provide a useful alternative to the RNA and DNA ligase-based protocols (22, 23).

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