VII. REPLICATION OF HOMOPOLYMERS

The initiator oligonucleotide is incorporated into product chains. When a multiplicity of initiator chains are present (more than 1 per template) then several chains grow simultaneously on each template. The structure and molecular weight distribution of the products may be predicted from the initiator to template ratio.

Calf thymus DNA polymerase catalyzes a template-directed synthesis on suitably initiated single-stranded polydeoxynucleotides. Since no extraneous degradative enzyme activities are present in this enzyme preparation (3, 4) as contaminants or associated with polymerase itself, certain mechanistic details of the polymerization are easily demonstrated. The work to be described concerns the details of the initiation process on simple homopolymer templates and shows how the initiation mechanics are reflected in the structure of the product.

Two basic structures result from the replication of simple polydeoxynucleotides. double-stranded products covalently connected to template, and double-stranded products not covalently connected to template. The first kind of product is formed by "covalent initiation" in the presence of terminal deoxynucleotidyltransferase and the second is formed by oligodeoxynucleotide initiation. In the second case the product chain length is determined by the relative number of initiator and template molecules present in the reaction, that is, the multiplicity of initiation or the molecular ratio of template to initiator. For the present discussion we define the multiplicity of initiator to template as zero when the initiator sequence is attached to the template, as in terminal transferase initiated reactions.

Most of the concepts presented in this paper have been known since 1964 and have been described in several publications and abstracts from this laboratory (1, 5-8). The availability of a DNA polymerase preparation that is free from terminal transferase contamination now provides a clearer demonstration of the initiator effect, and has been used in most of the present work. The findings presented demonstrate the mechanism of action of calf thymus polymerase. The products synthesized and template systems described are also of considerable practical use.

MATERIALS AND METHODS

Deoxynucleoside triphosphates were synthesized by the morpholodate procedure (9) and purified as previously described (10). Radioactive dNTPs were purchased from Schwarz-Mann. Oligodeoxynucleotides (11) and polydeoxynucleotides (12) are the standard preparations of this laboratory. Terminal deoxynucleotidyltransferase and replicative deoxynucleotidyltransferase (6 to 8 S DNA polymerase) from calf thymus gland are prepared as described (13) or by slight modifications of published procedures (4).

Radioactive polydeoxynucleotides formed in polymerization reactions, or isolated from CsCl or sucrose gradients, were worked up for analysis as acid-insoluble precipitates on filter paper or glass fiber (Whatman GF/C) discs essentially as previously described (14).

Neutral sucrose gradients were prepared as described by Britten and Roberts (15). The gradients were made up to contain 5 to 20% sucrose (w/w) in 0.1 M NaCl, 10 mM Tris-Cl pH 8.0, and 10 mM EDTA. Alkaline sucrose gradients were also used to contain 0.1 M NaOH and 10 mM EDTA. All sucrose gradients were centrifuged at 48,000 rpm for 20 hours at 4°C in the SW-56 rotor of the Spinco centrifuge. Neutral CsCl gradients were made up to \( \rho = 1.68 \) with solid paraffin.
CsCl in 83 mM Tris-Cl at pH 8.0. Alkaline CsCl gradients were made up at $\bar{p} = 1.71$ and contained 50 mM glycine-NaOH at pH 11.55. All CsCl gradient centrifugations were carried out for 72 hours at 40,000 rpm at 25° in the SW-50 rotor of the Spinco centrifuge. The gradients were fractionated from the bottom of the tubes by pumping out at constant rate. Sucrose gradients were fractionated into 35 equal volume fractions and 45 equal volume fractions were taken from CsCl gradients. In order to obtain quantitative precipitation of poly(dT) on glass fiber discs, all alkaline gradient fractions were collected into about one-fifth of the fraction volume of 0.4 mM poly(da) (nucleotide concentration) in 1 M potassium phosphate at pH 6.0.

Radioactivity data were obtained as punched tape from the Packard scintillation counter and processed on a Wang 700 computer. Programs for conversion of count data to nanomoles for single isotope and double isotope experiments were written in this department.4

RESULTS

**Requirement for Initiator Molecules**—Single chain homopolymers are inactive templates for calf thymus DNA polymerase. The only exception is found in cases where double chain structures are formed, as in poly(dC) at neutral pH (16), and in this case some self-initiation can occur (see Fig. 2 below). The activity of complementary oligodeoxynucleotides as initiators of enzymatic polydeoxynucleotide chain growth is demonstrated in Fig. 1. This experiment shows that oligodeoxythymidylates with chain length 6 or greater are effective in initiating poly(da) replication and oligodeoxadenylates of 5 or more nucleotides initiate poly(dT) replication. Chain lengths of 9 to 10 nucleotides are within 20% of maximal initiation, measured in this experiment as the initial rate of polymerization.

The results presented should be considered with regard to two other studies related to this initiation process. The first comparison is with physical studies on oligopolymer interaction in the deoxyadenylate-deoxythymidylate system (17). Physical interaction between hexamers and polymers is extremely weak at the temperature (35°) used for the enzyme reactions. The melting temperature for a dA₆-dT₆ complex is 33.7° in 40 mM potassium phosphate, 8 mM MgCl₂, while a dT₆-dA₆ complex has $T_m = 35.0°$ (only 2:1 complexes are found). The slopes of these transitions are about 0.1-0.2 degree⁻¹. We find it difficult to mount an argument for simple physical complexes as sufficient for the initiation event, particularly in the enzymatic replication of poly(da). We prefer to think that the enzyme plays some role in stabilizing the complex even before chain growth commences. There is, however, an effect of temperature on the minimum chain length required for initiation. This data is presented in Table I, and shows that a 10° drop in temperature lowers the chain length requirement for initiation by 1 to 2 nucleotides. At 34°-35°, the usual reaction temperature, the concentration of stable physical complex for poly(da) and oligothymidylates with chain length less than 10 is negligible, although oligothymidylates with chain length longer than 6 can initiate poly(da) replication quite efficiently at all temperatures tested.

The argument against a simple physical complex as the initiation complex is supported by a second kind of result, and this concerns a similar study conducted with a different DNA polymerase (2). The low molecular weight DNA polymerase from

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4 We are indebted to Dr. G. W. Schwert for programming assistance and use of the computer.

**TABLE I**

*Relative rate of poly(da) replication as a function of temperature and chain length of oligothymidylate*

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Initiator chain length</th>
<th>Reaction rate at 70°C</th>
<th>80°C</th>
<th>90°C</th>
<th>100°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>3</td>
<td>0.04</td>
<td>0.44</td>
<td>1.14</td>
<td>1.04</td>
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<tr>
<td>31</td>
<td>2</td>
<td>0.02</td>
<td>0.22</td>
<td>0.74</td>
<td>0.91</td>
</tr>
<tr>
<td>34</td>
<td>1</td>
<td>0.00</td>
<td>0.00</td>
<td>0.44</td>
<td>0.84</td>
</tr>
<tr>
<td>38</td>
<td>0</td>
<td>0.01</td>
<td>0.03</td>
<td>0.22</td>
<td>0.50</td>
</tr>
</tbody>
</table>

$T_m(°C)$

<table>
<thead>
<tr>
<th>Initiator chain length</th>
<th>Reaction rate at 70°C</th>
<th>80°C</th>
<th>90°C</th>
<th>100°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;5</td>
<td>14.5</td>
<td>21.0</td>
<td>29.2</td>
<td></td>
</tr>
<tr>
<td>&gt;5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
rabbit bone marrow requires oligothymidylates with chain length greater than 8 nucleotides for initiation. It may be beneficial to restate the fact that neither of these two enzymes has any exonuclease activities (2-4), therefore, the requirement for different chain lengths cannot be attributed to degradation or modification of the oligonucleotides by the enzyme preparation used. Since different enzymes require different chain lengths for initiation under similar solution conditions, we believe that the initiation complex is a ternary complex, consisting of oligomer, polymer, and enzyme, and that the enzyme has a specific effect in determining the minimum length of oligomer required for initiation.

**Enzymatic Initiation**—The presence of terminal deoxynucleotidyltransferase (10) in DNA polymerase reactions will also bring about initiation of homopolymer templates if the complementary dNTP is present. The resultant product is then covalently bonded to the template, and will not separate from template in alkaline CsCl gradients. This type of product is described later in this report and is used as evidence for the absence of endonuclease activity in the DNA polymerase preparations used. Typical time courses for poly(dA), poly(dT), and poly(dC) replication are shown in Fig. 2. They illustrate the points described above under oligonucleotide initiation, enzymatic initiation, and in the poly(dC) case, self-initiation. The extent of synthesis reached in all cases is one complete complement of template.

Initiation in heteropolymeric systems (denatured DNA) may also be facilitated by 3',5'-exonuclease activity as has been demonstrated by Englund (18). This kind of result is not possible in homopolymer systems.

**Preferences for Divalent Ions**—Replication of homopolymers by calf thymus DNA polymerase proceeds optimally in Mn++, although DNA templates work somewhat better in Mg++. The metal ion participation in systems of this kind is very complex; involving dNTPs, polynucleotides (and interacting oligonucleotides), PPi, produced, buffers used, and the enzyme itself. When template concentration is 0.1 to 0.4 μM (that is 100 to 200 μM in total nucleotide in a polymer 500 to 1000 nucleotides in length), initiator oligonucleotide at about 0.1 to 4 μM (oligonucleotide concentration), dNTPs at 100 to 200 μM, and phosphate buffer at 20 mM we find that 200 to 500 μM MnCl₂ works well for poly(dT) and poly(dA) replication. MgCl₂ at 3 to 8 mM works 60% as well as Mn++ for poly(dA) replication. MgCl₂ at 0.5 mM (optimum) works only 10% as well as Mn++ for poly(dT) replication.

**pH Optimum**—The optimum pH for replication of a particular homopolymer system is a function of the initiator-template system itself in addition to the enzyme used. In the presence of 20 mM potassium phosphate the optimum for replication of poly(dA) is at pH = 6.6; while for poly(dT) and activated DNA it is at pH = 7.2 for the 6 to 8 species of calf thymus DNA polymerase. The 3.4 S DNA polymerase (2) has all pH optima displaced by about 1 pH unit toward the alkaline range. The pH optima are also affected by ionic strength.

**Structure of Products-Poly(dA) Replication**—When poly(dA) replication is initiated by any of a variety of oligonucleotides at any of a variety of initiator-template ratios, the product is always poly(dA)-poly(dT). In the complex all of the constituent chains sediment together in neutral sucrose gradients (Fig. 3 A, B, and C), and band at the same density in neutral CsCl (Fig. 4 A, B, and C). These figures, representative of many similar experiments with different initiator chain lengths and 1:T ratios, also demonstrate the nucleotide equivalence between template and product. The slight reduction in the amount of radioactive product at higher 1:T ratios is due to the contribution of non-radioactive nucleotides from initiator chains.

If products of equivalent sedimentation rate, prepared identically except for initiator to template ratio, are sedimented in alkaline sucrose the results of Fig. 3 D, E, and F, are produced. These figures show that identically sedimenting template chains (poly(dA)) have product chains (poly(dT)) in the complex with variable sedimentation rates. The rate of sedimentation of the product poly(dT) is inversely related to the initiator to template ratio, and indicates that high ratios bring about multiple initiation. The multiplicity of product chains is also seen in alkaline CsCl gradients (Fig. 4 C), where a high multiplicity of initiation causes an increased band width at the density of the poly(dT) chain. In all cases deoxyribonucleotide initiation results in product chains that separate from the template, that is, they are not covalently linked to the template.

**Structure of Product-Poly(dT) Replication**—The general structure of products formed in reaction mixtures containing a poly(dT) template and an oligo(dA) initiator is completely analogous to the poly(dA) results described above (see Fig. 4 F). The principal demonstration of products in this system therefore concerns only the result of covalent initiation by terminal deoxynucleotidyltransferase (13). Fig. 5 shows the result obtained upon alkaline CsCl equilibrium centrifugation of a poly(dT) replication mixture initiated by addition of terminal transferase. In this experiment the template is about 50% replicated, and the gradient shows unused poly(dT) as well as poly(dA) and poly(dT) banding at identical positions at an intermediate density in the gradient. No free poly(dA) is present in the gradient. The only reasonable interpretation of this finding is that the product poly(dA) is covalently connected to the template poly(dT). Covalent products of this kind were demonstrated some years ago with DNA templates (3) and can...
Fig. 3. Sucrose gradient analysis of the products of poly(dA) replication. Reactions (0.5 ml) were carried out as described in Fig. 1 except [3H]poly(dA) was used as template, [methyl-3H]dTTP as monomer, and d(pT), as initiator. After incubation for 14 hours at 35°C the reactions were terminated by addition of 50 μl of 0.2 M EDTA. Part of each reaction (0.15 ml) was analyzed directly on a neutral sucrose gradient, and a second part (0.15 ml) was adjusted to 0.2 N NaOH and 20 mM EDTA and analyzed on an alkaline sucrose gradient. The procedures for analysis of the products of poly(dA) replication on alkaline and neutral gradients are described under “Materials and Methods.” The direction of sedimentation is from right to left with T representing the top of the gradient and B representing the bottom of the gradient. O—O, [3H]poly(dA) template; ▲—▲, [3H]poly(dT) product.

Fig. 4. CsCl gradient analysis of the products of homopolymer replication. Reactions were carried out under conditions as described in Fig. 1 with the variations noted. Incubation was for 10 hours at 35°C. The analysis of the reaction products in the gradients is described under “Materials and Methods.” T represents only be isolated from enzyme reactions that are free from all endonuclease contaminants.

Initiator Incorporation—The analysis of the products of poly(dA) replication described above demonstrate that the multiplicity of oligodeoxyxynucleotide initiator per template molecule has an inverse relation to the sedimentation rate of the product chain. The implication is that initiator oligonucleotide molecules are the points of chain growth and most probably are incorporated into the product chain. In the homopolymer systems we have studied, this supposition is readily demonstrated. Table II shows the incorporation of oligodeoxyadenylate chains into products separated from reactants on Sephadex G-50 columns. The results show that oligonucleotides with 6 to 14 nucleotides are incorporated. At the lower chain lengths the incorporation is not very efficient and is not improved by increasing the multiplicity of initiation. At higher chain lengths the efficiency is high, approaching 100% at I:T of 1 to 4, but seems to saturate at an I:T of about 4. This limit indicates
that a growing point consists of a template chain, which is still in excess, an initiator chain, the number of which can be increased up to template saturation, and an enzyme molecule, which is probably limiting the number of initiation points to 4 per template molecule. Since the enzyme used in this study is not pure we cannot provide proof of this idea at the present. An initiation point cannot grow without an enzyme molecule and while it is probably true that enzyme molecules can dissociate from an initiation point, earlier studies (7) on kinetics of chain growth indicated that chains grow to completion before the enzyme dissociates. The number of growth points in the current study is probably fixed shortly after polymerization commences and initiator chains that do not begin growth at the onset of polymerization are probably displaced from the template by growing chains.

This demonstration of initiator chain incorporation provides the starting point for an analysis of the number distribution of product chains.

**Analysis of Product Chain Distribution**—Using a poly(dT) template chain with \( n = 500 \), varying multiplicity of \([^{14}C]d(pA)_{12} \) initiator, and \([^{3}H]dATP \) results in poly(dA)-poly(dT) products that sediment to identical positions on neutral sucrose gradients. Sedimentation of such products on alkaline sucrose gradients (Fig. 6) allows an analysis of the number average molecular weight of the product chain. That is, from the amount of \([^{3}H] \) and \([^{14}C] \) in each gradient fraction the number average chain length in that fraction is calculated. These results show a distribution of chain lengths consistent with the input multiplicity at all multiplicities of initiation and a normal distribution of chain lengths in the product.

This experiment demonstrates the stoichiometric behavior of the systems described and the kind of analysis that can be made in this model system for initiation polymerization on templates.

**TABLE II**

<table>
<thead>
<tr>
<th>Input I-T</th>
<th>Radioactivity in polymer</th>
<th>Nanomoles of initiator incorporated/nanomole of template</th>
</tr>
</thead>
<tbody>
<tr>
<td>([^{3}H]dATP)</td>
<td>0.0</td>
<td>1.00</td>
</tr>
<tr>
<td>([^{3}H]dATP)</td>
<td>0.0</td>
<td>1.00</td>
</tr>
<tr>
<td>([^{3}H]dATP)</td>
<td>0.0</td>
<td>1.00</td>
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<tr>
<td>([^{3}H]dATP)</td>
<td>0.0</td>
<td>1.00</td>
</tr>
<tr>
<td>([^{3}H]dATP)</td>
<td>0.0</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The principal virtue of studying initiation mechanisms for replication of polydeoxynucleotides is that they provide simple physical models for analysis and suggest possibilities for understanding the more complex biological initiation process. We have been concerned with these systems for some time. Our initial studies were carried out as soon as pure polydeoxynucleotides became available (1, 5), and demonstrated the need for a complementary initiator molecule in homopolymer replication. The role of the initiator in providing a free 3'-OH was then examined using initiator oligonucleotides with an acetylated 3'-OH (6, 7). Finally a study of the kinetics of polymerization (7) demonstrated that the homopolymer replication is continuous on a replicating chain, rather than distributive. That is, once a molecule begins to replicate enzymatically the molecule is fully replicated before the enzyme dissociates and starts a second replication. A major experimental finding in the present study is that the 3'-OH group of the initiator oligonucleotide is used for growth of chains on templates, ending up as a covalent part of the product chain. The means whereby this initiation point is provided will be reflected in the detailed structure of the product,
containing 10 mM EDTA, and analyzed by alkaline sucrose gradient centrifugation as described under "Materials and Methods." The polymer products isolated from the colicin were precipitated in 70% ethanol, redissolved in 0.2 N NaOH containing 10 mM EDTA, and analyzed by alkaline sucrose gradient centrifugation as described under "Materials and Methods." The polymer products isolated from the colicin were precipitated in 70% ethanol, redissolved in 0.2 N NaOH containing 10 mM EDTA, and analyzed by alkaline sucrose gradient centrifugation as described under "Materials and Methods.”

The experiments described have not been presented as a detailed physical analysis from the standpoint of the statistics of initiation in poly(dA) versus poly(dT) templates, which should be reflected in the molecular weight distribution in the product chain. For example, we note that in the alkaline sucrose gradients of poly(dA) replication, the poly(dT) product formed at 1:T ratios of about one sediment at about the same rate as the template. Reflecting on this observation, one wonders why this is so, since it would seem more reasonable that a statistical distribution of initiator oligonucleotides over the template should result in a product with a mean chain length about half that of the template. Since the product distribution does not reflect this simple picture we assume that in this case some migration of initiator occurs during synthesis, with the net result that all product chains appear to be of length equivalent to the measurement of chain length at 1:T = 1 in this case (Fig. 6) leads to an average product chain length of about half the template length, more in line with statistical expectations.

Several kinds of initiation model have been suggested during the course of this work. For example, the limited addition of complementary nucleotides to a homopolymer template through the action of terminal transferase forms a covalent initiation site on the 3' terminus of the polymer. The structure of the DNA polymerase products of this template system is demonstrated in Fig. 5 of this report. Alternatively, if the terminal transferase is used to add noncomplementary nucleotides to a homo- or heteropolymer chain then the region of repeating nu-

...and even in our system prepared from defined chemical structures, product structre is the essential analysis. If the polymerization enzymes were capable of initiating new chains or if degradation reactions were occurring, the structure and stoichiometry in the product would be quite different.

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Lucy M. S. Chang, G. R. Cassani and F. J. Bollum


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