Enzymatic multiplication of a chemically synthesized DNA fragment

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ABSTRACT

A synthetic DNA fragment of 19 residues was enlarged by the enzymatic addition of deoxyadenylate residues to its 3'-end with calf thymus terminal deoxynucleotidyl transferase. The 3'-terminus of this elongated DNA strand was blocked with 2',3'-dideoxyadenylate to prevent hydrolysis by the 3'-exonuclease function of E. coli DNA polymerase I. This elongated and 3'-blocked fragment was annealed to an oligomeric primer and used as a template for the synthesis of a complementary copy of the synthetic 19-mer. The product of such a repair synthesis was separated by gel filtration and analyzed by nearest neighbor techniques. All template strands were copied with complete repair in over 90% of the chains. Facile recovery of the elongated template by virtue of its size permitted repetition of the copy process, thus allowing accumulation of the desired strand.

INTRODUCTION

The synthesis of a DNA duplex coding for an S-peptide analog of bovine pancreatic ribonuclease A (1) led to the study of its enzymatic multiplication. There is need for a method to increase the original quantity of a chemically synthesized DNA sequence after its laborious assembly. In principle, a synthetic DNA duplex can be reproduced indefinitely by enzymatic means. However, at the present time this multiplication can only take place within a system analogous to the living cell. All in vitro systems using the known DNA polymerases have requirements that make them basically "repair enzymes". The mode of action of these polymerases (especially those from E. coli) has been studied by a number of investigators (2,3,4) and is well characterized.
Repair synthesis requires, in addition to the polymerase, substrates and appropriate metal ions, a primer with a 3'-hydroxyl terminus hydrogen-bonded in the usual antiparallel fashion to a protruding template strand. Polymerization then starts at this terminus and proceeds until the template has been copied in the Watson-Crick sense to its 5'-terminus.

Our approach to the enzymatic joining of chemically synthesized deoxyriboöligonucleotides yields each strand of a duplex separately. In principle, such strands can be multiplied via repair synthesis as discussed above. In this paper, we present a scheme which overcomes certain technical difficulties, such as enzymatic destruction of the template, and permits facile separation of product and, indeed, template for repeat use. DNA polymerase I is used in the following fashion (figure 1): (1) a template is elongated from its 3'-terminus; (2) the elongated template is protected against degradation by blockade with 2',3'-dideoxyribonucleotide; (3) the desired sequence is produced by

![Diagram](image-url)
appropriately-primed repair synthesis; (4) the resulting transcript is separated from the elongated template by virtue of size difference; (5) the cycle resumes with (3).

In the experiments here described, the scheme was validated by appropriate model experiments. A modified template was generated from a chemically synthesized nonadecamer as described in Figure 1, and an appropriate primer, also of chemical provenance, was repaired enzymatically so as to constitute a complete and faithful complement of the nonadecamer. The product was separated from the template, and the latter was again used to direct the synthesis of further product.

MATERIALS AND METHODS

Enzymes. Highly purified *E. coli* DNA polymerase I was prepared by the procedure of Jovin et al. (5). Terminal deoxynucleotidyl transferase from calf thymus was purified as described (6), except that the final hydroxyapatite chromatography was omitted. The enzyme from the Sephadex G-100 column was further purified by isoelectric focusing (LKB 8100; pH 7 to 9). The final preparation had no detectable exo- or endonucleolytic activity under the conditions of use. Micrococcal nuclease, venom phosphodiesterase and spleen phosphodiesterase were obtained from Worthington Biochemical Corporation. Polynucleotide kinase was obtained as previously described (7). Calf intestinal alkaline phosphatase was obtained from Sigma Chemical Company and analyzed as previously described (8).

were described earlier (8).

2',3'-Dideoxyadenosine triphosphate was prepared by allowing 5 mmoles of anhydrous pyridinium S-ethyl phosphorothioate to react with 2 mmoles of N-dimethylaminomethylene-2',3'-dideoxyadenosine and 11 mmoles of dicyclohexylcarbodiimide in anhydrous pyridine. After 16 hr at room temperature, the reaction was stopped with water, filtered, and the filtrate chromatographed on a 5 x 90 cm column of DEAE-cellulose with 6 liters of a linear buffer gradient varying from 0.005 to 0.2 molar triethylammonium bicarbonate (pH 7.5). The product, eluted at 0.09 M buffer, was shown to be homogeneous by paper chromatography. After removal of the N-dimethylaminomethylene group with concentrated ammonia the S-ethyl nucleoside phosphorothioate (1.3 mmoles) was allowed to react with pyridinium pyrophosphate (6.7 mmoles) and iodine (11 mmoles) in anhydrous 2-picoline for 16 hours at room temperature. The reaction was quenched with water, extracted with ether, and the aqueous phase chromatographed on a 2 x 50 cm column of DEAE cellulose. Elution was carried out with a 4 liter linear gradient of 0.005 to 0.5 molar triethyl ammonium bicarbonate. The 2',3'-dideoxyadenosine triphosphate eluted at 0.175 molar buffer, was characterized by paper chromatography and enzyme degradation.

Elongation of nonadecamer template. The template was extended in a reaction mixture containing 0.2 M potassium cacodylate (pH 7.2), 4 mM MgCl₂, 1.2 mM 2-mercaptoethanol, 0.52 nmol nonadecamer and 56 nmol [³¹⁴C]dATP (specific activity 24 cpm/pmol) in a volume of 1 ml. The reaction was incubated at 37° for 16 hours with 128 units of terminal deoxynucleotidyl transferase.

The elongated template was separated from unreacted starting material and excess dATP by chromatography on a column of G-75 Sephadex superfine (0.9 x 100 cm). The column was equilibrated and eluted with 0.1 M triethylammonium bicarbonate (pH 8.0) buffer.
Preparation of [14C] labeled d(pT)₃-d(pA)₇₀. The oligomer d(pT)₃ was extended with [14C]deoxyadenylic acid residues as described for elongation of nonadecamer template. The labeled oligomer was divided into two parts; one was used for the 3'-end addition of 2',3'-dideoxyadenylic acid.

Addition of 2',3'-dideoxyadenylic acid to elongated template. Elongated template, 0.10 nmol, was incubated in 0.2 M potassium cacodylate (pH 7.2), 4 mM MgCl₂ and 1.2 mM 2-mercaptoethanol with 32 nmol 2',3'-dideoxyadenosine 5'-triphosphate in a total volume of 0.20 ml. The solution was incubated with 32 units deoxynucleotidy1 transferase for 1 hour at 37°C and heated for 10 min at 75°C to denature the enzyme. Excess triphosphate was removed by passage of the reaction through a Sephadex G-75 column (0.9 cm x 45 cm) using 0.1 M triethylammonium bicarbonate (pH 8) as the eluent. The buffer was removed from the product by rotary evaporation and the product dissolved in water.

Preparation of the Complementary Strand. A copy of the template was made according to the scheme shown in Fig. 2a in a reaction mixture of 0.1 ml containing 120 mM potassium phosphate (pH 6.9), 8 mM MgCl₂, 5 mM dithiothreitol, 10 nmol each of four deoxyribonucleoside triphosphates (ᵣ200 CPM/pmol of [α³²P]TTP), 20 pmol of template and 80 pmol of tridecamer d(pT-T-A-A-T-C-C-A-T-A-T-G-C) primer. The mixture was annealed by heating to 70°C for 2 minutes and cooled slowly to 15°C over the course of 1 hour. 30 Units of E. coli DNA polymerase I were added and the resulting solution was incubated for 4 hours at 15°C. The ³²P-labeled product was separated from excess [α³²P]TTP by spotting on a DEAE-cellulose paper strip (Whatman DE-81) followed by development with 0.5 M triethylammonium bicarbonate buffer (pH 7.6) for 4 hours.

5'-End Group Labeling with Polynucleotide Kinase. If the oligomer contained a 5'-PO₄, the latter was removed in a reaction
mixture (30 µl) containing 5 µmol of Tris (pH 7.6), 2.5 µg of serum bovine albumin, and 5 µg of calf alkaline phosphatase. After incubation at 37° for 1 hour, the phosphatase was inactivated by heating at 100° for 3 minutes. The 5'-OH oligomer was labeled with $^{32}$P by bringing the reaction mixture up to 50 µl by addition of 0.5 µmol MgCl$_2$, 0.2 µmol dithiothreitol, 500 pmol $[^{32}$P] ATP (≈500 CPM/pmol) and 10 units polynucleotide kinase. The reaction mixture was incubated 1 hour at 37° and applied to a 3 cm wide strip of DEAE-cellulose paper (Whatman DE-81). The strip was developed in descending fashion with 0.3 M ammonium formate (pH 7) for 3 hours. If necessary, the area containing 5'-$^{32}$P labeled oligomer was eluted with 2M-triethylammonium bicarbonate (pH 7.6). When elongated template was labeled with 5'-$^{32}$P, the phosphorylated product was separated on a G-75 Sephadex column as described for elongation of nonadecamer template.

**RESULTS**

Conditions for completion of copy. It should require 80 pmol of $[^{32}$P]TTP to complete the copy of 20 pmol of nonadecamer template with the tridecamer as primer (Fig. 2a).

\[
\text{AAGACAGCATATGGATTAA } 3' \\
\text{GTAATCATTGC} \\
(a.)
\]

\[
\text{G'TAATCATTGC} \\
\text{AAAGACAGCATATGGATTAAAAAA(A)$_n$} \\
(b.)
\]

(a) Tridecamer primer annealed to nonadecamer template. 
(b) After elongation.
As seen in Table 1, this amount was actually incorporated. A section of the tridecamer primer is seen to be self-complementary for six base pairs (Fig. 3) and thus capable of a repair synthesis after initial removal of the 3'-cytidylic acid by the 3'-exonuclease function of E. coli polymerase (10). However, no evidence was found for polymerization without template (Table 1).

The kinetics of a DNA polymerase repair reaction with a nonadecamer as template and a tridecamer as primer are illustrated in Fig. 4. The double stranded duplex was more than 90% completed within 20 minutes as shown by the incorporation of four labeled thymidylic acid residues. The continued incubation of the reaction caused a slow completion of the strand without any noticeable hydrolysis of the product.

Fig. 3. Possible self-annealing of tridecamer primer.
TABLE 1. Incorporation of TTP with a nonadecamer as template and tridecamer as primer

<table>
<thead>
<tr>
<th>Template</th>
<th>Primer</th>
<th>pmoles incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>3.5</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>4.5</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>4.6</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>81.4</td>
</tr>
</tbody>
</table>

The reaction mixture and procedure are given in Materials and Methods. At end of incubation the reaction was spotted on DEAE-cellulose paper and developed as described.

![Figure 4](image)

Fig. 4. Kinetics of complementary strand synthesis. Composition of the reaction mixture and procedure is given in Materials and Methods. Aliquots of 10 µl were separated on DEAE-paper as described.

Incubation temperatures of 10° and 15° were equally effective, (Fig. 4). When the temperature was raised to 20° or 25°, the kinetics of TTP incorporation were similar to the lower tem-
peratures, (not shown) but it was found by "nearest neighbor"
analysis that a true copy had not been obtained.

It would be advantageous to use as short a primer as possible
to minimize the chemical synthesis needed for the multiplication
scheme. A primer of nine nucleotides complementary to the 3'-end
of the template strand was found to provide sufficient binding
for the DNA polymerase to finish the copy of nearly 100% of the
template strands (Table 2). It was necessary to increase the
primer concentration four fold when using the nonamer as primer.
Use of the hexamer as primer was unsuccessful: little or no syn-
thesis was found even at increased primer concentrations or lower
temperature. These results on the required primer length are
similar to those of Wu et al. (11) and Kleppe et al. (9).

TABLE 2. Minimum number of nucleotides required for primer

<table>
<thead>
<tr>
<th>Primer Length</th>
<th>% of Template Strands Copied</th>
<th>Temp.</th>
<th>Primer Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>97</td>
<td>15°</td>
<td>4 x Template</td>
</tr>
<tr>
<td>9</td>
<td>90</td>
<td>10°</td>
<td>16 x Template</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>10°</td>
<td>16 x Template</td>
</tr>
</tbody>
</table>

Procedure and reaction mixture for copying nonadecamer template
are given in Materials and Methods. The primer length and con-
centration were varied. The incorporation was assayed on DEAE-
paper.

**Fidelity of DNA copy.** A detailed analysis was carried out
to ascertain that the copy made was a true complement of the
template used. First, a partial copy was made by restricting
the triphosphates to only [α^{32}P]TTP. Only one thymidylic acid
residue should be added to the tridecamer primer. After hydroly-
sis with micrococcal nuclease and spleen phosphodiesterase, the
[\textsuperscript{32}P] label should have been transferred to deoxycytidylic acid. As shown in Table 3A, this was the result found. However, if both [\alpha\textsuperscript{32}P]TTP and dGTP were present, then two [\textsuperscript{32}P] labeled thymidylic acid residues should be added to the primer. These [\textsuperscript{32}P] labels should be transferred to deoxycytidylic and deoxyguanylylidylic acid; this was the result observed.

When forming a complete complement of the template, all four deoxyribonucleoside triphosphates were included in the reaction mixture (even though only three were required). The nucleotide ratios found after a nearest neighbor analysis were close to those expected (Table 3B). The ratio of the dTp/dGp indicates the completion of the copy. The [\textsuperscript{32}P] label from the [\alpha\textsuperscript{32}P]TTP is transferred to thymidylic acid only if the final nucleotide has been added. These results indicate that synthesis went to completion and that a true complement of the template was formed.

<table>
<thead>
<tr>
<th>TABLE 3. Nearest neighbor analysis of template copy</th>
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<tbody>
<tr>
<td><strong>Part A. Partial copy</strong></td>
</tr>
<tr>
<td>Nucleotides Present</td>
</tr>
<tr>
<td>dTTP</td>
</tr>
<tr>
<td>dTTP, dGTP</td>
</tr>
<tr>
<td><strong>Part B. Complete copy</strong></td>
</tr>
<tr>
<td>Nucleotide Ratio</td>
</tr>
<tr>
<td>dCp/dGp</td>
</tr>
<tr>
<td>dCp/dTp</td>
</tr>
<tr>
<td>dTp/dGp</td>
</tr>
</tbody>
</table>

The reaction mixture and procedure for preparing the copy of nonadecamer template is given in Materials and Methods. The tridecamer was used as primer. Part A contained only those deoxyribonucleoside triphosphates indicated, while Part B contained all four triphosphates. [\alpha\textsuperscript{32}P]TTP was present as label in all reactions. After incubation, the mixture was separated on DEAE-cellulose paper. The peak at the origin was eluted with 2M-triethylammonium bicarbonate and a nearest neighbor analysis carried out as described earlier (8).
Elongation and blocking of the 3'-terminus of the template.
The formation of a template copy yields a DNA duplex which cannot easily be separated for reuse of the template strand. In order to recycle the template, its size was increased, thus making separation feasible following the copy reaction. This procedure was carried out by extending the nonadecamer template from the 3'-terminus with $[^{14}\text{C}]$-labeled deoxyadenylic acid residues, using terminal deoxynucleotidyl transferase from calf thymus (Fig. 1b). Separation of the elongated template from excess $[^{14}\text{C}]$dATP by gel chromatography is described under Materials and Methods. The first peak, which was found at the void volume, was pooled and a sample used to determine its length by $5'-[^{32}\text{P}]$ incorporation with polynucleotide kinase. An average of 70 deoxyadenylic acid residues were found for template strand as determined by the ratio of $[^{32}\text{P}]$ molecules added by polynucleotide kinase to $[^{14}\text{C}]$ labeled adenylate residues.

The 3'-exonuclease function of DNA polymerase I is known to hydrolyze single stranded DNA chains starting from the 3'-terminus (10). For this reason, the elongated template was blocked on the 3'-terminus with 2',3'-dideoxyadenylate which was shown by Atkinson et al. (12) to inhibit this 3'-nuclease activity.

Before blocking the elongated template, a model compound $d(pT)^{7-d(pA)}$ was prepared, as described in Methods. Some of these model DNA chains were terminated with 2,3-dideoxyadenylate using terminal deoxynucleotidyl transferase. Figure 5 illustrates that, under our conditions of DNA polymerase synthesis, little or no digestion of the strand blocked with 2',3'-dideoxyadenylate was seen.

Copy and separation of the elongated template. The nonadecamer template, after elongation with $[^{14}\text{C}]$ labeled deoxyadenylic acid residues, was blocked at the 3'-terminus with 2',3'-dideoxyadenylate. This elongated and 3'-blocked template was used for a multiplication reaction with DNA polymerase I and the four
deoxynucleoside triphosphates. TTP was labeled with \( {\text{[d}}^{32}\text{P]} \). The reaction mixture was separated by gel chromatography after completion of the copying process. As seen in Fig. 6, the template labeled with \( {\text{[}}^{14}\text{C]} \) was well separated from the product labeled with \( {\text{[}}^{32}\text{P]} \).

Fig. 5. Hydrolysis of \( {\text{[}}^{14}\text{C]} \) labeled \( \text{d(pT)}_3\text{-d(pA)}_7 \). The reaction mixture was the same as used for the preparation of complementary strand, except primer and template were omitted. Instead, 15 pmoles of chains of (a) \( \text{d(pT)}_3\text{-d(pA)}_7 \) or (b) \( \text{d(pT)}_3\text{-d(pA)}_7 \) blocked at the 3'-terminus with 2',3'-dideoxyadenylic acid were used. The reaction mixture was separated on DEAE-cellulose paper at indicated times.
Fig. 6. Separation of elongated template from its complement. After preparation of the complementary strand as described in Materials and Methods, the reaction was added to a jacketed column of 0.9 x 99 cm Sephadex G-75, superfine. The column was developed with 0.1 M triethylammonium bicarbonate (pH 8.0) at 75° (7).

Characterization of the product and reuse of template. The product (complement of 19-mer template) was taken from the second peak in Fig. 6 and analyzed by nearest neighbor techniques as described in Table 3. As expected, the results (not shown) were similar to those of Table 3, part B, thus, confirming that the strand was completed. As further proof of the integrity of the completed product strand, a sample was separated by high pressure chromatography (Fig. 7). The markers included were the tridecamer primer and the nonadecamer template minus its 5'-phosphate. When the 5'-phosphate was replaced on the nonadecamer by polynucleotide kinase with a [32P] label and again chromatographed on the same column, the nonadecamer eluted at the same position as the completed product.
Fig. 7. Characterization of completed copy by ion exchange chromatography on Pellionex AL-WAX. The 0.12 x 50 cm column was eluted at 50°C with a linear gradient of ammonium sulfate, 0.001 to 1 M, 40 ml each, buffered to pH 4.4 with 0.001 M ammonium acetate. A flow rate of 1.1 ml min⁻¹ at 500 psi was used (1).

A 5'-endgroup analysis of the product is shown in Table 4. Only thymidylic acid was found. These results show that no hydrolysis had taken place at either the 3' or 5'-terminus of the product during the polymerase reaction. Table 4 also contains the 5'-end analysis of the elongated template. Again the correct nucleotide (deoxyadenylic acid) was found. As a final verification of the method, the elongated template was reused and the new product analyzed as before. Similar results were obtained.
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TABLE 4. 5'-Endgroup analysis of elongated template and the complement of the nonadecamer template after one reaction.

<table>
<thead>
<tr>
<th></th>
<th>% distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpA</td>
</tr>
<tr>
<td>Elongated template</td>
<td>100</td>
</tr>
<tr>
<td>Nonadecamer product</td>
<td>0</td>
</tr>
</tbody>
</table>

The 5'-phosphate of the DNA strand was removed with phosphatase and replaced with \(^{32}P\) using polynucleotide kinase as described in Materials and Methods. Approximately 10,000 CPM of the labeled oligomer were digested in a total volume of 0.2 ml containing 0.2 A\(_{260}\) of calf thymus DNA, 50 mM triethylammonium bicarbonate buffer (pH 8.6), 5 mM MgCl\(_2\), 3 μmol potassium phosphate buffer (pH 6.9), 0.5 μg pancreatic DNase, 5 μg venom phosphodiesterase (both enzymes from Worthington Biochemical). The reaction was incubated 1 hour at 37°, boiled 3 minutes to stop and spotted on Whatman 3 MM paper. The 5'-nucleotides were separated by electrophoresis at pH 3.9 and examined for radioactivity.

DISCUSSION

The chemical synthesis of deoxyribonucleotides of defined sequence, and their joining in vitro into larger arrays, has become possible in recent years, largely because of the work of Khorana (13). Nevertheless, such synthetic efforts are still far from trivial, and considerable expense in manpower and material is required. It would be desirable, therefore, to have such material permanently available, and to generate copies whenever needed for biochemical experimentation.

The use of DNA polymerase in generating complementary copies of preexisting templates would permit realization of this aim, provided that the template could be recovered intact from the enzymatic copy reaction. In order to attain this goal, a scheme was devised wherein a template is rendered separable from newly synthesized product by elongation and protected against enzymatic degradation by blockade. The scheme was tested and there does not
appear to be any reason why it would not be applicable to more complex fragments, or workable on a larger scale.

A deoxyribooligonucleotide of known sequence, nineteen monomers in length, was elongated by means of terminal transferase until it contained some seventy deoxyadenylate residues. It was then annealed to a primer complementary to the 3'-terminal region of the original oligomer, so that a structure was formed in which a long single-stranded 3'-terminal tail protruded beyond the short double-stranded region produced by primer and template. This type of structure would be expected to be degraded by the 3'-exonuclease activity of the polymerase; repair of such degraded regions would be impossible due to the absence of a required guiding complementary strand. Our experiments show that this hydrolysis without repair does indeed take place. The lack of such structures in nature may be explained by the ease with which the protruding sequence can be lost.

The 3'-termination of the DNA chain with 2',3'-dideoxyadenylate effectively blocked the 3'-exonuclease action of DNA polymerase I, as had been described by Atkinson et al. (12). Other methods might be used for accomplishing the same end. Thus, termination with arabinosyl nucleotides (12) or even termination with a 3'-phosphate (14) could be expected to inhibit or prevent the 3'-exonuclease action.

The elongated, blocked primer-template complex was then subjected to limited and exhaustive repair synthesis with the polymerase. Analysis of the incorporated nucleotides showed that a true copy of the template had been made. Furthermore, the results illustrated that the last nucleotide was added in over 90% of the template strands. The procedure used here was similar to that of Kleppe et al. (9) and confirmed their results. In our experiments, it was found further that the primer concentration and primer length were related. By increasing the concentration of primer, it was possible to decrease its length
and still copy substantially all template strands. This was true until a minimum length was reached which would not prime at any concentration.

Finally, separation of the newly synthesized oligomer from the much larger template proceeded without difficulty. The latter was again annealed with primer and repair synthesis undertaken with the same results as before. The repeated use of such templates thus facilitates the availability of DNA fragments of defined sequence.

ACKNOWLEDGMENTS

We wish to thank Dr. C. McGregor for isoelectric focusing of calf thymus terminal deoxynucleotidyl transferase. Also we are grateful to Dr. A.L. Nussbaum and Dr. C.C. Richardson for many helpful suggestions.

ADDENDUM

Very recently, an alternate method for accomplishing the objective of this paper has been described (15). These authors attached the template covalently to a cellulose support and separated the product after denaturation.

REFERENCES

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