Rolling Circle DNA Synthesis: Small Circular Oligonucleotides as Efficient Templates for DNA Polymerases

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Abstract: We report that small, single-stranded circular DNA oligonucleotides 26 to 74 nucleotides (nt) in size can behave as catalytic templates for DNA synthesis by several DNA polymerase enzymes. The DNA products are repeating end-to-end multimeric copies of the synthetic circular DNAs, and range from 1 000 to >12 000 nucleotides in length. Several aspects of this reaction are unusual: first, the synthesis proceeds efficiently despite the curvature and small size of the circles, some of which have diameters significantly smaller than that of the enzyme itself. Second, the synthesis can proceed hundreds of times around the circle, while rolling replication of larger circular plasmid DNAs requires other proteins for processive synthesis. Finally, the synthesis scheme produces multiple copies of the template without the requirement for either heating or cooling cycles and requires less than stoichiometric amounts of primer, unlike other DNA synthesis methods. We report on the scope of this reaction, and demonstrate that the multimeric products can be cleaved enzymatically to short, sequence-defined oligodeoxynucleotides. This new approach to DNA synthesis may be a practical way to produce useful repeating DNAs, and combined with DNA cleavage strategies, it may represent a useful enzymatic approach to short, sequence-defined oligodeoxynucleotides.

Introduction

DNA polymerase enzymes serve to copy genetic information by elongation of short primer oligonucleotides and proceeding along a template strand, using Watson−Crick complementarity encoded by the template to incorporate nucleotide triphosphates into the polymer.[1] Here we report the use of an unusual class of synthetic DNA templates as substrates for DNA synthesis by DNA polymerase enzymes. Small chemically synthesized circular oligodeoxynucleotides are shown to be efficient substrates for a number of polymerases, producing repeating multimeric DNA strands which are end-to-end copies of the circular templates.

We have previously studied circular oligonucleotides as unusual ligands for high-affinity recognition of DNA and RNA sequences.[2−5] As part of that study we have developed methods for efficient synthesis of circular DNAs and RNAs ranging in size from 26 to 74 nucleotides.[6,7] Since nucleic acids serve not only as recognition elements but also as encoders of genetic information, we have begun a new program to examine whether such circular molecules might serve as enzyme substrates. We recently reported that RNA polymerase enzymes could utilize circular 34-nucleotide DNAs as templates for *de novo* synthesis of repeating RNA strands,[8] despite the fact that the circles do not contain RNA promoter sequences, and despite the small size and curvature of the circles. The processive synthesis, termed "rolling circle RNA synthesis", produces product RNA strands up to 9000 nucleotides in length, and may be a useful approach to the synthesis of biologically relevant RNAs.

We describe here studies directed at the use of small circles as substrates for DNA polymerases. We find that circles as small as 26 nucleotides can serve as efficient templates, and that repeating DNA strands as long as 12 000 nucleotides are produced. Variation of conditions establishes optimal concentrations of primer, circle, enzyme, and dNTP's for best polymer synthesis. We further demonstrate enzymatic cleavage of the multimeric products into unit-length oligodeoxynucleotides. This "rolling circle DNA synthesis" process represents a potentially useful method for generation of repeating DNAs and, combined with cleavage strategies, a new method for generation of short, sequence-defined oligodeoxynucleotides.

Experimental Section

Oligodeoxynucleotides. The procedure used for synthesis of DNA circles **3**, **4**, and **5** has been described elsewhere.[7] The method used for construction of the other circles was essentially the published procedure;[3] The linear precursor for circle **1** was 5′-pdmCTTTTm-CTTTTTTmCTTTmCTTTTTmCTTT (where mC denotes 5-methylcytosine), and the template used for cyclization was the duplex formed from 5′-dCCGGATCCTGGATGTTTAAAGAAAGAAAAGATT-CCGGAATTCCG and its complement. The linear precursor for circle **2** was 5′-pdTTTCTTCCCTCTTCTTTCTTTTCCACCCCTTTTC, and the template used for cyclization was 5′-dAAGAAAGAAAAG. The cyclization reactions contained 50 μM circle precursor, 50 μM template, 100 mM $NiCl_2$, 200 mM imidazole·HCl (pH 7.0), and 125 mM BrCN, and it was allowed to proceed 10 h at 23 °C. After dialysis and lyophilization the product was purified by preparative denaturing 20% polyacrylamide gel electrophoresis, and the product band was isolated by excision, crushing, and eluting into 0.2 M NaCl. The salts were removed by dialysis against distilled deionized water, and the DNA was quantitated by absorbance at 260 nm, using the nearest neighbor method to calculate molar extinction coefficients.

Linear oligonucleotides (circle precursors, linear controls, templates, and primers) were synthesized on an Applied Biosystems 392 DNA

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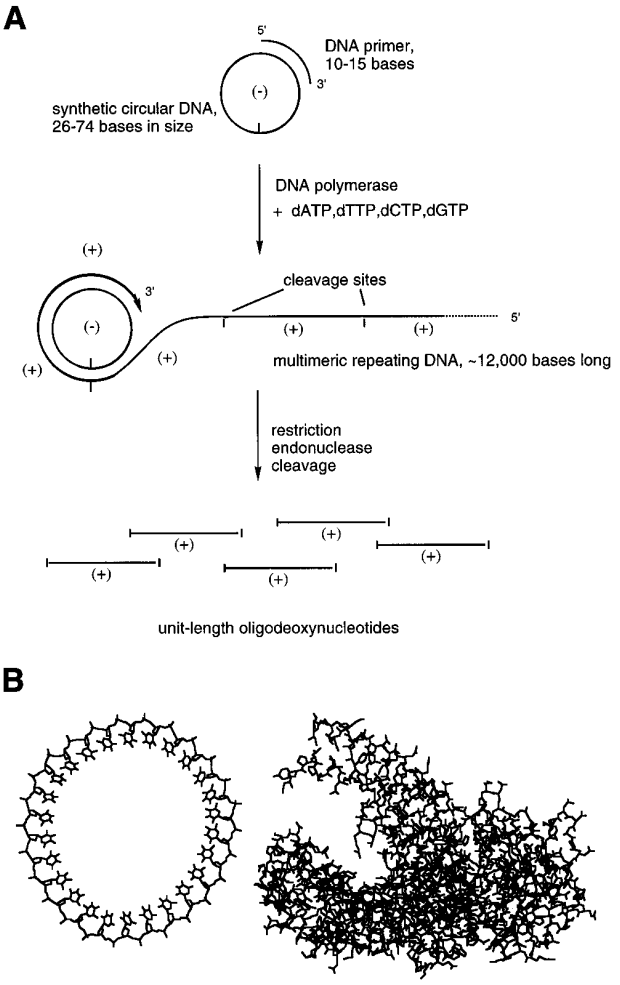

Figure 1. (A) Schematic of rolling circle DNA synthesis, in which small circular oligodeoxynucleotides act as templates for DNA polymerases. Multimeric product can also be cleaved into monomer-length oligonucleotides if desired, as shown. (B) Model of a circular 26-base oligodeoxynucleotide next to the crystal structure of the KF polymerase,[15] showing relative sizes. The internal diameter of the circle is ~40 Å; the enzyme dimensions are ~65 × 65 × 85 Å.

synthesizer using the standard DNA cycle. Deprotection in concentrated NH_4OH was carried out at 55 °C for 12 h. After lyophilization the DNA was purified by preparative denaturing polyacrylamide gel electrophoresis and quantified as described above.

Standard Conditions for Rolling Circle DNA Synthesis. Unless noted otherwise in the text, the conditions for DNA synthesis using the Klenow fragment (KF) of DNA Polymerase I were as follows: 10 nM circle, 10 nM primer, 1mM each of dATP, dTTP, dCTP, and dGTP (Boehringer Mannheim), and 4 units of KF (United States Biochemical) were incubated in 20 μL of Klenow reaction buffer (Tris·HCl 50 mM (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mg/mL BSA) for 1−24 h. The reaction was stopped by addition of 10 μL of a 40% formamide, 50 mM EDTA stop solution.

For other enzymes, the conditions used were as follows: T4 DNA Polymerase (GIBCO BRL) 0.25 units/μL, 10 nM circle, 10 nM primer, 1 mM of each of the four dNTP's in a buffer containing 33 mM Tris·acetate (pH 7.9), 66 mM sodium acetate, 10 mM magnesium acetate, 100 mg/mL BSA, 0.5 mM DTT, and with incubation at 37 °C for 3 h. T7 DNA Polymerase and Sequenase 2.0 (T7 Pol exonuclease free) obtained from United States Biochemical: 0.3 units/μL, 10 nM circle, 10 nM primer, 10 nM of each of the dNTP's in a buffer containing 40 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, and with incubation at 37 °C for 3 h. *Taq* DNA Polymerase (GIBCO BRL) 0.25 units/μL, 10 nM circle, 10 nM primer, 1 mM of each of the dNTP's in a buffer containing 20 mM Tris·HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, and with incubation at 65 °C for 3 h. E. Coli DNA Polymerase I (United States Biochemical) 0.5 units/μL, 10 nM circle, 10 nM primer, 1 mM of each of the dNTP's in a buffer containing 20 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, and with incubation at 37 °C for 3 h. Reactions were stopped as described above.

Time Course Dialysis Experiments. The polymerase reactions for quantitation by dialysis were carried out using 24 units of KF in 120 μL of a buffer containing 10 nM circle, 10 nM primer, 1 mM of each of the dNTP's, 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 50 mg/mL BSA. Aliquots (10 μL) were removed at desired time points and added to 5 μL of a 40% formamide, 50 mM EDTA stop solution. Distilled deionized water (90 μL) was added and the solution was dialyzed against distilled deionized water (23 °C, 4 changes, 1 L each) in a cellulose membrane with molecular weight cutoff 1000 (Schleicher and Schuell). The solution remaining in the dialysis tubing was diluted to 0.5 mL and quantitated by absorbance at 260 nm using an extinction coefficient of 7500 per nucleotide.

Enzymatic Cleavage of Multimers into Unit Length Oligonucleotides. The rolling circle reactions for restriction enzyme cleavage were carried out with internal labeling so that cleaved products would retain their labels. The reaction mixture was the same as the standard conditions described above except with 1 mM each of dATP, dCTP, and dGTP, 0.165 mM of α-[^{32}P]-dTTP (specific activity: 3000 Ci/mmol). Reaction products generated after 3 h of synthesis were precipitated by adding two volumes of precipitation buffer (50 mM KCl in ethanol) and were then resuspended in 20 μL of Taq Iα reaction buffer: 10 mM NaCl, 1 mM $MgCl_2$, 100 mM Tris·HCl (pH 8.4), 10 mg/mL BSA, with 5 u/μL Taq Iα (New England Biolabs). After 12 h of incubation at 65 °C, the products were analyzed by 10% denaturing polyacrylamide gel electrophoresis.

Sequencing of Rolling Circle Reaction Products. Sanger sequencing of a rolling circle product was carried out using the ^{32}P-end-labeled primer 5′-pdTTTCTTCCCTCTTCTTTCTTTTCCACCCCTTTC, which is the linear precursor to the circular template used in the synthesis of the polymer. The sequencing itself was carried out using the Sequenase 2.0 kit provided by United States Biochemical.

Results

Processing of Circles by Various DNA Polymerases. The scheme for rolling synthesis reaction with circular oligonucleotides is displayed in Figure 1. We first tested the polymerase reaction with a 34-nucleotide circular oligodeoxynucleotide using the Klenow fragment (KF) of *E. Coli* DNA polymerase I. Figure 2 shows the sequences of various circles used in this study and Figures 3 and 4 display results with the 34-nucleotide circle **2**.

We used standard conditions for enzymatic synthesis, with circle and primer at 10 nM each and 1 mM of each of the four dNTP's. The reactions were carried out at pH 7.5, 37 °C, for 3 h. A 12-nt primer complementary to the circle was used, and it was ^{32}P end-labeled; alternatively, the products were internally labeled by incorporation of a ^{32}P-labeled nucleotide triphosphate.

Early experiments using circle **2** showed that long DNAs were generated from the short labeled primer; these were too long to be resolved by a 10% polyacrylamide gel (Figure 3). Interestingly, when an unlabeled primer was used and labeled limiting dTTP was added, this resulted in a regular banding pattern of longer DNAs in the gel lane (Figure 3); size markers showed that the repeat unit was 34 nucleotides, the same size as the circle. Since an A occurs only once in the circle, the synthesis must pause to incorporate the label once per cycle around the template. This banding pattern provided good evidence that the circle was acting as a true template.

Further control reactions were run to examine the reaction (see Figure 4). Results of denaturing gel analysis of the products of primer extension show that the circular structure is required

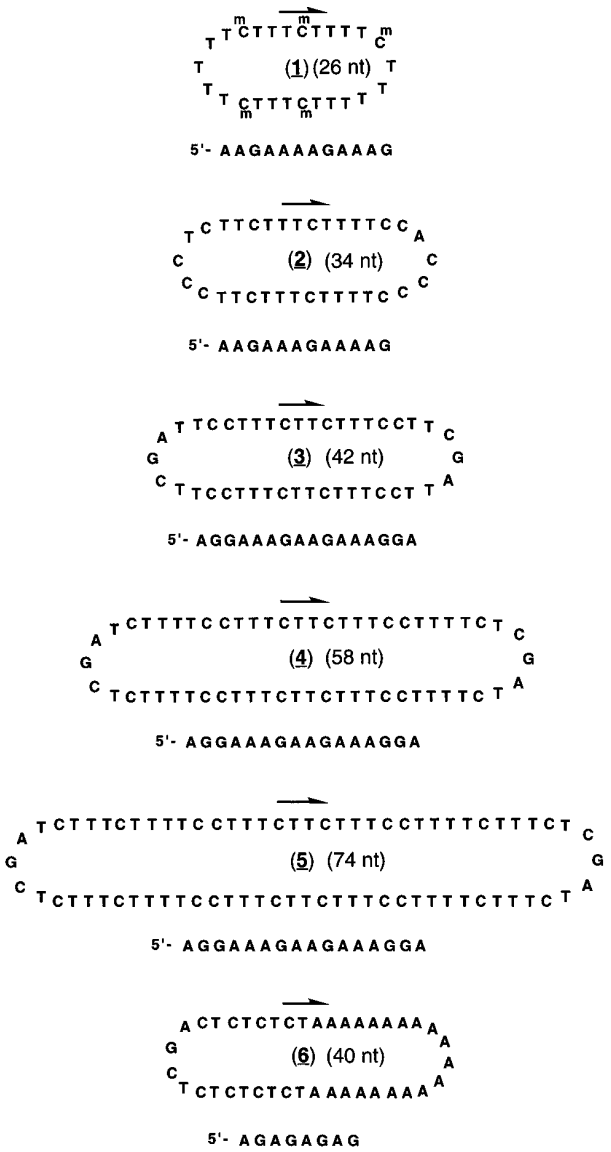

Figure 2. Sequences of the circular oligodeoxynucleotides used as templates for polymerases in this study. Shown underneath each circle is the corresponding primer used to initiate synthesis. Arrows indicate 5′ to 3′ strand orientation; mC (used in circle **1**) refers to 5-methylcytosine.

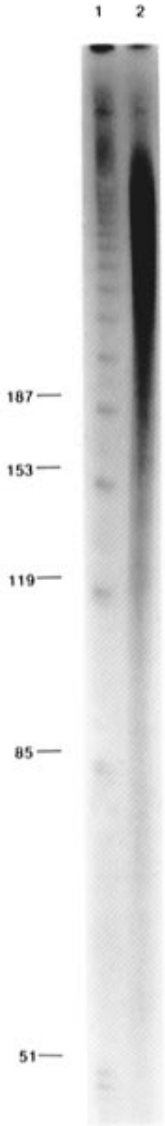

Figure 3. Rolling circle DNA synthesis with 34mer circular DNA template **2** and KF enzyme. Shown is an autoradiogram from 20% denaturing PAGE gel analysis of products, which are radiolabeled either by (lane 1) incorporation of α-^{32}P-dTTPs or (lane 2) by initiating with ^{32}P-labeled primer. Banding patterns produced during synthesis (lane 1) arise from limiting concentration of dTTP in reaction, causing pauses once per turn, since only one A is present in the circle.

for synthesis of long DNAs: A linear 34-nt oligonucleotide having the same sequence as the circle gives very little extension of the primer (Figure 4), while the closed circle gives a large amount of long products. Other experiments show an absolute requirement for dNTP's, primer, circle, and enzyme in the primer extension reaction.

We then tested a number of commercially available DNA polymerase enzymes for their ability to utilize this same circle as a template (Figure 5). The same radiolabeled primer was used, and recommended reaction conditions were chosen from suppliers of the enzymes. Results analyzed by agarose gel electrophoresis show that several DNA polymerases give long products by extension of the primer. The Klenow enzyme, DNA Pol I holoenzyme, T4 DNA Polymerase, and T7 DNA Polymerase and its (exo-) variant all appear to give significant amounts of products predominantly longer than ∼2000 nucleotides as judged by size markers. The Taq Polymerase at 72 °C was the exception, giving only small amounts of extended products. The Klenow enzyme and T4 DNA Polymerase were the most successful at generating the longest and most abundant products, and DNA Polymerase I holoenzyme was next most proficient. The two T7 variants gave smaller amounts of labeled long products, at least under the conditions studied.

Reaction Time Course. To investigate the time dependence of the rolling circle reaction, we carried out a time course experiment using the Klenow enzyme and 42mer circle **3**. Figure 6 shows the appearance of products over time as analyzed by agarose gel electrophoresis. The results of this experiment show that the amount of radioactivity in product bands increases over a period of ca. 3−6 h, and that the length of the products increases from 1 kb at 10 min to 2 kb at 30 min, and then to at least 12 kb at 3 h. Judging by the 10- and 30-min bands, the product length increases at a rate of ca. 50 nucleotides per minute. This is slow relative to the known rate of nucleotide incorporation by Pol I of ca. 600 nucleotides per minute.[1]

We also quantitated the number of nucleotides being taken up as a function of time. This was carried out by stopping the

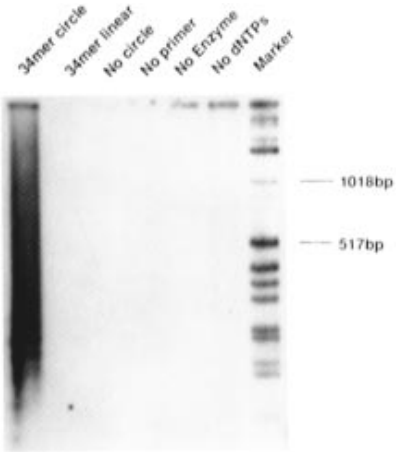

Figure 4. Rolling circle DNA synthesis from 34mer circle **2** (lane 1) and control experiments (lanes 2−6) showing the requirement for circularity, template, primer, enzyme, and dNTP's for successful reaction. Shown is an autoradiogram from 1% agarose gel analysis of products, which are radiolabeled by initiating with ^{32}P-labeled primer. Residual counts at the origin (lanes 5 and 6) are artifacts arising from ethanol precipitation.

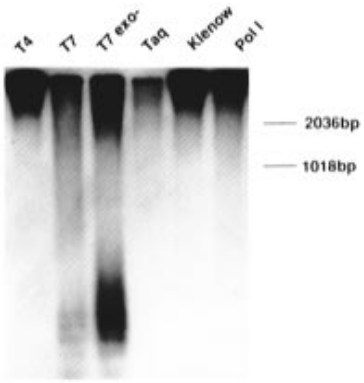

Figure 5. Comparison of the abilities of several DNA polymerases to process 34mer circular DNA template **2**. Shown is an autoradiogram from 1% agarose gel analysis of products, which are radiolabeled by initiating with ^{32}P-labeled primer. See Experimental Section for details.

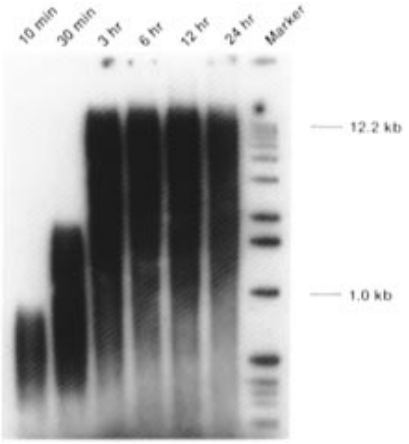

Figure 6. Time course showing the increase in length of rolling circle products. Shown is an autoradiogram from 1% agarose gel analysis of products at several time points in one reaction with 42mer circle **3** as template. Products are radiolabeled by initiating with ^{32}P-labeled primer.

reaction with EDTA at specific time points and dialyzing away unreacted dNTP's. The remaining nucleotides in polymeric strands were then quantitated by absorbance at 260 nm. Figure 7 displays results of this analysis. Under standard conditions we find that the reaction approaches ∼90% completion after ca. 6 h. At the plateau level, the enzyme has incorporated approximately 1% of the dNTP's into polymer.

Effect of Circle Size and Sequence. Since it seemed likely that there exists a circular oligonucleotide which is too short and curved to serve as a DNA polymerase substrate, we investigated whether the size of the circle over the range we have synthesized would have an effect on the rolling circle synthesis. To this end, we examined circles 26, 34, 42, 58, and 74 nucleotides in size (see Figure 2 for sequences) as templates for the Klenow enzyme. The results are shown in Figure 8. The results of this experiment show clearly that all the circle sizes tested are successful as templates for the Klenow enzyme. The 26mer, 42mer, and 74mer all show similar amounts of radioactivity and similar lengths of products, indicating that circle size over this range does not have a large effect on the polymerization. Interestingly, the 34mer shows considerably shorter products (up to ∼2 kb in length) than do the other circles.

The circles **1**−**5** are all pyrimidine-rich in sequence. To test whether this sequence bias might affect the ability to act as a rolling template for the Klenow enzyme, we then examined circle **6** (Figure 2), a compound having 22 purines of 40 total nucleotides. It was compared to circle **3**, a 42mer containing 38 pyrimidines. Treatment under standard polymerase reaction conditions revealed (data not shown) that both circles produced very long bands by agarose gel electrophoresis, with very little difference between the two circles. Thus the data indicate that circles of quite varied sequence composition can behave as efficient templates.

Product Length and Sequence. Agarose gel analysis of the lengths of the products shows (Figure 8) that most of the circles give product lengths greater than 2000 nucleotides. Some cases (particularly the 26mer and 42mer) give products longer than can be resolved on this gel; the lower limit is 12 000 nucleotides for those cases. For a 26mer circle, this length corresponds to at least 460 times around the template, while for the 74mer, it is ca. 160 times around.

There have previously been a few examples in the literature in which a linear strand of DNA can act as template in the synthesis of product DNA strands that are longer than the template.[9,10] Mechanisms proposed for this are strand slippage, which is most likely to occur with repetitive template sequences, and "fold-back" synthesis, in which strands template themselves by forming hairpins and reversing direction. In the present case we have shown (see above) that linear precursors to our circles do not serve as efficient templates, while the circles do. This is consistent with a rolling mechanism for synthesis which produces the very long products, and is not supportive of the other mechanisms. However, we wished to gather separate evidence which might support this mechanism.

One experiment which confirms a rolling synthesis mechanism arises from the use of α-^{32}P-dTTP as the source of radioactivity rather than radiolabeling the primer. If a circle contains a single copy of one of the four bases, then use of a limiting amount of the labeled complementary nucleotide will result in a pause before its incorporation each time around the circle. Since the primer starts at a unique site, the enzymatic synthesis will give predictable bands which begin at the distance from primer 5′ end to first pause site, and then progress upward on the gel with a repeat length exactly the size of the circle.

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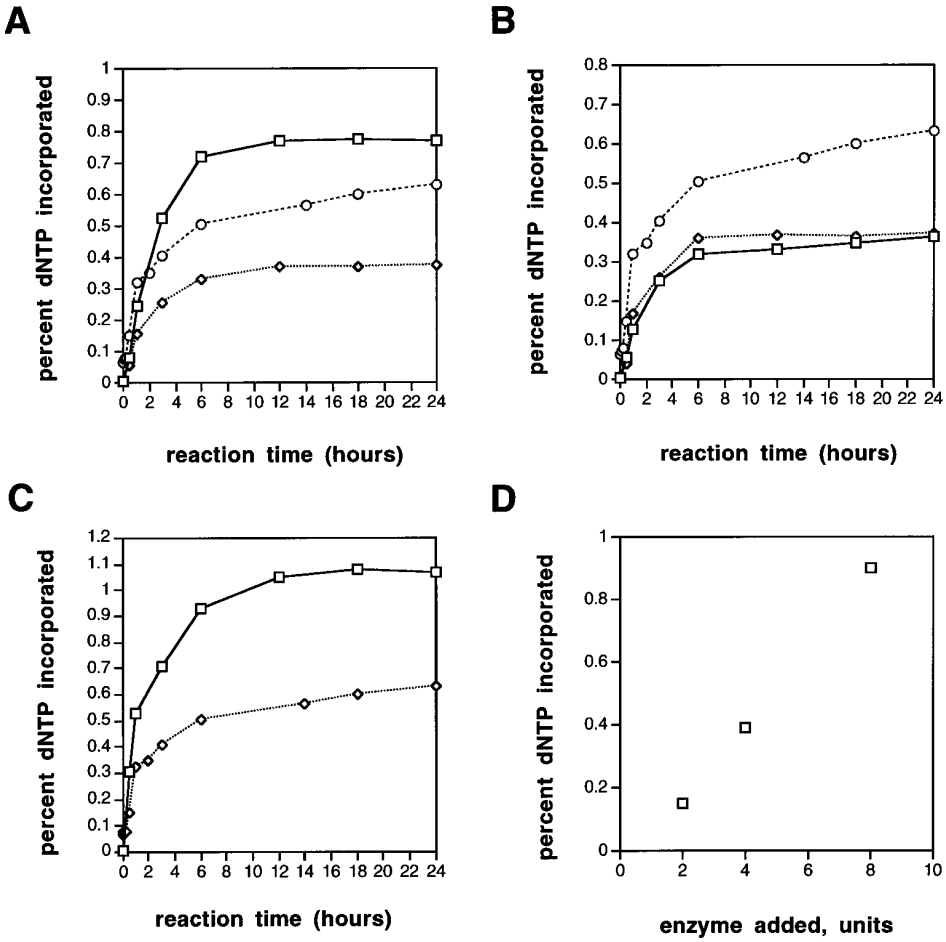

Figure 7. Quantitative analysis of nucleotide uptake into polymeric products, as a function of circle and primer concentrations and ratios, dNTP concentrations, and enzyme activity. (A) The effect of [circle•primer] concentration on nucleotide uptake, using 1:1 circle:primer ratio. Legend: [circle•primer] = 1 nM (◇), 10 nM (○), 100 nM (□). (B) The effect of varied circle:primer ratio on nucleotide uptake. The ratios are 5:1 (□), 1:1 (○), and 1:5 (◇). (C) The effect of lowering dNTP concentration on nucleotide uptake. [dNTP] = 1 mM (◇) and 0.5 mM (□). (D) The effect of initial enzyme amount on polymer yield (as percent nucleotide uptake) at 3 h of reaction. The nucleotide fraction incorporated into polymeric strands was quantitated by UV absorbance of reaction products after removal of free nucleotides by equilibrium dialysis (see Experimental Section).

Figure 3 displays the result of such an experiment. Circle **2** is a 34mer with a single A residue. We carried out the enzymatic synthesis using cold primer, cold dCTP, dATP, dGTP, and labeled dTTP in limiting concentrations. Results show a very regular banding pattern in the gel lane, giving a repeat unit of exactly 34 nucleotides, as mentioned above. The fastest band has length of 17 nucleotides, also as predicted based on the distance from primer to the first A in the circle (i.e., the first pause site). It should be noted also that the multimeric products under these conditions are not, on average, as long as those using radiolabeled primer. This is expected, since use of a limiting nucleotide causes pauses which will tend to shorten the average strand length.

Sanger sequencing was also carried out to confirm further the proposed rolling mechanism. For this we used the precursor of circle **2**, a linear 34mer, as the radiolabeled primer, and we carried sequencing on the rolling circle product from circular template **2**. Results show that the sequence is as predicted; the multimer product is a regular repeating multimer copy of the circular template. Thus we conclude that, at least for this circle and this enzyme, a true rolling mechanism is operative for the majority of the observed product.

Although the evidence points strongly to a rolling circle mechanism for the production of the observed long DNA products, this does not rule out a combination of rolling synthesis and some self-priming "fold-back" synthesis. The first mechanism alone would produce only single-stranded DNA, while the addition of the second mechanism would tend to convert it to double-stranded products. To test whether this was occurring, we ran a standard reaction using circle **3** as template and with internal radiolabeling. The products were then exhaustively cleaved with the restriction endonuclease *Taq* Iα, which has a unique site in the repeating sequence, and then analyzed on a nondenaturing polyacrylamide gel. We used authentic oligonucleotides (single- or double-stranded) having the expected sequence as markers for comparison. The autoradiogram (not shown) revealed that the cleavage yielded one principal band which co-migrates with authentic single-stranded product, and little or no band which has the mobility of the duplex expected from "fold-back" synthesis. Quantitation of radioactivity in these two positions in the gel showed a ratio of at least 4 to 1 single-stranded to double-stranded products. This provides additional support that (at least for this sequence) the products arise chiefly, if not completely, from a rolling circle mechanism.

Also providing supporting evidence of a rolling mechanism for three of the circles are additional experiments in which the multimeric products are cleaved into predicted oligonucleotide products by a restriction endonuclease. Those experiments are described below.

Optimization of DNA Yields. Since this mechanism is nontypical for most DNA synthesis carried out previously, we felt it prudent to attempt to quantify the products of the reaction

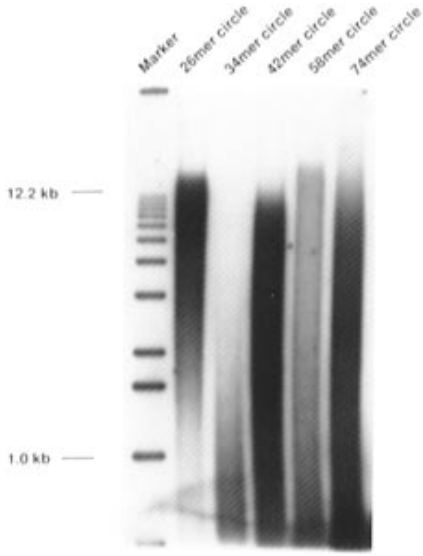

Figure 8. The effect of size of circular oligodeoxynucleotides on rolling synthesis products with the KF enzyme. Shown is an autoradiogram from 1% agarose gel analysis of products, which are radiolabeled by initiating with ^{32}P-labeled primer.

relative to the reagents used at the start, and to begin to optimize the conditions for the reaction. To this end we used reactions run without radiolabeling and quantified polymeric products by dialysis followed by measurement of UV absorbance, as described above.

To investigate possible effects of concentrations of the species involved in the reaction, we followed reactions using the 42mer circle over a 24-h period (see Figure 7) were run. Early results indicated that the products reached a plateau after ca. 6 h, although dNTP's and primer were not completely consumed. We varied concentration of one of the constituents and observed the result on the nucleotide uptake into polymer. The nucleotide uptake was plotted as a percentage of total [dNTP] (see Figure 7). In separate experiments we varied (circle + primer) concentration, circle:primer ratio, dNTP concentration, and enzyme amount. The results show (Figure 7) that all of these factors affect polymer yield. Increasing the (circle+primer) concentration over a three-decade range resulted in a ~3-fold increase in yield. The results also indicate that a circle:primer ratio of 1:1 may be optimum; addition of extra equivalents of circle or primer was seen to decrease yield by approximately 2-fold. Lowering dNTP concentration by 2-fold did not change the amount of product, thus increasing the effective yield by 2-fold. Finally, the addition of increasing amounts of enzyme at the start of the reaction resulted in a nearly linear increase in yield after 3 h of reaction (Figure 8D).

The plots of yield as a function of time show that the reaction reaches a plateau after a few hours even though dNTP's have only been consumed to the level of ca. 1%. In addition, we observed that only ca. 10% of the primer is consumed. Several explanations are possible for the slowing of the reaction. First, the enzyme may lose activity after a few hours; second, the dNTP's might be unstable in the reaction conditions; or third, the circle might bind strongly to the polymeric product strands and thus the reaction might be inhibited by products.

To test these possibilities we carried out a standard reaction, quantifying products over 24 h, and then added a second portion of enzyme after this time and continued monitoring product formation. The experiment is displayed in Figure 9. Results show clearly that addition of the second amount of enzyme resulted in a second, almost identical growth in product

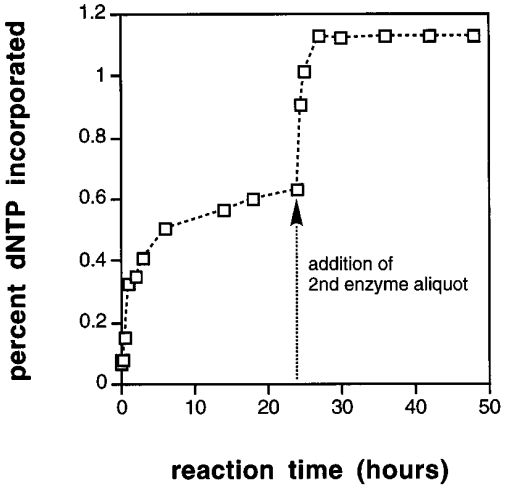

Figure 9. Effect of addition of a second aliquot of KF polymerase to a reaction mixture with 42mer circle **3** after 24 h. Quantitation of nucleotides taken up during rolling circle synthesis was performed by equilibrium dialysis (see Experimental Section).

formation. Thus we conclude that the enzyme loses activity under the reaction conditions, having a half-life of approximately 2 h. The results also establish that dNTP's are still viable well after 24 h and that any binding of products by the circular template does not appear to inhibit product formation under these conditions.

Cleavage of Multimers into Unit-Length Oligonucleotides. The enzymatic processing of the small circular templates results in the synthesis of long repeating multimeric copies of the circular template. Some potential uses may exist for such a repeating sequence (see Discussion section). In addition, for some applications it might be desirable to cleave the multimer into unit lengths since oligodeoxynucleotides of defined length and sequence have many uses. In addition, if such a cleavage were successful this would provide further evidence that the long DNA strands being synthesized are true repeating copies of the circle.

To test this we carried out enzymatic reactions using KF and three circular templates of successively larger sizes of 42, 58, and 74 nucleotides. These three circles were each synthesized by end-to-end dimerization of one half-length oligonucleotide,[7] and so the 36mer contains two 17mer repeats, the 58mer two 29mer repeats, and the 74mer two 37mer repeats. To visualize the products we carried out reactions using cold primer and labeled the products by use of α-^{32}P-dTTP, which could be incorporated once per half-cycle around a template. Figure 10 shows the polyacrylamide gel analysis of the products of the three reactions. The reactions show regular banding patterns progressing upward on the gel, as expected for circles with two specific priming sites and two pause sites.

We then tested the possibility of cleavage of these internally labeled products. All three of these circles contain two TCGA sites, which correspond to the palidromic recognition site of the endonuclease *Taq* Iα. This enzyme has single-strand cleavage activity as well as the expected double-strand activity. Reactions were run as above with internal labeling, and then the products were cleaved using this enzyme. Results show (Figure 10) that the long products are almost completely cleaved to the expected products. For example, with the 42mer circle there are two major products of cleavage seen: a 21mer band (corresponding to the repeat unit) and an 18mer band (corresponding to the first unit from the primer to the first cleavage site). One or two minor products are also visible at 42 and 39

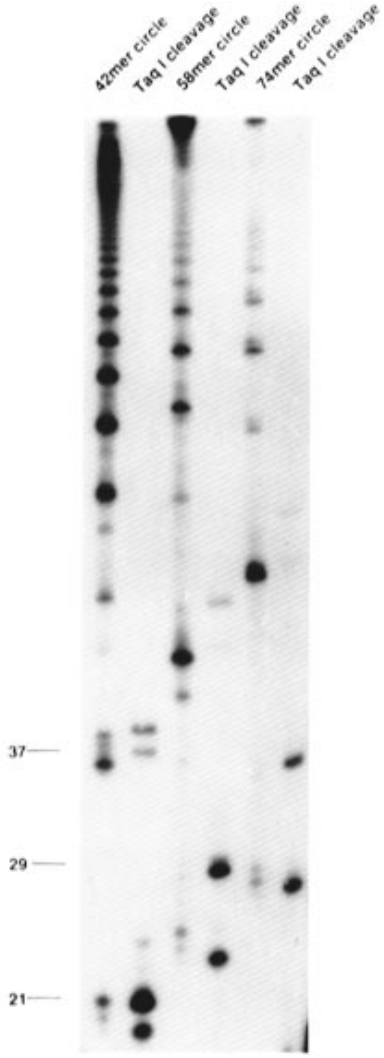

Figure 10. Cleavage of multimer products arising from three different circles (**3**−5) with restriction endonuclease *Taq* Iα. Shown is an autoradiogram from denaturing PAGE gel analysis of products, which are radiolabeled by incorporation of α-^{32}P-dTTP. Banding patterns produced during synthesis (lanes 1, 3, 5) arise from limiting concentration of dTTP in reaction. After cleavage, predicted products are half-length copies of the circles, which contain two copies of the same sequence.

nucleotides; these correspond to products arising from incomplete cleavage of one half-circle unit from the major products. Similar product bands are observed for the other two circles: the 58mer circle produces a major 29mer band after cleavage, and the 74mer circle produces a 37mer band after cleavage.

Thus, the results show that the repeating multimers can indeed be cleaved using a commercially available restriction enzyme, and this produces the expected unit-length oligonucleotides as the chief product. Since the recognition site in the circular template is palindromic, the multimer copy contains the same sequence once per unit. This serves as strong evidence that the enzymatic synthesis is a true rolling progression of the enzyme around the circle.

Discussion

Unusual Polymerase Substrates. The successful utilization of very small circular DNAs as polymerase templates poses interesting structural and topological problems. It seems surprising at first glance that circles as small as 26 nucleotides can be replicated efficiently. We estimate the diameter of a circle this size to be ca. 40 Å; this is smaller than the diameters of common polymerase enzymes themselves (see Figure 1B). It is the business of a DNA polymerase to synthesize a duplex from a single-stranded template, and yet it is likely not possible to form a stable duplex all the way around a 26mer circle because of the tight radius of curvature. Indeed, most studies aimed at cyclization of short duplexes by ligation have shown that it is very difficult to cyclize fragments shorter than ca. 120 base pairs long.[11] This is much longer than any of the circles in this study.

We hypothesize, therefore, that with these synthetic circular templates the nascent DNA strand is not hybridized completely around the circle during enlongation. This implies that as nucleotides are being added at the 3′ end of the new strand, the DNA is unwinding itself spontaneously from the circular template a number of nucleotides behind the enzyme. This therefore implies an unusual mechanism for the small circles and leads to a testable prediction, that as a circle becomes larger and larger there will come a point where self-unwinding will not occur because of lack of strain in the curved duplex. Rolling replication of large plasmid-sized circles is known to require unwinding and single-strand binding activities to aid in displacement of the duplex in front of the polymerase.[12] In the present experiments the rolling replication occurs very readily (indeed, more than 300 rounds) even in the absence of any proteins other than the polymerase itself.

Our finding that polymerization occurs rather slowly relative to that on linear templates is consistent with an unusual mechanism. For example, a slow rate of progression might arise from the additional strain which would tend to be imposed when a nucleotide is added to the chain, thus lengthening the duplex within a bent structure. Another possible explanation for slow progression is that the enzyme dissociates and re-initiates multiple times during the elongation of a given strand; this might also be favored by the geometric constraints of the templates. It is also possible that the circle itself may switch to a different product strand during elongation; further experiments will be necessary to test these possibilities.

Future studies will investigate further the lower and upper size limits on this unusual circular template ususage. On the small side, it is clear that there exists a circular DNA too small to be efficiently replicated, since single-stranded nucleic acid circles are known as small as two nucleotides in length. The lower limit is, therefore, somewhere between 26 and 2 nucleotides. Reaching the upper limit (if indeed there is one) may be dependent mostly on the ability to produce synthetic single-stranded circles large enough for this purpose. We have begun investigating convergent strategies for synthesizing such longer oligonucleotide molecules.[7] If there is a special "self-unwinding" mechanism involved with these very small templates, then this leads to the prediction that there will be an upper size limit which corresponds to the smallest relatively unstrained circular duplex.

Another factor which arises in the rolling replication is a topological one: as the right-handed duplex is being synthesized, there is a tendency for the new strand to be pulled through the center of the circle once per turn. It is clear that this does not occur, since most of the newly synthesized strands are hundreds of turns in length, and thus would stall out the synthesis at much shorter lengths than we observe. Two factors may negate this topological problem: First, since the circular template is single

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(12) Eisenberg, S.; Scott, J. F.; Kornberg, A. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3151−3155.

stranded it can turn itself "inside out" by rotating about any of the phosphodiester bonds, erasing any twisting tendency. Second, it is possible that the duplex unwinds itself less than a turn behind the polymerase complex, thus requiring no alleviation of winding tendency.

After we completed the majority of this work, another report was published on rolling replication of small circular DNAs.[13] It describes the use of DNA circles as small as 34 nucleotides as templates for DNA synthesis, and they report examining three DNA polymerases for this activity. Our study is in agreement with the finding that *E. Coli* DNA polymerase I efficiently utilizes small circles; however, their study found no other enzymes that operate as efficiently, although T7 and Klenow fragment were also examined. Our results show, however, that at least three polymerases process small circular templates efficiently, and we find that both T4 polymerase and Klenow are more efficient than Pol I holoenzyme. It is possible that differences in the reaction conditions or in enzyme activity may explain part of this difference.

A number of other differences exist in the two studies. Our study has established that the lower circle size limit for replication is at least eight nucleotides shorter than the smallest compound in the earlier study.[13] Another difference is that the previous study utilized circles cyclized enzymatically, while our templates were cyclized chemically; thus the mode of cyclization has no apparent effect on the reaction. We have also succeeded in showing that under optimum conditions the multimer product strands can be longer than 12 kb in length, while the previous report did not resolve the lengths of the products. Finally, the present results show that the single-stranded multimer products can be directly cleaved into monomer oligonucleotides, while the previous study performed cleavage only after the multimer was made double stranded in a separate enzymatic polymerization.

DNA Synthesis/Amplification Method. The previous study utilizing small circles as templates did not investigate the optimization of synthesis in terms of maximizing product and minimizing catalyst (circle, enzyme) use.[13] We felt it worthwhile to investigate these factors since the reaction can be considered an amplification of a DNA sequence and thus might have practical application.

If one examines the stoichiometry of the rolling reaction one notes that only the dNTP's are consumed stoichiometrically. This is unusual for DNA amplification: the polymerase chain reaction, for example, consumes both dNTP's and one primer per DNA copy. In the present reaction the primer is used once to begin a strand and then hundreds of repeating copies are produced; in effect, the strand is self-priming after initiation. The circular template is, of course, catalytic since it turns over (quite literally) hundreds of times making one product strand, and it is possible that it may then be reused for a new strand if a new primer is available.

An analysis of product yields under our standard reaction conditions shows that, using a 42mer template, ~3 ng of multimer DNA is produced for each picomole of circle•primer complex. We have also shown that this can be at least doubled by addition of greater amounts of enzyme. With the assumption that all primers are consumed, each primer is used to make 240 copies of the template sequence; however, the true number may be closer to 2000 copies, since we estimate that roughly 10% of the primer is in fact consumed under our standard reaction conditions.

Thus, the rolling circle reaction has a number of characteristics which make it worth study as a method for synthesis of DNA. It would seem to be the method of choice for preparation of repeating DNA sequences. As a simple amplification method, of course, PCR is superior because of its exponential amplification and its sensitivity.[14] On larger scales, PCR may be less economical because of its stoichiometric primer use. For the rolling circle synthesis, larger scales are less of a factor because of the low primer use and the lack of heating−cooling cycles. Practical application on larger scales would, of course, require determination of limiting factors and analysis of reagent and catalyst cost. Our results show that the enzyme itself is the limiting factor in converting dNTP's to useful DNA sequences. Two strategies for addressing this are to find ways to enhance the stability and lifetime of the enzyme and to overexpress the enzyme in the laboratory to lower its cost.

Conclusions. The present study explores the finding that very small synthetic circular oligodeoxynucleotides can serve as efficient templates for DNA polymerase enzymes. The results establish that circles ranging from 26 to 74 nucleotides in size act as rolling templates, and that the product multimeric strands can be longer than 12 kB in size. The results also show that a number of enzymes, including *E. Coli* Pol I, the Klenow enzyme, and T4 DNA Polymerase, utilize these synthetic templates efficiently. Our optimization studies reveal conditions which yield largest amounts of the repeating product strands, and point out that polymerase stability is the factor currently limiting yields.

It seems possible that rolling circle DNA synthesis may become a useful method for preparing specific DNA sequences. Repeating DNA sequences can be useful as hybridization probes for nucleic acids and as affinity probes for DNA-binding proteins. If part of the repeating sequence is randomized, the circle itself or the multimer products can be useful for *in vitro* selection experiments.[13] In addition, repeating sequences can be used to encode engineered repeating peptide sequences. Finally, it is possible that, combined with efficient DNA cleavage strategies, the new method may be used to generate unit-length oligonucleotides in amplified amounts. Studies exploring these possibilities are currently underway.

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