Unusual promoter-independent transcription reactions with bacteriophage RNA polymerases

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ABSTRACT

Efficient transcription reactions of DNA-dependent RNA polymerases require the presence of a specific promoter sequence. This report shows that in the absence of their cognate promoter, two bacteriophage RNA polymerases are capable of performing unusual transcription reactions: (i) the DNA template serves also as a primer for RNA synthesis and this leads to hybrid DNA/RNA molecules, (ii) if the DNA template forms a hairpin structure, the linear DNA can be transcribed via the 'rolling circle' mechanism.

INTRODUCTION

For efficient and specific RNA synthesis, the transcription initiation requires the presence of a promoter sequence which is recognized by its cognate RNA polymerase. Such promoter structures can be located upstream of the transcribed segment (1) or internal to it, as it was observed for the eukaryotic RNA polymerase III (2). The RNA polymerases from the bacteriophages SP6, T7 and T3 have a stringent requirement for their cognate 17-mer promoter sequences (3). Recently it was shown, that single stranded oligodeoxyribonucleotides without any promoter can serve as templates for the bacteriophage RNA polymerases and in some cases the major reaction product was an RNA resulting from end-to-end transcription of the DNA template (4,5). In this report, it is shown that oligonucleotides can serve as template as well as primer in a novel 'fold-back' mechanism for RNA synthesis which results in the formation of unusual DNA/RNA hybrids. In a different reaction type, one oligonucleotide gave rise not only to template-length RNA transcripts but also to longer RNAs, which appeared like a ladder of multimeric transcripts (5). These products proved to be bona fide multimers, and in contrast to the imprecise selection of the transcription initiation site, the sequence junctions between monomer units were precise; this means, no information from the template was lost. This behavior was exactly as expected for a circular template, transcribed by the rolling circle mechanism (6).

It is discussed that the reported properties of RNA polymerases might be related to the functions of primordial polymerases.

MATERIALS AND METHODS

Preparation and Labeling of Oligonucleotides. The oligonucleotides 24K and BI were prepared with a 381A DNA synthesizer from Applied Biosystems; Ph31 was a gift from Pharmacia. They were purified by gel electrophoresis (5).

For [5'-^{32}P]-labeling, 100 ng oligonucleotide were incubated for 30 min at 37°C with 160 μCi [gamma-^{32}P]-ATP (7000 Ci/mmol, 'crude' product; obtained from ICN) and 5 U

of polynucleotide kinase (from NE Biolabs) in 10 µl of 100 mM Tris-HCl (pH 8), 20 mM $MgCl_2$, 3 mM spermidine, 10 mM DTT. The labeled products were again gel-purified and used for transcriptions.

The trinucleotide dATC (from Pharmacia) and the mononucleotide deoxy-cytidine-3′-phosphate were labeled by the same procedure, however, 10 µg were used. For a chase reaction, 1 µl of 100 mM ATP was added after 30 min, and the incubation was continued for 30 min more. In this reaction, the mononucleotide was converted to the diphosphate [5′-^{32}P]-pdCp. The 3′-terminal phosphate was removed by adding 100 ng nuclease P1 in 10 µl 100 mM ammonium acetate (pH 4.5) and incubating for 2 h at 37°C. The mononucleotide and the trinucleotide were purified by thin layer chromatography on cellulose plates with isobutyric acid/ammonia as solvent (7). After autoradiography, cellulose containing the products was scraped off and sucked into glass capillaries (sealed at one end with cotton wool and connected to an aspirator). The labeled material was eluted with two volumes of water (100 µl) into Eppendorf vials. After drying in a dessicator, the products were used as potential primers in transcription reactions.

Transcription Reactions. All reaction conditions were as described previously (5). 100 ng template oligonucleotide (about 10 pmoles) were incubated for 5 h at 37°C in 10 µl containing 40 mM Tris-HCl (pH 8), 20 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 1 mM of each NTP, 8 % polyethylene glycol (m.w. 6000), 0.01 % Triton X-100 and 20 units of RNA polymerase (SP6 or T7; obtained from NE Biolabs).

Labeled products were produced by either including [alpha-^{32}P]-GTP (obtained from Amersham with a specific activity of 400 Ci/mmol and diluted to 0.5 Ci/mmol) or using [5′-^{32}P]-labeled template oligonucleotides (about 200 µCi/µg). For priming, about 50 µCi of [5′-^{32}P]-dATC (0.5 mM in the reaction) or 50 µCi [5′-^{32}P]-pdC (1.25 mM in the reaction) were added.

For DNase treatment, one unit of RQ DNase (from Promega) was added and the incubation was continued for 15 min more.

The reactions were terminated by ethanol precipitations, adding an equal volume of 4 M ammonium acetate and 2.5 volumes ethanol. The dried pellets were dissolved in 15 µl 8 M urea, 0.03% dyes (bromophenol blue and xylene cyanol FF) and subjected to electrophoresis on 20% denaturing polyacrylamide gels. The products were isolated as described (5, 7).

For labeling with [α-^{32}P]-dATP, 5 µCi dATP (3000 Ci/mmol; obtained from Amersham) and 1 mM each of dCTP, dGTP, dTTP (from Boehringer Mannheim) were used, replacing the ribonucleoside triphosphates. In a different reaction, 1 mM dATP was added to compete with the incorporation of [α-^{32}P]-dATP.

Sequence Analyses. Prior to the sequencing reactions, the isolated products were dephosphorylated and end-labeled with [gamma-^{32}P]-ATP and polynucleotide kinase (7). The [5′-^{32}P]-labeled transcripts were purified by gel electrophoresis (7). Enzymatic RNA sequencing and conditions for partial acid cleavage were described previously (7). Partial alkali cleavage: End-labeled RNA with 5 µg tRNA as carrier was heated for two minutes at 80°C in 4 µl 6 M urea, 50 mM NaOH, 0.03% dyes (bromophenol blue and xylene cyanol FF). The reaction was terminated by freezing in dry ice. The thawed samples were applied immediately on 20% polyacrylamide gels.

Chemical DNA sequencing was performed essentially as described by Eckert (8). However, the ethanol precipitations were done by adding sodium acetate, which had been adjusted to pH 5 with acetic acid. In addition, the lyophilisation after piperidine treatment

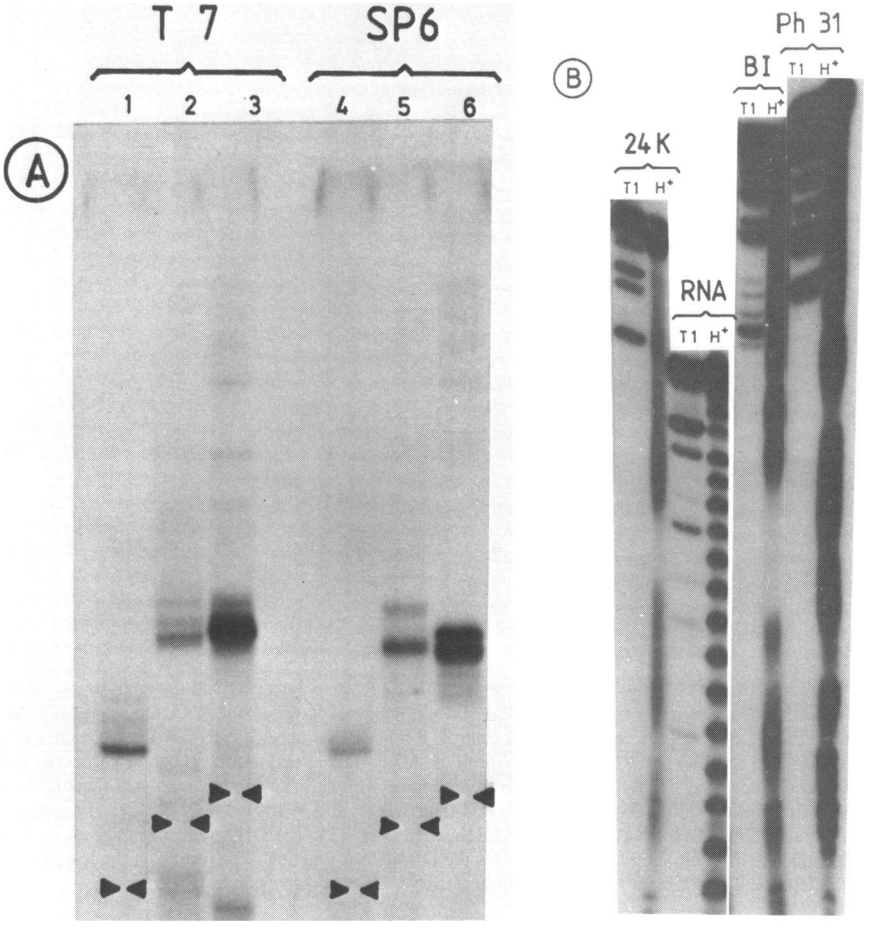


Fig. 1. Transcription reactions with oligonucleotides as templates and an attempt to characterize the products. (A) The specified RNA polymerases (T7 or SP6) were used with the oligonucleotides 24K (TGG CGA CAC CAG CAG GAT TTG AA) in lanes 1 and 4, BI (ACC ACT CAG GCA TAG TGT CCT CTA TAG TG) in lanes 2 and 5, and Ph31 (TAT CGT CTT CGA CAC CAG CAG GAT TTA AAC C) in lanes 3 and 6. [alpha-^{32}P]-GTP was included in the reactions. Arrowheads indicate the positions of the respective template oligonucleotide. (B) For sequence analysis, the isolated transcripts shown in panel A were [5'-^{32}P]-labeled. They were partially digested with RNase T1 (lane T1, G-specific) or sulfuric acid (lane H^+, random cleavages generate a sequence ladder) and analyzed on a 20% sequencing gel. The respective template oligonucleotides are specified above the lanes and an authentic RNA sample is included for comparison.

were replaced by ethanol precipitation: 0.1 volume of 2 M sodium acetate (pH 5) and 2.5 volumes of ethanol were added. After centrifugation (10 min in an Eppendorf centrifuge), the pellet was washed with 200 µl of 70% ethanol. The dried pellet was dissolved in 15 µl 8 M urea, 0.03% dyes (bromophenol blue and xylene cyanol FF) and subjected to gel electrophoresis.

Nuclease Treatments.

About 10 ng of the [5'-^{32}P]-labeled product (10000 cpm) was used in the reactions. Treatment with RQ DNase (from Promega) was with 0.5 U enzyme in 10 µl 40 mM Tris-

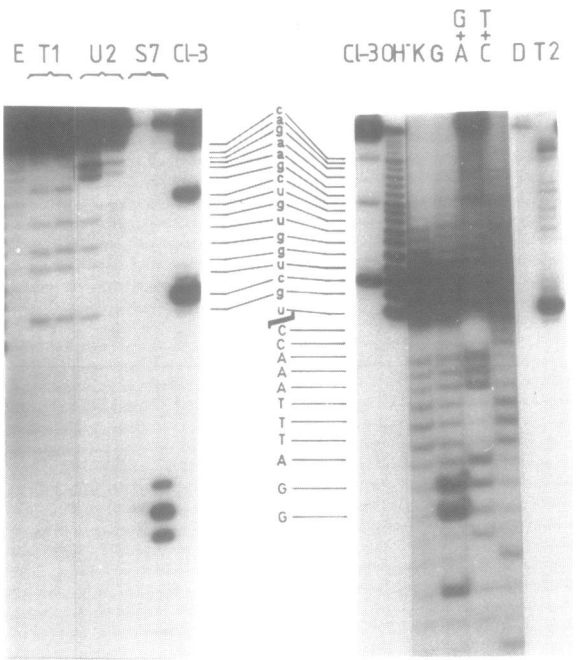


Fig. 2. Oligonucleotide Ph31 was used as the template for T7 RNA polymerase and the analysis of one major [5′-^{32}P]-labeled product is shown here. The nucleic acid was subjected to enzymatic RNA sequencing (left panel), using the following enzymes: E, no enzyme as control; RNase T1 (G-specific), RNase U2 [A(+G)-specific], nuclease S7 (A+U-specific) and RNase Cl-3 (C-specific). Alkali (OH^-) was used to generate a sequence ladder. For DNA sequencing, the sample was treated with piperidine without prior modification step (lane K) or after treatment with dimethyl sulfate (lane G), formic acid (lane G+A) and hydrazine (lane T+C). The sample was also treated with RNase-free DNase (lane D) and RNase T2 (lane T2). The DNA sequence is given in capitals, the RNA sequence in small letters. A line indicates the DNA/RNA junction. The results on the left panel were from an independent experiment to obtain a complete RNA sequence analysis.

HCl (pH 8), 6 mM $MgCl_2$ for 15 min at 37°C. Cleavage by RNase T2 (from BRL) was with 0.3 U in the presence of 2 μg carrier tRNA in 10 μl 10 mM ammonium acetate (pH 4.5) for 60 min at 37°C.

The samples were pipetted onto 10 μl urea/dye mix (8M urea, 0.03% bromophenol blue, 0.03% xylene cyanol) which had been dried in a dessicator. After boiling for 2 min and chilling on ice, the material was loaded on a 20% denaturing polyacrylamide gel.

End Group Analyses. The isolated transcripts were labeled at their 5′- or 3′-termini, respectively (7). Aliquots (approx. 1000 cpm; 100 ng carrier RNA was added) were digested to completion and the products were analyzed by cochromatography with authentic marker nucleotides on cellulose thin layer plates, as described previously (7). The digestions were performed as follows.

For the 3′-termini: with 0.3 units of RNase T2 (obtained from BRL) in 10 μl 10 mM ammonium acetate (pH 4.5) for 1 h at 37°C.

For the 5′-termini: with 50 ng nuclease P1 (from Pharmacia) in 10 μl 50 mM ammonium acetate (pH 5.3) for 2 h at 50°C.

Identification of the Internal Ribonucleoside at DNA-RNA Junctions. 'Fold-back' transcripts

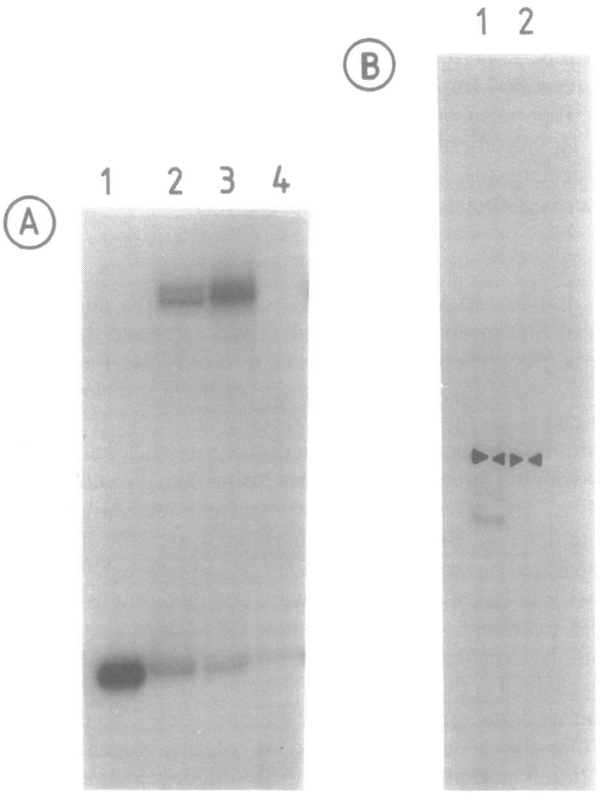
which had been internally labeled with [α-^{32}P]-GTP were used. First, the RNA chain was removed by exhaustive degradation: Approx. 10 ng product (10 000 cpm) were incubated in 100 μl of 1 M piperidine for 5 hours at 95°C. The material was recovered by ethanol precipitation as described for chemical DNA sequencing (above). Purified product was isolated after electrophoresis on a 20% denaturing polyacrylamide gel (7). An exonuclease digestion with 2 μg spleen phosphodiesterase (from Boehringer Mannheim) in 10 μl 50 mM ammonium acetate (pH 5.3) for 1 h at 37°C liberated the labeled nucleotide. It was identified as described above for end group analysis.

RESULTS

RNA Extensions Attached to Single Stranded Oligodeoxyribonucleotides.

From the results of Sharmeen and Taylor (4) it was expected that essentially any single stranded oligodeoxyribonucleotide could serve as a template for SP6 RNA polymerase. However, in a later study (5), the apparent size of the transcripts matched the expected length for end-to-end transcription products for only one of the four oligonucleotides used. In this case, a faithful RNA copy of the oligodeoxyribonucleotide was synthesized. The reaction products with the three other templates, using both T7 and SP6 RNA polymerases, are shown in Fig. 1A. The products are considerably larger than the respective oligonucleotide templates. For a preliminary analysis, the isolated [5′-^{32}P]-labeled transcripts were analyzed on sequencing gels after partial cleavage with RNase T1 or sulfuric acid, respectively (Fig. 1B). Clear patterns were obtained only with RNase T1; there was a smear after acid treatment.

This smear could be explained if these 'transcripts' contained a DNA moiety. This DNA could be partially cleaved at depurinated nucleosides, since acid treatment of DNA results in a removal of purine bases, generating labile apurinic sites. This conclusion was supported by the finding that the 5′-terminal nucleotide of this nucleic acid was exclusively deoxythymidine (the 5′-terminus of the template oligonucleotide Ph31) and it could be liberated only with nuclease P1, whereas RNase T2 did not yield any mono- or dinucleotides (not shown). The detailed analysis of a [5′-^{32}P]-labeled transcript is shown in Fig. 2. The product was degraded by RNase-free DNase (lane D), whereas RNase T2 generated a product which was exactly one ribonucleotide longer than the initially added oligodeoxyribonucleotide (lane T2). The same fragment was also obtained by piperidine treatment of the unmodified nucleic acid (lane K). The DNA sequence was determined by chemical sequence analysis and it matched exactly the expected sequence for the intact synthetic oligonucleotide (capital letters in Fig. 2). The RNA sequence was analyzed with the RNA-specific RNases T1, U2 and Cl-3. They cleaved only in the 3′-terminal extension (RNA sequence in small letters). Nuclease S7 (specific for A and U; see Fig.5) can also cleave DNA and shorter fragments were observed. The DNA part should be completely resistant to alkali. In contrast to sulfuric acid (Fig. 1B), sodium hydroxide treatment generated an RNA sequence ladder (lane OH^-; Fig. 2). The nucleotide at the junction between DNA and RNA was identified in the following way. The transcript was labeled with [alpha-^{32}P]-GTP. The second ribonucleotide after the DNA−RNA junction is guanosine (see Fig. 2). This means, after exhaustive treatment with piperidine, the product contained the complete DNA moiety with a 5′-hydroxyl group and a single [^{32}P]-labeled ribonucleotide at its 3′-end. The 3′-terminal [^{32}P]-phosphate was derived from the previously neighbouring guanosine. As expected for this product, nuclease P1 produced only [^{32}P]-phosphate. The [3′-^{32}P]-labeled mononucleotide was liberated by exonuclease



C

5' TAT CGT CTT CGA CAC CAG CAG G A T T
3' aua gca gaa gcu gug guc guC C A A A T

D

5' TAT CGT CTT CGA CAC CAG CAG GAT TTA AAC C 3'
3' aua gca gaa gcu gug guc guC CTA 5'

treatment with spleen phosphodi-esterase, and this required a free hydroxyl group at the 5′-end of the DNA moiety. The nucleotide was identified as uridine-3′-phosphate by thin layer chromatography (data not shown).

From these results it can be concluded that the template oligodeoxyribonucleotide itself was extended in the transcription reaction. This was confirmed by using a 5′-labeled template oligonucleotide (Fig. 3A). In the absence of ribonucleoside triphosphates, the oligonucleotide was not changed in the transcription reaction (lane 1). Extended products were formed after the addition of nucleoside triphosphates (lane 2). No major additional transcripts were detected when all synthesized RNAs were labeled additionally with [alpha-^{32}P]-GTP (lane 3). All products were sensitive to RNase-free DNase (lane 4).

A model for the formation of the observed products is shown in Fig. 3C. The 3′-end of the oligodeoxyribonucleotide folds back to form a very short hairpin (two G:C pairs can form; with oligonucleotide BI, a single G:C pair seems sufficient). The 3′-end forms the primer for the bacteriophage RNA polymerases and the oligonucleotide serves also as the template in the extension reaction.

A Trideoxyribonucleotide Serves as a Primer.

It is well known that ribodinucleotides and deoxyoligonucleotides can serve as primers in promoter-dependent transcription reactions (9−12). In the promoter-independent reaction described above, the template served also as primer. By adding high amounts of a deoxytrinucleotide, it was possible to separate the functions of template and primer. The trinucleotide [5′-^{32}P]-pdATC was added to oligonucleotide Ph31 in the transcription reaction and it could function as a primer (see Fig. 3B). However, only a small amount of product (about one mol transcript per ten moles template) was formed by priming at the expected site, possibly due to competition with the intramolecular hairpin formation. The product partially sequenced with RNase T1 and an alkali ladder (not shown; see also Fig. 3). The sequence of the product is given in Fig. 3D.

It was shown recently, that even the ribonucleoside monophosphate pG can serve as primer for T7 RNA polymerase (13). However, no priming was observed with [5′-^{32}P]-cytidine-5′-monophosphate (lane 2 in Fig. 3B).

Finally it was confirmed that the commercial T7 RNA polymerase is an authentic RNA polymerase, since [alpha-^{32}P]-dATP was not incorporated into reaction products. A low level was observed, but this was probably due to ribonucleotide contamination in the labeled deoxyadenosine triphosphate; excess dATP did not compete with the synthesis of [^{32}P]-labeled material (data not shown).

Transcription of a Linear Template by the 'Rolling Circle' Mechanism.

The synthesis of multimeric RNAs with SP6 RNA polymerase and the template GK25 is shown in Fig. 4A, lane 2 (for the sequence see Fig. 6). At each step of the product ladder, several RNA bands occur. They seem to result from imprecise starts (no unique

Fig. 3. (A) The [5′-^{32}P]-labeled oligonucleotide Ph31 was used as template for T7 RNA polymerase. Lane 1, no NTPs added; lane 2, with 1 mM NTPs; lane 3, with 1 mM NTPs containing [alpha-^{32}P]-GTP (0.5 Ci/mmol); lane 4, as lane 3, but followed by treatment with RNase-free DNase. (B) Transcriptions with unlabeled Ph31 and labeled DNA primers. Lane 1, with the trinucleotide [5′-^{32}P]-pdATC; lane 2, with the mononucleotide [5′-^{32}P]-pdC. Arrowheads indicate the positions of the template oligonucleotide. (C,D) These schemes describe the extension reactions observed in (A) and (B), respectively. A line indicates the DNA/RNA junctions. The DNA sequence is given in capitals, the RNA sequence in small letters. Only the base pairs in the internal hairpin loop or with the external primer trinucleotide are indicated by hyphens.

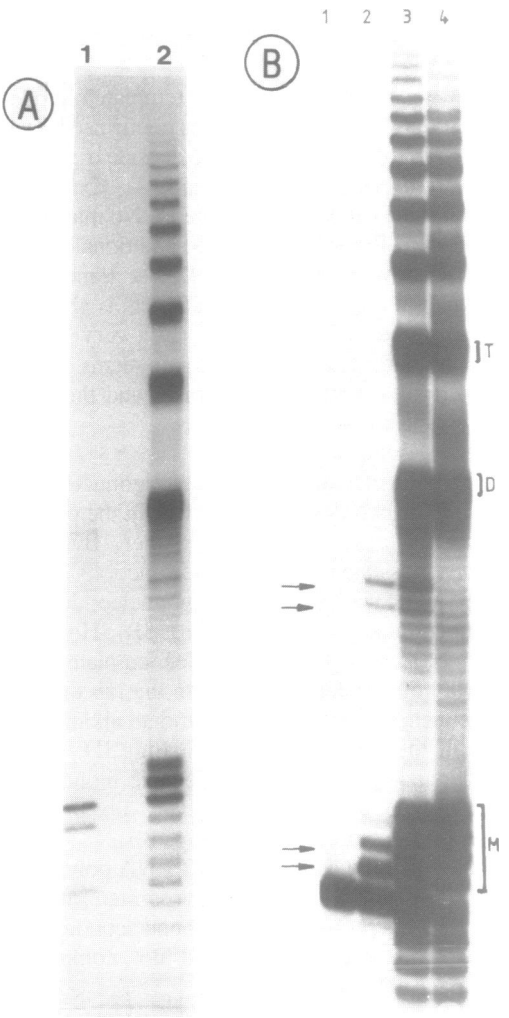


Fig. 4. Transcriptions with the linear, single stranded oligodeoxyribonucleotide GK25 (see Fig. 6). (A) The products were labeled with [alpha-^{32}P]-GTP. Lane 1, synthesis with T7 RNA polymerase; lane 2, with SP6 RNA polymerase. (B) [5′-^{32}P]-labeled GK25 was used with SP6 RNA polymerase. Lane 1, incubation in the absence of NTPs; lane 2, including 1 mM unlabeled NTPs; lane 3, in the presence of 1 mM NTPs and [alpha-^{32}P]-GTP; lane 4, treatment of the products from lane 3 with RNase-free DNase. At the right margin, the positions of monomeric (M), dimeric (D) and trimeric (T) products are indicated.

transcription initiation site) and the partial addition of one or two extra, unencoded nucleotides at the 3′-end (5, 14−16). The RNA products were analyzed further. A DNase treatment (Fig. 4B, lane 4) did not affect these transcripts, i.e., they did not contain a DNA moiety. The monomer-size products (marked 'M' in Fig. 4B) almost move together with the template oligonucleotide. Their sequence was identical with the comigrating transcripts obtained with T7 RNA polymerase, which had been characterized previously

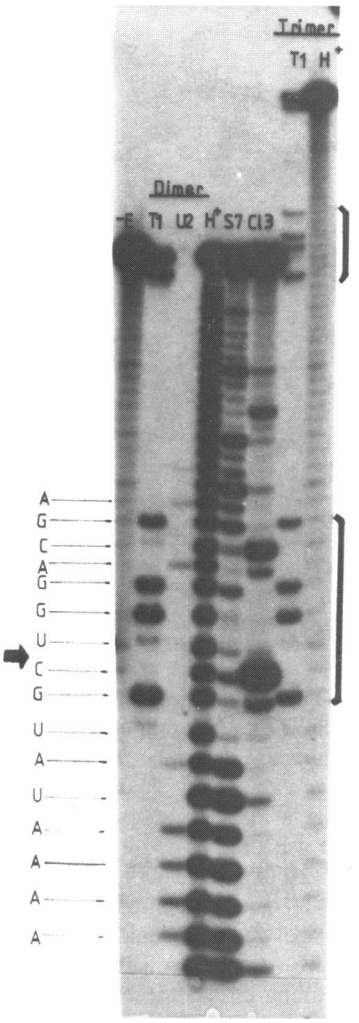


Fig. 5. Sequence analysis of an isolated, [5′-^{32}P]-labeled dimeric and trimeric transcript. The arrow indicates the expected 3′-end of a full-size monomeric transcript (see also Fig. 6). The brackets at the right margin specify the characteristic RNase T1 pattern at the junction between the monomer units. Individual lanes: −E, no enzyme as control; RNase T1 (G-specific); RNase U2 [A(+G)-specific]; H^+, sulfuric acid generates a ladder; nuclease S7 (A+U-specific; please note that cleavage occurs at the phosphodiester bond preceding A or U); RNase Cl-3 (C-specific).

(5). The RNAs start predominantly with guanosine corresponding to the penultimate cytosine at the 3′-end of the template DNA. With SP6 RNA polymerase, about one third of the monomer-size RNAs ends with the cytosine complementary to the 5′-terminal guanosine of the template (see Fig.6). With a similar abundance, products with one and two extra, unencoded nucleotides were observed (Fig. 4A, lane 2). Similar to other examples (14−16), these extra positions contained all four nucleosides with some preferance for adenosine

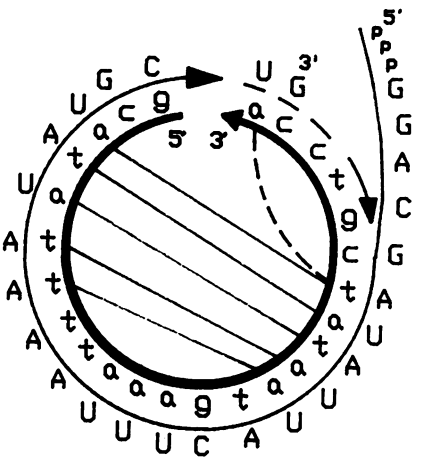


Fig. 6. Model for the formation of multimeric RNA transcripts. The sequence of the template GK25 is given in the central open circle (DNA in small letters). A nascent transcript is shown, encircling it (RNA in capital letters). The 5'-3' orientation is indicated by solid arrows. The broken arrow indicates the growing RNA chain, displacing the previously synthesized RNA. The sequence of the monomeric RNA with the preferred initiation site and two extra 3'-terminal residues (UG) is shown. Potential base pairs in the template DNA are indicated by the central straight lines and the curved broken line.

and cytidine (data not shown). The enzymatic RNA sequence analysis of the dimeric and trimeric products (Fig. 5) showed that the junction between two monomer units is absolutely precise and includes the uridine which was skipped completely at the transcription initiation site (see Fig. 6; the RNAs have exclusively guanosine as 5'-terminal nucleoside).

To exclude the possibity that a circular template is formed during the reaction, a [5'-^{32}P]-labeled oligonucleotide was used. The more slowly moving products (arrows in Fig. 4B, lane 2) are not circularized oligonucleotides. They are short 'fold-back' transcripts and were analyzed by chemical DNA sequencing (not shown). Circular oligonucleotides would contain an internal [^{32}P]-label and this would result in an obscured sequence ladder. A clear pattern was obtained, i.e., they were [5'-^{32}P]-end-labeled, linear products.

A model for the apparent 'rolling circle' mechanism for the transcription of a linear template is shown in Fig. 6. The transcription starts with a guanosine near the 3'-end of the DNA. However, it does not stop completely at the 5'-end of the template but one or two nucleotides are added additionally. In addition to the more abundant adenosine and cytidine, uridine and guanosine do also occur and these products can give rise to the multimers (Fig. 6). Already from the product sizes, observed with SP6 and T7 RNA polymerase, respectively, it seems that SP6 RNA polymerase yields more product with two additional nucleotides (Fig. 4A; confirmed by analysis of the 3'-termini). This different behaviour may explain why the multimeric transcripts are fairly abundant with SP6 RNA polymerase and occur at a much lower level with T7 RNA polymerase (Fig. 4A). However, it was surprising to find that only one template of the four tested was transcribed as described here, whereas three other oligonucleotides were extended at their 3'-termini by the 'fold-back' mechanism (see above). An inspection of possible secondary structures for the template GK25 is presented in Fig. 6. For the 'fold-back' mechanism, the 3'-terminal

adenosine would have to base pair with the next sterically available thymidine (dotted line) and this interferes with the formation of a stem structure (5 base pairs, straight lines). This means, the template GK25 forms a short hairpin with two protruding bases at the 5′-end and a single stranded 3′-terminal CCA-end. The bacteriophage RNA polymerases prefer guanosine as initiating nucleoside and they bind and initiate transcription at the 3′-end of single stranded DNAs. That means, a protruding CCA-end meets these requirements. The CCA-end has to be completely single stranded, since a template for 'fold-back' transcription (Ph31, see Fig. 3) could not be activated for end-to-end transcription by the addition of an adenosine to the already existing, but base-paired CC-end (data not shown).

DISCUSSION

The mechanisms for the synthesis of nucleic acids suggest that the initiation reaction is most difficult. *De novo* synthesis is observed only for RNA, whereas DNA synthesis depends on preformed primers (17). In general, RNA polymerases interact efficiently with specific promoter sequences and do not use primers. Some exceptions are known, like the sequential synthesis of corona and influenza virus RNA (18) or the *in vitro* transcription reactions with bacteriophage RNA polymerases where cap analogs or short oligonucleotides can be used as primers (9−12). In this report it is shown, that the template DNA itself can be used as primer if no specific promoter sequence is present.

These reactions result in 'fold-back' transcripts and they are the predominant products of promoter-independent transcription reactions. Alternatively, the formation of 'end-to-end' transcripts becomes the major route, if the template structure interferes with the formation of 'fold-back' transcripts. This means, the 3′-terminal nucleoside finds no free partner for base-pairing. This is the case with the template GK25 (Fig.6) and the C-rich CCA-end permits initiation with the highly preferred guanosine triphosphate (5). With SP6 RNA polymerase, a ladder of multimeric RNAs was observed in addition to the template-size monomeric transcripts. Presumably, these multimers result from base pairs between the two 'extra' nucleotides at the 3′-end of the RNA and the 3′-end of the template (Fig. 6). In consequence, the same products are formed which are expected from a rolling circle mechanism.

Possible Development of Promoters for primitive RNA Polymerases.

One may speculate that the reported properties of RNA polymerases are not mere artefacts but they might be related to the properties of primitive polymerases. For the development of promoter functions, it was suggested previously for the 'RNA world', that primitive RNA polymerases which use RNA as template, recognize 3′-terminal tRNA-like structures as the first promoter-like elements (19). Considering the results presented here, similar promoter elements might have been used in the early DNA world.

In the absence of their cognate promoter, also the very efficient DNA-dependent bacteriophage RNA polymerases with their stringent promoter recognition can produce free RNA transcripts with a template DNA containing a 'tRNA-like structure'. The most reduced form of the 'tRNA' element could be a hairpin with a 3′-protruding CCA-end, as in the template GK25 (Fig. 6). Such reduced 'tRNA-like' structures in RNA molecules can be recognized and cleaved by the tRNA-specific RNase P (20,21).

In addition to this primitive recognition of a DNA template, primordial polymerases could have aquired an additional domain which recognized a specific DNA sequence, the promoter. The results of Butler and Chamberlin (22) are in agreement with this concept. They had shown that the promoter recognition of SP6 RNA polymerase was lost after

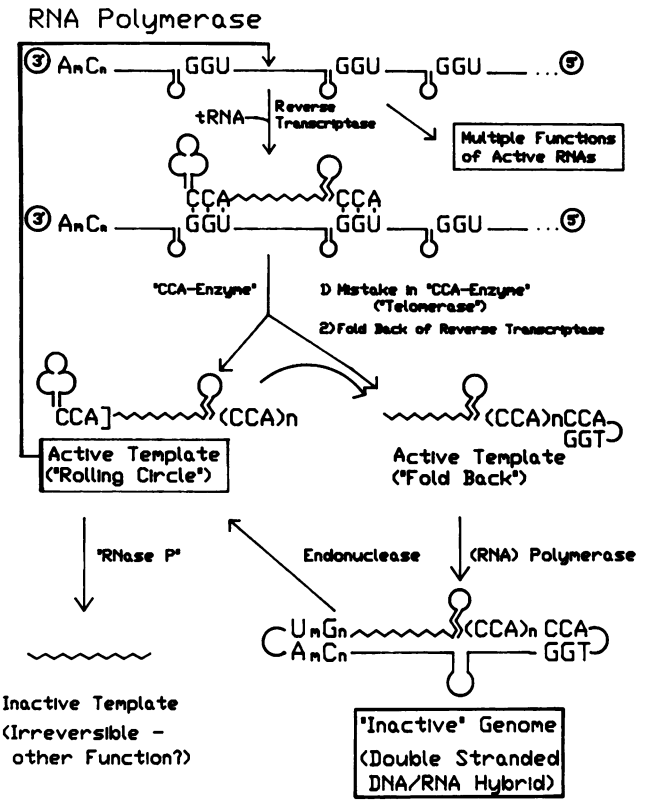


Fig. 7. Hypothetical scenario at the threshold of the emerging DNA world. The multimeric RNA transcript is shown as a straight line, punctuated by (5')UGG(3') triplets joined to 'tRNA motifs' (short hairpins). The tRNA primer for reverse transcriptase is shown as a cloverleaf structure, DNA as a zigzag line. The primer tRNA [with a bracket] is still included in the synthesized DNA template on the left, whereas it was removed from the template on the right. For details see text.

a limited protease treatment, whereas promoter-independent, catalytic activities were retained [transcription with poly(dI)·(dC) as template].

The inefficient and imprecise initiation at these 3'-terminal 'tRNA' elements was a disadvantage. This could be compensated by a rolling circle mechanism: a single and rate-limiting initiation event is sufficient for the production of many unit-length RNAs and the monomer transcription units do not lack any sequence information, regardless of the imprecise initiation site selection. Processing (self-cleavage ?) could generate the free monomers or the multimers could result in gene amplification, like in amphibian oocytes where extrachromosomal rRNA genes are amplified via a rolling circle mechanism (23). However, in promoter-independent transcriptions, a free 3'-end seems essential for the binding of RNA polymerase, prohibiting the use of a circular template. This limitation can be overcome, if a linear template can be used for a 'rolling circle' transcription reaction.

Hypothetical Scenario for a Genome Organization at the Threshold of the DNA world. Darnell and Doolittle (24) have proposed concepts for the molecular evolution at the very

early steps in the development of life. Very likely, the first stage was an 'RNA world' and the central importance of 'tRNA-like motifs' has been suggested recently by Weiner and Maizels (19). The next stage included proteins in the 'RNP world' and finally the DNA genomes were introduced.

A hypothetical scenario at the threshold to the DNA world is shown in Fig. 7. A multimeric RNA transcript (as produced in Fig. 6) is the starting point. The 'rolling circle' mechanism seems plausible also for larger linear template DNAs if the structure permits contacts between the two termini. This RNA may be processed and fulfill the multiple functions of RNAs in the 'RNA world' (25).

Remarkably, the bacteriophage RNA polymerases tend to add C and A after the template-dependent RNA synthesis has been completed (14–16). This activity creates a potential transcription start site (a CA-end) at the 3′-end of the transcript, assuring that a previously lost 5′-terminal UG sequence in the template does not lead to inactive progeny. This means, the RNA polymerase acts like a primitive 'CCA-enzyme'. For the transition to DNA, the multimeric RNA can be reverse transcribed into DNA and the CCA-end of a tRNA could serve as primer, like in viral reverse transcriptase reactions (26). In a simple model, only the 3′-terminal CCA is used in the hybrid formation. Only the synthesis of a monomer DNA is shown, including the 3′-terminal CCA. It is likely, that the priming tRNA will be removed from the DNA product. In Fig. 7, this tRNA element is not yet removed from one of the DNA products.

As already suggested for the 'RNA world' (27), also here the CCA-end has to be preserved. An enzyme may act on DNA, adding several 'CCA-like' repeats (as found in chromosomal telomere structures). This maintained the template structure, required for the 'rolling circle' type transcription (see also Fig. 6). The template could be inactivated by simply removing the 'tRNA-like motif', e.g., it is cleaved off by a DNase analog of RNase P. This mechanism was suggested by Weiner and Maizels (19) for RNA templates and RNase P can remove the 3′-terminal tRNA-like structure from TYMV RNA (21). However, the removal would be irreversible and another mechanism for silencing the template is illustrated in Fig. 7. Reverse transcriptase tends to fold back after the template-dependent DNA synthesis has been completed, generating a short hairpin structure (28). The same product could result from errors of the 'CCA-enzyme', leading to the incorporation of T and G. As shown in Fig. 6, the C and A adding RNA polymerases add also U and G.

The 'telomerases' may have evolved from mutants of the 'CCA-enzyme' which specifically added the originally 'wrong' nucleotides, G and T (29). Both reaction types lead to the template for the 'fold back' transcription reaction, resulting in an inactive double stranded 'genomic' nucleic acid. It has no free 3′-end at the DNA strand, a minimal requirement for transcription initiation with the primitive DNA-dependent RNA polymerases. However, a protruding CA-end is created at the 3′-end of the RNA moiety, a potential start site for RNA-dependent RNA polymerases. Again this template can be silenced by a fold-back reaction, i.e. the addition of U (or T in DNA) and G. These reactions lead to a linear double stranded nucleic acid which has terminal structures very similar to chromosomal telomeres (30). The 'silent genomic nucleic acid' can be reactivated by an endonuclease cut in the CCA-repeat near the 3′-terminus of the 'tRNA-like' structure. This liberates a protruding 3′-terminal CCA sequence, i.e., a potential transcription start site. It is interesting to note that a similar endonuclease might act on the telomere ends in chromosomes, leading to the nicks found in the CA-repeats (30).

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