Studies of the Ribonucleic Acid Polymerase from Escherichia coli

IV. EFFECT OF OLIGONUCLEOTIDES ON THE RIBONUCLEIC ACID POLYMERASE REACTION WITH SYNTHETIC POLYRIBONUCLEOTIDES AS TEMPLATES*

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Several laboratories have reported on the properties and characteristics of RNA polymerase reactions with synthetic polyribonucleotides as templates (1–3), with general agreement that the synthetic polyribonucleotides are efficient templates for the synthesis of complementary polyribonucleotides. The use of synthetic polyribonucleotides of different base composition and size is of particular value for certain studies of the mechanism of action of RNA polymerase. The present communication reports results with synthetic polyribonucleotides of known composition and size. Characteristics of the stimulation of reactions involving polyadenylic acid and polyuridylic acid by complementary oligonucleotides suggest that oligonucleotides may act as chain initiators for the RNA polymerase. A preliminary communication on the effect of complementary oligonucleotides on the RNA polymerase reaction has already been reported by us (4).

EXPERIMENTAL PROCEDURE

Materials and Methods—ATP-8-14C, GTP-8-14C, and UTP-2-14C were obtained from Schwarz BioResearch, Inc., and were purified by paper chromatography in several solvents before use. ATP, UTP, CTP, and GTP were obtained from Pabst Laboratories and, if necessary, purified before use by paper chromatography in several solvents. The synthetic polyribonucleotides used have been described in the accompanying paper (3). The methods of preparing phage T2 DNA and heated phage T2 DNA have also been mentioned in the accompanying paper (3).

The purification of RNA polymerase for Escherichia coli B has been described previously (5). All the studies reported here were performed with the final density gradient fraction (Table I of Stevens and Henry (5)). Assay of enzyme activity was performed by the Millipore filtration technique described previously (5).

Adenine oligonucleotides of type pApA were made from poly A by the action of a nuclease from Azotobacter agilis (6); those of type ApA were obtained by the action of E. coli alkaline phosphatase (Worthington Biochemical Corporation) on type pApA; and type ApAp by controlled alkaline hydrolysis of poly A (7).

Uracil oligonucleotides of type UpUp were prepared by the controlled action of pancreatic ribonuclease on poly U (8), and those of type UpU by the action of E. coli alkaline phosphatase on type UpU. The oligonucleotides of different chain length were separated and purified by paper chromatographic procedures (8–10). The solvent that gave best results is l-propanol-concentrated NH₄OH-H₂O (55:10:35, v/v/v). Whatman No. 3MM paper was used. Guanine oligonucleotides of type GpGp were kindly furnished by Dr. Marie Lipsett. Oligonucleotides of type GpG were obtained by the action of E. coli alkaline phosphatase on type GpG.

2-14C-Labeled adenine oligonucleotides were prepared from 14C-poly A. The 14C-poly A was prepared from 14C-labeled ADP by the action of polynucleotide phosphorylase. ADP-14C was purchased from Schwarz BioResearch, Inc. Polynucleotide phosphorylase was prepared from Micrococcus lysodeikticus according to Singer and O'Brien (11).

RESULTS

Stimulation of Polyribonucleotide-directed Reactions with Complementary Oligonucleotides—Adenine oligonucleotides (dinucleotide to hexanucleotide) of type pApA and ApA (with a free 3'-hydroxyl end) greatly stimulate the formation of poly A when poly U is used as a template for RNA polymerase. (5'-AMP, 3'-AMP, and ADP do not stimulate the reaction.) The stimulation occurs at a very low concentration of oligonucleotide, even at 1 μM. The effect of oligonucleotide concentration will be presented in a later section. The stimulation at oligonucleotide concentration of 5 μM is shown in Table I, Reaction A. The amount of stimulation increases with the chain length of the oligonucleotide. Oligonucleotides of type ApAp (with a 3'-phosphate end) do not stimulate (Table I), indicating the necessity of a free 3'-hydroxyl end for stimulatory activity. Some inhibition is usually observed at high concentration of the latter oligonucleotide. Under the conditions of incubation, the adenine oligonucleotides alone do not serve as templates for poly A formation; this has been tested both by acid insolubility and by the paper chromatographic assay procedure of Falaschi, Adler, and Khorana (12). Little or no effect of the adenine oligonucleotides was observed on reactions with poly A or poly C as a template. Similarly, no stimulatory effect was observed on the formation of heteropolymer (5) or homopolymer (13) with native phage T2 DNA and heated phage T2 DNA, respectively (Table I). Actually, heteropolymer formation is somewhat inhibited by
oligonucleotides. UpUpU, for example, stimulates the formation of poly U only with poly A as a template. No stimulation is observed with oligonucleotides of type UpUp. These results are shown in Table II. It may also be noted (Table II) that UpU can exert its stimulatory effect even in presence of UpUp which does not stimulate.

Studies with guanine oligonucleotides have been somewhat disappointing. GpGpG, for example, does not stimulate poly G formation with poly C template at 37°. At higher temperatures (45°, 50°, and 55°), there is some stimulation. The situation is, however, complicated by the fact that the rate of reaction falls off rapidly after 45°. GpGpG has no effect on the reaction at any temperature. The guanine oligonucleotides have been found to have no effect on reactions with other templates.

The effect of oligonucleotides on heteropolyribonucleotide-directed reactions will be presented in another section.

Incorporation of Oligonucleotides into Chain Ends of Product—
The stimulation of the reactions outlined above by complementary oligonucleotides with a free 3'-hydroxyl end (and the lack of stimulation by those with a 3'-phosphate end) suggested that the oligonucleotides might be acting as primers or chain initiators. Thus, it was of interest to determine whether the oligonucleotides themselves were incorporated into the polyribonucleotide product. Table III shows the results of studies of the incorporation of oligonucleotides. Labeled oligonucleotides (14C-PApApA and 14C-PApApApApA) were added to reaction mixtures similar to those described in Table I, with the use of un-

Table I

<table>
<thead>
<tr>
<th>Oligonucleotide added</th>
<th>14C-AMP incorporated</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Poly A formation with poly U template</td>
</tr>
<tr>
<td>None</td>
<td>0.27</td>
</tr>
<tr>
<td>pApA</td>
<td>0.41</td>
</tr>
<tr>
<td>pApApA</td>
<td>0.26</td>
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<tr>
<td>pApApApA</td>
<td>0.46</td>
</tr>
<tr>
<td>pApApApApA</td>
<td>0.97</td>
</tr>
<tr>
<td>ApApA</td>
<td>0.83</td>
</tr>
<tr>
<td>ApAp</td>
<td>0.28</td>
</tr>
<tr>
<td>ApApAp</td>
<td>0.23</td>
</tr>
</tbody>
</table>

+ The concentrations of the oligonucleotides were calculated by assuming that the molar extinction coefficients of 15,400 for AMP, 9,900 for UMP, and 11,700 for GMP at 260 mμ, pH 7.0.

Table II

Effect of uracil oligonucleotides on formation of poly U with poly A as template

The reaction mixture (0.2 ml) contained: 14C-UTP, 70 mμmoles, 5 X 10^6 cpm per μmole; poly A, 10 μg; Tris buffer, pH 7.8, 4 mμmoles; MnCl₂, 0.5 mμmoles; β-mercaptoethanol, 4 mμmoles; and protein, 15 mμg (specific activity, 300). Quantity of each oligonucleotide added was 1 mμmole except where shown. Isotope incorporated into acid-insoluble material was measured after a 10-min incubation period at 37°.

<table>
<thead>
<tr>
<th>Oligonucleotide added</th>
<th>14C-AMP incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mμmoles</td>
</tr>
<tr>
<td>None</td>
<td>0.20</td>
</tr>
<tr>
<td>UpU</td>
<td>0.38</td>
</tr>
<tr>
<td>UpUpU</td>
<td>1.54</td>
</tr>
<tr>
<td>UpUpU (3 mμmoles)</td>
<td>1.56</td>
</tr>
<tr>
<td>UpU</td>
<td>0.21</td>
</tr>
<tr>
<td>UpU + UpUp</td>
<td>0.39</td>
</tr>
<tr>
<td>UpU (3 mμmoles) + UpU</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Results analogous to those above were obtained with uracil adenine (and also uracil) oligonucleotides, especially at higher concentration.

Distribution of radioactivity in alkaline hydrolysate of product

<table>
<thead>
<tr>
<th>Labeled oligonucleotide</th>
<th>Distribution of radioactivity in alkaline hydrolysate of product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenosine</td>
</tr>
<tr>
<td></td>
<td>mμmoles adenine</td>
</tr>
<tr>
<td>pApApA</td>
<td>0.0</td>
</tr>
</tbody>
</table>
labeled ATP. At the end of the reaction period, the poly A formed was isolated by acid precipitation and degraded to the component nucleotides by alkaline hydrolysis. Adenosine, 3'-AMP, and adenosine 3',5'-diphosphate were isolated from the alkaline hydrolysates by paper chromatography and the label in each was determined. If the 14C-oligonucleotide was acting as a primer for the formation of long chain poly A, one would expect to find label in adenosine 3',5'-diphosphate and 3'-AMP, the ratio of the amounts in each being dependent on the chain length of the oligonucleotide. With both 14C-pApApA and 14C-pApApApApA, label was found in the poly A formed, and in adenosine 3',5'-diphosphate. However, in both cases, particularly with 14C-pApApA, more label was found in 3'-AMP than could be expected from only terminal incorporation of oligonucleotide. The discrepancy could be explained by the presence of a contaminating phosphatase; however, no phosphatase activity could be detected with p-nitrophenyl phosphate as a substrate, according to Caren and Levinthal (14). It is possible that oligonucleotides below a minimum size might be incorporated inside the chain of poly A.

**Effect of Oligonucleotide Concentration**—The amount of stimulation with adenine or uracil oligonucleotides increases with the concentration of the oligonucleotide. Concentration curves for pApApApA in the poly U-directed reaction at 37° are shown in Fig. 1. The \( K_m \) value for pApApApA is calculated to be about \( 1.4 \times 10^{-4} \) M, indicating a high affinity. At 0°, where the adenine oligonucleotides inhibit the formation of poly A with poly U template, the \( K_m \) for pApApApA has been calculated to be about 1.3 \( \mu \)M, indicating a 10-fold higher affinity than that at 37°.

**Figure 1**

A, concentration curve for pApApApA in poly A formation with poly U and ATP. B, reciprocal plot of above. The reaction mixture (0.2 ml) contained: \( \frac{14}{m} \)-ATP, 50 mmoles, \( 4 \times 10^4 \) cpm per \( \mu \)mole; poly U, 10 \( \mu \)g; Tris buffer, pH 7.8, 4 \( \mu \)moles; MnCl\(_2\), 0.5 \( \mu \)mole; mercaptoethanol, 4 \( \mu \)moles; protein, 22 \( \mu \)g (specific activity, 230); and pApApApA in the concentrations shown. Isotope incorporated into acid-insoluble material was measured after a 10-min incubation period at 37°.

**Figure 2**

A, concentration curve for UpUpU in poly U formation with poly A and UTP. B, reciprocal plot of above. The reaction mixture (0.2 ml) contained: \( \frac{14}{m} \)-UTP, 70 mmoles, \( 4 \times 10^4 \) cpm per \( \mu \)mole; poly A, 10 \( \mu \)g; Tris buffer, pH 7.8, 4 \( \mu \)moles; MnCl\(_2\), 0.5 \( \mu \)mole; \( \beta \)-mercaptoethanol, 0.5 \( \mu \)mole; protein, 30 \( \mu \)g (specific activity, 170); and UpUpU in the concentrations shown. Isotope incorporated into acid-insoluble material was measured after a 10-min incubation period at 37°.

**Figure 3**

Reciprocal substrate concentration plot for UpUpU in poly U formation with poly A and UTP. Reaction conditions same as those described in Fig. 2.

Concentration curves for UpUpU in the poly A-directed reaction at 37° are shown in Fig. 2. The curved plot of \( 1/V \) versus \( 1/S \) suggests a complex reaction and makes it impossible to obtain the true \( K_m \) of UpUpU. A straight line plot is obtained when \( 1/V \) is plotted against \( 1/S^2 \) (Fig. 3). The \( K_m \) value for UpUpU is calculated to be about \( 6 \times 10^{-4} \) M, which is slightly higher than that determined for pApApApA at 37°.

**Effect of Oligonucleotides on Substrate (Nucleoside Triphosphate) Concentration Curves**—The results shown above were obtained at suboptimal concentrations of nucleoside triphosphate (low substrate, see accompanying paper (3)). The oligonucleotides have been found to have a profound influence on the nucleoside triphosphate concentration curves. (No effect was found on template affinity.) Such curves for the poly U- and poly A-
ATP, mM

FIG. 4. Effect of oligonucleotides on nucleoside triphosphate concentration curves. A, formation of poly A with poly U template in the absence and presence of pApApApA. Reaction conditions similar to those described in Table I, with different concentrations of $^{14}$C-ATP. Amount of pApApApA where added was 1 mpmole. B, formation of poly U with poly A template in the absence and presence of UpUpU. Reaction conditions similar to those described in Table II, with different concentrations of $^{14}$C-UTP. Amount of UpUpU where added was 1.6 mmoles.

FIG. 5. Effect of oligonucleotides on reciprocal substrate (nucleoside triphosphate) concentration curves. A, formation of poly A with poly U template in the absence and presence of pApApApA. Reaction conditions similar to those described in Table I (Reaction A), with different concentrations of $^{14}$C-ATP. Amount of pApApApA where added was 1 mpmole. B, formation of poly U with poly A template in the absence and presence of UpUpU. Reaction conditions similar to those described in Table II, with different concentrations of $^{14}$C-UTP. Amount of UpUpU where added was 1.6 mmoles.

directed reactions are shown in Figs. 4 and 5. As discussed in the accompanying paper (3), the nucleoside triphosphate concentration curves for these reactions (minus oligonucleotides) are complex and resemble those described for reactions in which the substrate also acts as an activator (15). Other interpretations, like a higher order reaction or allosteric effects, can be proposed. It is impossible to determine the true $K_m$ values of these reactions by the usual extrapolation methods. In the accompanying paper (3), the application of the Hill equation has been discussed. In the presence of stimulatory oligonucleotides, the more usual type of substrate curves is obtained (Figs. 4 and 5). The plots of $1/V$ versus $1/S$ become straighter, and extrapolation is possible to give $K_m$ values that are of the same order of magnitude (5 to $7 \times 10^{-4}$ M) as those obtained (with the help of the Hill equation) for the reactions minus oligonucleotides.

Effect of Temperature—Since the stimulation occurs only with oligonucleotides complementary to the template, some form of interaction between the two might be expected to occur. This interaction, possibly through hydrogen bonding, could be expected to be influenced by temperature. The effect of temperature on the reactions with synthetic polynucleotides (minus oligonucleotides) has been presented in the accompanying paper (3). This effect is dependent both on the nature of the polynucleotide template and the nucleoside triphosphate concentration. The temperature studies here were performed at low nucleoside triphosphate concentration since the effect of oligonucleotides is maximal under such conditions. The influence of pApApApA and UpUpU on the temperature curves of the poly U and poly A reactions, respectively, are shown in Fig. 6.

Poly A formation with poly U template (minus oligonucleotide) proceeds best at 25°, although appreciable reaction takes place at lower temperatures (even at 0°); the reaction proceeds slowly at 37° and 45°. In the presence of pApApApA, the rate is highest at 37° and the stimulation is greatest at 37° and 45°; some stimulation occurs also at 25°, but at 15° and lower, the oligonucleotides inhibit the formation of poly A (Fig. 6A).

The rate of formation of poly U with poly A template (minus oligonucleotide) is highest at 25°, although not much higher than that at 37°. Stimulation by UpUpU is maximal at 37° and 45°, although there is appreciable stimulation at lower temperatures (Fig. 6B).

The effect of chain length of the oligonucleotide on the optimal temperature of stimulation has also been studied. The results with adenine oligonucleotides and poly U template are shown in Table IV. At higher temperatures (37° and 45°), oligonucleotides with a longer chain length stimulate the reaction (formation of poly A) better than those with a shorter chain length. The

FIG. 6. Effect of oligonucleotides on temperature dependency curves. A, formation of poly A with poly U template in the absence and presence of pApApApA. Reaction mixture similar to that described in Table I (Reaction A). Amount of pApApApA where added was 1 mpmole. B, formation of poly U with poly A template in the absence and presence of UpUpU. Reaction mixture similar to that described in Table II. Amount of UpUpU where added was 1.6 mmoles.
results support the idea of complex formation between the polyribo-
ucleotide template and the oligonucleotide.

*Time Course and Extent of Reaction*—Fig. 7A shows time curves
for poly A formation with poly U at low ATP concentration in
the absence and presence of pApApApA. The lag displayed in
the absence of pApApApA is largely overcome by the presence
of the oligonucleotide. Similar results were obtained with poly
U formation in the absence and presence of UpUpU. It is
tempting to propose that the lag is due to chain initiation that
proceeds at a slow rate. However, other possibilities cannot be
ignored. No time lag is displayed at high ATP concentration
even in the absence of oligonucleotide (Fig. 7B).

The maximum amount of poly A formed at low ATP concentra-
tion seems to be about equal to the amount of poly U present.
Similar results are obtained at high ATP concentration in the
absence of oligonucleotide; in the presence of pApApApA, the
amount of poly A formed is about twice the amount of poly U
present. The reasons for these differences are not clear.

*Effect of Oligonucleotides on Heteropolyribonucleotides*—Adenine
oligonucleotides have no effect on the formation of either poly A
or poly U with poly AU (base ratio, 1:1) as template; the forma-
tion of poly AU is slightly (about 20%) stimulated. Similarly,
uracil oligonucleotides do not stimulate the formation of either
dpoly A or poly U with poly AU template, but poly AU formation
is stimulated to a small degree (about 20%).

With poly CU (base ratio, C:U = 1:10) as a template, the
formation of poly A is markedly stimulated by adenine oligo-
10:1) poly G formation at 37° is not affected by GpGpG.
At higher temperatures, for example 45°, there is a small stimulation.
GpGpGp has no effect.

### Discussion

The results reported in this paper show the stimulation of
reactions involving poly U and poly A by complementary oligo-
nucleotides; poly A formation with poly U template is stimulated
by adenine oligonucleotides while poly U formation on poly A
template is stimulated by uracil oligonucleotides.

A free 3'-hydroxyl end has been shown to be essential for
stimulatory activity of the oligonucleotides; oligonucleotides with a
3'-phosphate end (of type AAp or UpUp) do not stimulate.
A free 5'-hydroxyl end is not required for stimulatory activity.
These results, along with the studies on the incorporation of the
oligonucleotides preferentially into chain ends (5'-phosphonomo-
ester end) of the product, suggest that the oligonucleotides stimu-
late the reaction by acting as primers or chain initiators.

The effect of the oligonucleotides on the ATP and UTP con-
centration curves is interesting. The substrate (nucleoside
triphosphate) curves for the poly U and poly A reactions are complex.
The Lineweaver-Burk plots (1/V versus 1/S) have an
upward curvature and resemble those for reactions in which the
substrate also acts as an activator (15). That more than one
substrate molecule is participating in the formation of an enzyme-
substrate (and in this case possibly also the template) complex is
suggested by the fact that in poly A formation the plot 1/V
versus 1/S is a straight line, indicating a bimolecular reaction.
One of the substrate molecules is probably acting as an activator
or chain initiator. In poly U formation, a straight line is ob-
tained with a plot of 1/V versus 1/S, indicating more complex
kinetics. In the presence of complementary oligonucleotides,
the usual type of substrate plot is obtained for either poly A or
poly U formation; the plots of 1/V versus 1/S become straighter,
suggesting that the oligonucleotides are acting as chain initiators
(furnishing preformed chain ends). The oligonucleotides do not
affect the K_m values of the templates, poly U or poly A. It
should be noted that poly G formation with poly C template
displays the usual type of substrate plots; the plot of 1/V
versus 1/S is a straight line. The guanine oligonucleotides have very
little effect on poly G formation.

The effect of oligonucleotides in relation to temperature is very
interesting. Poly A formation (minus oligonucleotide) with poly
U template at low substrate concentration proceeds best at 25°
and quite well at lower temperatures (even at 0°) while the rate
is quite slow at 37° and 45°. In the presence of adenine oligo-
nucleotides, the optimal temperature is shifted upward to 37°.
The effect suggests that a chain initiation step is temperature
sensitive or that the oligonucleotide promotes secondary structure in the template, favoring its reactivity. That an increase in the ATP concentration also shifts the optimal temperatures upward favors the chain initiation idea in view of the substrate curves for ATP discussed above. The inhibition by adenine oligonucleotides at temperatures lower than 15° is somewhat surprising. It is possible that the oligonucleotides bind too strongly at these temperatures, thus essentially blocking an essential site or sites; this is suggested by the fact that the K_m for pApApApA at 0° is about 10 times smaller than that at 25° or 37°. The formation of poly U on poly A template (minus oligonucleotide) is not particularly sensitive to temperature, although the optimum falls at 25°. Maximal stimulations with uracil oligonucleotides occurs at 37° and 45° and also appreciably at lower temperatures. The formation of poly U and poly A with poly AU as template is optimal under all conditions of substrate concentration at 37°, and the ATP reaction shows no complex substrate curve. One must then suspect that the secondary structure of poly AU favors the reaction at higher temperatures or that the mechanism of homopolymer formation on a heteropolymer template is different in some manner. Poly G formation on poly C template proceeds best at 37° and 45° (probably reflecting the strength of the G-C hydrogen bond). A small stimulation by guanine oligonucleotides occurs only at temperatures above 45°, but the enzyme loses a lot of activity at these temperatures.

The temperature studies with oligonucleotides of different chain length (Table IV) further suggest a binding of the oligonucleotide to the template by complementary base pairing. Such polynucleotide-oligonucleotide complexes have been shown to be formed under appropriate conditions (16, 17).

None of the oligonucleotides tested (adenine, uracil, and guanine) have been found to stimulate RNA formation with phage T_2 DNA as a template. This could possibly be explained on the basis that the DNA (or any DNA) might have a definite starting point (or points) for RNA formation which could be stimulated by adding the right oligonucleotides. For example, if CCU is the initiation point for RNA synthesis, the oligonucleotide GGA should stimulate the reaction; any other oligonucleotide should inhibit the natural reaction by providing false starting signals for RNA synthesis. This could explain the inhibition (at higher concentrations) by adenine, uracil, or guanine oligonucleotide of RNA synthesis on T_2 DNA. Thus, studies of this nature open up possibilities of exploring starting points for RNA synthesis on various deoxyribonucleic acids. The studies of Gros et al. (18) with E. coli DNA and various oligonucleotides are suggestive in this regard.

**SUMMARY**

In studies undertaken to investigate the mechanism of chain initiation by ribonucleic acid polymerase, the reactions involving polyuridylic acid or polyadenylic acid as a template have been found to be stimulated by complementary oligonucleotides. A free 3'-hydroxyl end has been shown to be essential for the stimulatory activity of the oligonucleotides. Temperature and other kinetic studies suggest that the stimulation is due to the oligonucleotides acting as chain initiators. Studies with labeled oligonucleotides show that these are preferentially incorporated into the 5'-phosphohomooester chain end of the polymer product, indicating that the oligonucleotides act as primers or chain initiators.

**REFERENCES**

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