Rolling-Circle RNA Synthesis: Circular Oligonucleotides as Efficient Substrates for T7 RNA Polymerase

Sarah L. Daubendiek, Kevin Ryan, and Eric T. Kool*

Department of Chemistry, University of Rochester Rochester, New York 14627

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The synthesis of ribonucleic acid strands by RNA polymerase enzymes has long been known to initiate specifically at well-defined promoter sequences.[1] For example, the T7 RNA polymerase enzyme initiates RNA strand synthesis with a pppG residue immediately downstream from a conserved ~17-base-pair duplex DNA sequence.[2] This fact has been used in the "runoff transcription" method for laboratory synthesis of sequence-defined oligoribonucleotides, in which DNA strands which contain the double-stranded promoter at one end serve as templates for RNA synthesis.[3]

Recent studies have shown, however, that RNA synthesis can in some cases be initiated at specific structures rather than at specific nucleotide sequences. It was recently shown, for example, that short oligoribonucleotides can prime further RNA synthesis from artificial unpaired "bubble"-like structures within duplex DNA.[4] Single-stranded "bubble" regions within duplex DNA can also serve as *de novo* initiation sites for RNA synthesis.[5,6] It has been proposed that such "bubble" structures mimic the open transcription complex formed by the polymerase.[5]

We report here, however, that small single-stranded circular oligonucleotides can serve as efficient templates for initiation and elongation of RNA sequences and that this occurs in the absence of added RNA primers, in the absence of RNA promoter sequences, and in the absence of any duplex structure at all. The synthetic circular DNAs are considerably smaller than the enzyme itself, yet they serve as highly efficient templates for the production of repeating RNA sequences thousands of nucleotides in length.

The circles initially studied are shown in Figure 1. We have previously described the synthesis and DNA-binding properties of such circular oligonucleotides.[7,8] These 34-base compounds have no internal self-complementarity and probably exist as open single-stranded structures in aqueous solution. Because they were initially designed to form triple-helical complexes, they are rich in T and C nucleotides and contain few purine residues. Initial testing of the circles under standard transcription conditions revealed the production of long RNAs; some of these showed a repeating banding pattern. This suggested that the circles were behaving as true templates for the enzyme, which was surprising since the circles have no sequence similarity to known RNA promoter sequences. Moreover, RNA promoters are double-stranded, while these circles are single-stranded.

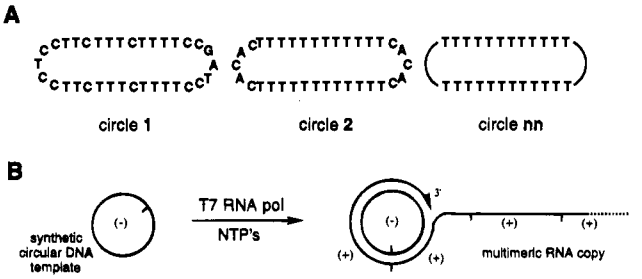

Figure 1. (A) The circular DNA oligonucleotides used as templates for RNA synthesis in this study; "circle **nn**" refers to a circle which contains the DNA nucleotides shown interrupted by two hexaethylene glycol linkers.[10] (B) The proposed "rolling-circle" mechanism for synthesis of long repeating RNAs from small circular templates.

After the initial findings we examined a number of controls and sequence/structure effects on this reaction. Figure 2 shows these results analyzed by polyacrylamide gel electrophoresis. Reactions were generally run under standard conditions commonly used for runoff transcription from linear synthetic DNA templates;[3] the RNA was radiolabeled for visualization by uptake of α-^{32}P-labeled UTP nucleotide. Results establish that a covalently closed circular structure is necessary for this efficient long-strand synthesis. Figure 2 shows that a linear precursor of circle **1** produces little RNA at all; the most abundant of the weak bands is ~30 nucleotides in length, or shorter than the template. By contrast, the closed circular version of this sequence produces much darker bands which are too long to be resolved by the gel. The large difference between linear and circular templates is worth noting, because there has been one report of linear oligonucleotides behaving as templates for RNA synthesis by a kind of "rolling" mechanism.[9] In this case it is clear that transcription is much more efficient from a true closed circle.

Other controls confirm (Figure 2) that ATP and enzyme are required for this synthesis. Separate experiments (not shown) demonstrate an absolute requirement for all four nucleotide triphosphates. Importantly, a second circle (sequence **2**), which has the same size but a different sequence, also behaves at least as well as circle **1** as a template for synthesis of long RNA strands. Thus, this mechanism is not specific only to one sequence of DNA. In addition, cyclic structure alone is necessary but not sufficient for this synthesis: circle **nn** contains sequences from circle **2** that are interrupted by hexaethylene glycol groups.[10] We find that it does not behave as a template for RNA synthesis (Figure 2), indicating either that initiation occurs only at the omitted sequences or that an uninterrupted DNA strand is required for continued synthesis. Simple curvature of a synthetic DNA oligonucleotide is apparently not sufficient for RNA synthesis.

We propose that this RNA synthesis occurs by a "rolling-circle" mechanism (Figure 1), in which after initiation the polymerase produces many true repeating RNA copies of the DNA strand, progressing around the circle multiple times. Since during transcription the appearance of RNA products longer than a linear DNA template has been noted previously,[11] it is important to show that in the present case the products are not simply random longer RNAs. We noted that, in cases where a greatly limiting concentration of UTP nucleotide was present, a repeating banding pattern was observed progressing upward on the gel. Circle **1** contains one A nucleotide, and as [UTP] decreases, pausing or termination prior to UTP incorporation

* To whom correspondence should be addressed.

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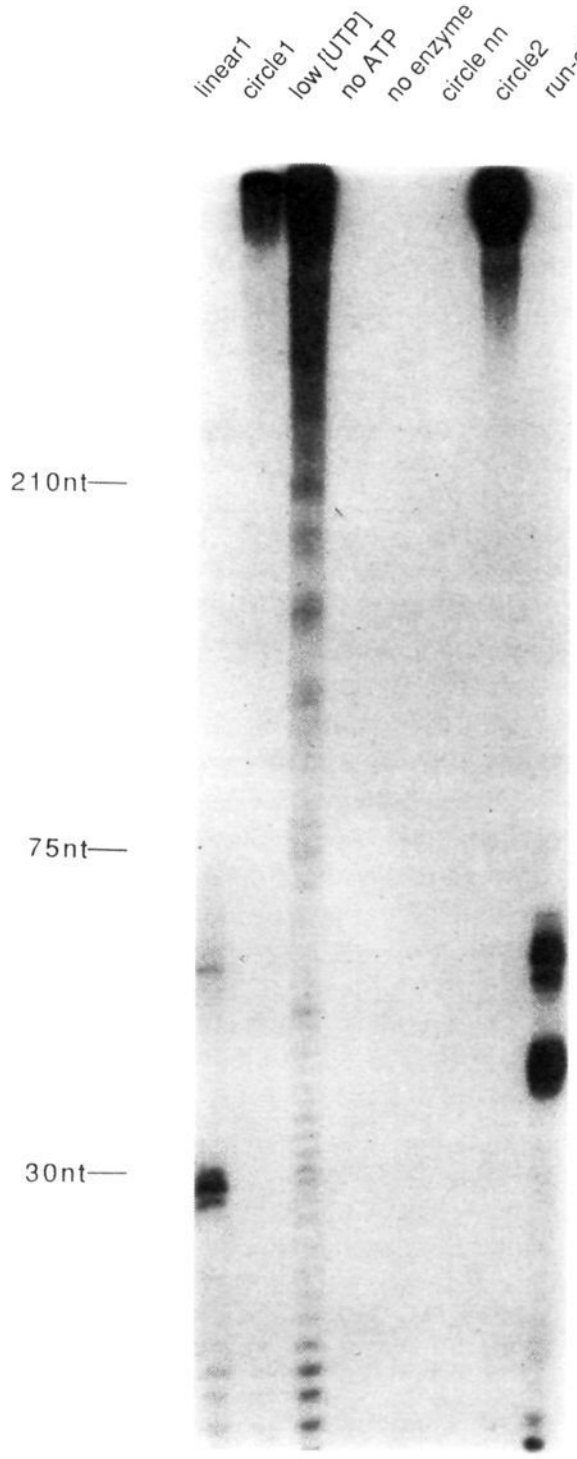

Figure 2. Autoradiogram from 10% denaturing polyacrylamide gel analysis of products from rolling-circle RNA synthesis and controls. The RNA is internally labeled by [α-^{32}P]UTP incorporation during synthesis. Lane 1: Linear precursor to circle **1** as template (sequence: 5′-pTTTCTTCCTCCTTCTTTCTTTTCCGATCCTTTTC). Lane 2: Circle **1** as template. Lane 3: Circle **1** as template with [UTP] lowered to 1.9 μM. Lanes 4 and 5: Circle **1** as template but with no ATP (4) or no enzyme (5). Lane 6: Circle **nn** as template. Lane 7: Circle **2** as template. Lane 8: Runoff transcription from linear T7 promoter template (identical conditions); template encodes monomer RNA which appears in multimer from circle **1**; template concentration is 1 μM. *Standard conditions for rolling-circle reaction:* 1 μM circle, 50 units of T7 RNAP (New England Biolabs), 0.5 mM ATP, GTP, CTP, 60 μM UTP, 0.27 μCi of [α-^{32}P]UTP in a pH 8.1 (25 mM Tris·HCl) buffer containing 20 mM NaCl, 15 mM $MgCl_2$, 0.4 mM spermine·4HCl, 100 μg/mL acetylated bovine serum albumin, 10 mM DTT, and 12.5 units/mL RNase inhibitor (Promega), in a total reaction volume of 15 μL. Reaction time is 1.5 h at 37 °C, and the reaction is stopped by addition of 1 volume of stop solution (30 mM EDTA, 8 M urea) and heating to 90 °C for 2 min, followed by chilling on ice prior to loading on the gel.

at this unique site is expected once per turn around the circle. Unique bands can occur, however, only if both initiation and termination occur at specific sites. Analysis of the prominent bands from circle **1** and related circles showed that they correspond very regularly to the relative positions of C residues in the circle (see Figure 2 for example of banding pattern). These bands can be explained only by initiation at these C's followed by partial termination at the UTP incorporation site. Thus, initiation of RNA synthesis with these circles occurs primarily with pppG, as has been seen for most RNA polymerases.[1] Importantly, this finding also serves as good evidence that the polymerase is making a true, sequence-specific repeating copy of the circle.

Analysis of the products by agarose gel electrophoresis with long size markers (data not shown) indicates that the products fall largely in the length range ~400−9000 nucleotides, which corresponds to ~12−260 turns around a 34-mer circular template. Thus, since a circle acts as a template for many repeating units, it can be in principle a catalytic template for RNA synthesis, incorporating many more nucleotides into RNA than are present as DNA. The synthesis is rapid; using 0.5 mM nucleotide triphosphates and 50 units of enzyme with a 1 μM circle, the reaction produces maximum product in less than 1 h at 37 °C. It remains to be seen whether the limiting factor under these conditions is consumption of monomers, enzyme stability, or other factors.

Comparison of the efficiency of nucleotide incorporation was then made with a standard linear "runoff" template for the monomer RNA in the repeating sequence from circle **1** (Figure 2). This template also contains a T7 promoter at its end, and a "top strand" was also annealed to the promoter to give the required duplex structure.[3] Results show that while this linear template does produce the 34-mer RNA monomer, the multimeric RNA products from the circular template appear considerably (several-fold) darker. While this is in some sense an indirect comparison since the products are different lengths, the results show that, in production of a given sequence, a circle can give even more RNA than can runoff transcription, the current laboratory standard. In a "rolling-circle" mechanism, the enzyme spends much more relative time in rapid chain elongation, rather than in the slower dissociation and reinitiation steps required for repeated copying of linear templates.[2]

A recent X-ray crystallographic study of T7 RNA polymerase shows that the protein has dimensions of 75 × 75 × 65 Å.[12] It is intriguing that a small 34-nucleotide circle, with an approximate diameter of only 36 Å, behaves as such an efficient substrate. There must be a strong tendency toward curvature in the template, and the RNA−DNA duplex being produced must undergo considerable bending and strain (or unwinding) during enlongation. It seems possible that such a substrate may in some ways resemble the natural transcription complex bubble which is formed during RNA synthesis from duplex DNA.[1]

Much more work will be needed to explore the limitations of circle sequence, size, and secondary structure which are viable in this new mode of RNA synthesis. Recent data (to be published elsewhere) indicate that a number of sequences and sizes are viable. We anticipate that this might become a generally useful method for the production of repeating RNA sequences which could be useful as biological probes. It is also possible to imagine combining this multimer synthesis with RNA cleavage strategies to produce amplified amounts of short, sequence-defined RNA oligonucleotides. Such studies are currently underway.

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