Enzymic Synthesis of Polynucleotides

OLIGODEOXYNUCLEOTIDES WITH ONE 3'-TERMINAL RIBONUCLEOTIDE AS PRIMERS FOR POLYDEOXYNUCLEOTIDE SYNTHESIS*

(Received for publication, February 4, 1972)

RANAJIT ROYCHOWDHURY

From the Institut für Biologie III (Genetik und Molekularbiologie), Universität Freiburg, Schauenstrasse 9-11, 7800 Freiburg, West Germany

SUMMARY

The formation of mono- and diaddition products resulting from incorporation of rAMP residue(s) at the 3' end of an oligodeoxynucleotide primer catalyzed by the enzyme terminal deoxynucleotidyltransferase is strongly influenced by the primer to substrate ratio in the reaction mixture. Low concentrations (1 to 100 μM) of primer (hexathymidilate) and an excess (1000 μM) of substrate (rATP) cause an exclusive synthesis of the diaddition product. The monoaddition product, undetectable at a very low primer to substrate ratio, however, becomes detectable when the primer concentration approaches one-fifth of the concentration of substrate. In the presence of an excess of primer, mainly the monoaddition product is formed.

The utilization of primer can be driven to completion using an excess (1 mM) of rATP and a limited (50 to 100 μM) concentration of primer. The optimum substrate utilization is noticed when there is a 4- to 5-fold excess of primer in the incubation medium.

All four common ribonucleotides (rAMP, rGMP, rCMP, rUMP) can be added at the 3' end of an oligodeoxynucleotide primer. Such ribonucleotide terminated primers can then be employed for the initiation of new chains at the ribo-3' terminus of the primer, using all four common deoxynucleoside triphosphates (dATP, dGTP, dCTP, dTTP) giving rise to polydeoxynucleotides covalently linked to the original deoxyoligonucleotide primer by a specific ribonucleotide linkage.

EXPERIMENTAL PROCEDURE

Materials

Nucleotides and Oligonucleotides—Ribonucleoside triphosphates, obtained from commercial sources, were used without further purification. Unlabeled and 3H-labeled deoxynucleoside triphosphates were obtained from Schwarz Bio-Research. The purities of the products specified by the above source for unlabeled dATP, dGTP, dCTP, dTTP were 100%, 97.5%, 99%, 97%, respectively. The radiochemical purity of [3H]dATP, [3H]dGTP, and [3H]dTTP was 97% each, and that of [3H]dCTP was 99%.

Unlabeled ribonucleoside triphosphates were obtained from Boehringer Mannheim, GmbH, with a chemical purity of 95 to
Radioactive ribonucleoside triphosphates were products of Radiochemical Centre, Amersham. [8-$^{3}$C]rATP, most extensively used in experiments reported in this paper, had a radiochemical purity of greater than 99%. The possibility that the terminal ribonucleotide incorporation into oligodeoxynucleotides is due to a contamination of [4-$^{3}$C]rAMP with [4-$^{3}$C]dATP was excluded by a chromatographic analysis. When developed in a solvent system (isopropanol-25%, NH$_4$OH-0.1 M boric acid, 7:2:1, v/v/v) which separates rATP from dATP for 11 hours, the internal markers of rATP and dATP migrated 0.9 cm and 4.6 cm from the origin, respectively. The spots were cut out and counted. Of the total radioactivity applied to the chromatogram 101,650 cpm and 40 cpm were detected in the spots of rATP and dATP, respectively. This accounts for a radiochemical purity of greater than 99%. The possibility that the radioactivity from unutilized triphosphates was completely removed from the products during chromatography because of their higher $R_f$ values after degradation by phosphatase. The material (three paper strips) was eluted from paper and evaporated as described earlier (2) and dissolved in 10 mM Tris-HCl (pH 7.5). Of the total of 1.4 $A_{260}$ units of d(pT-T-T-T-T)-[14$^{3}$C]rG in each incubation, 1.15 $A_{260}$ units of d(T-T-T-T-T)-[14$^{3}$C]rG (A$_{260}$ 260 nm, $R_f$ with respect to d(pT)G = 0.67) and 0.9 $A_{260}$ unit of d(T-T-T-T-T)-[4$^{3}$C]rC (A$_{260}$ 266 nm, $R_f$ with respect to d(T)G = 0.79) were recovered after final purification.

**Methods**

**Preparation of Ribonucleotide-terminated Oligodeoxynucleotides**—The incubation mixture for preparation of d(pT-T-T-T-T)-[4$^{3}$C]rU contained 40 mM potassium cacodylate (pH 6.8), 8 mM MgCl$_2$, 200 $\mu$M dithiothreitol, 300 $\mu$M d(pT)$_2$, 500 $\mu$M [4-$^{3}$C]rUTP (22,400 cpm per nmole), and 40 $\mu$g of terminal transferase in a final volume of 500 $\mu$l. The incubation period was 8 hours at 37°C. The incubation mixture was then diluted 10-fold with water and subjected to chromatography on a DEAE-cellulose column as described earlier (2). The monoaddition product obtained from the column was further purified by chromatography in Solvent System I (n-propyl alcohol-concentrated NH$_4$OH-H$_2$O, 55:10:65, v/v/v) for 70 hours during which d(pT)$_2$ separated clearly from d(pT-T-T-T-T)-rU so that purified material contained no trace of d(pT)$_2$. The purified material had an absorption maximum at 264 nm (pH 8.0) and its $R_f$ with respect to d(pT)$_2$ was 0.68.

The incubation mixture for preparation of d(T-T-T-T-T)-[4-$^{3}$C]rG and d(T-T-T-T-T)-[4-$^{3}$C]rC contained buffer system as described above, 500 $\mu$g d(pT)$_2$, 5 mM [3$^{14}$C]rGTP (8,900 cpm per nmole), or [4$^{3}$C]rCTP (7,700 cpm per nmole) in two separate tubes and 4 $\mu$g of terminal transferase in final volumes of 50 $\mu$l. The incubation period was 12 hours at 37°C. Under these conditions almost complete utilization of primer is achieved, leading to the formation of mainly the diaddition product. The reaction mixture was treated with alkali and phosphatase according to the method (3) described earlier so that a single end product of reaction was obtained. These were then subjected to paper chromatography in Solvent System I for 40 hours, during which d(T-T-T-T-T)-[4-$^{3}$C]rG and d(T-T-T-T-T)-[4-$^{3}$C]rC migrated 23 and 27 cm from the origin, respectively, compared to the internal marker d(T-T-T-T-T) which migrated 34 cm from the origin so that the products were completely free from unutilized primer. The positions of the radioactive products were detected by counting 1-cm paper strips cut serially. The terminal phosphate at the 5'-end of the products was removed during the phosphatase treatment. The excess of radioactive triphosphates unutilized during the terminal transferase reaction was completely degraded into inorganic P$_1$ and 14$^{3}$C-ribonucleosides so that the radioactivity from unutilized triphosphates was completely removed from the products during chromatography because of their higher $R_f$ values after degradation by phosphatase.

The material (three paper strips) was eluted from paper and evaporated as described earlier (2) and dissolved in 10 mM Tris-HCl (pH 7.5). Of the total of 1.4 $A_{260}$ units of d(pT-T-T-T-T) used as primer in each incubation, 1.15 $A_{260}$ units of d(T-T-T-T-T)-[14$^{3}$C]rG (A$_{260}$ 260 nm, $R_f$ with respect to d(T)G = 0.67) and 0.9 $A_{260}$ unit of d(T-T-T-T-T)-[4$^{3}$C]rC (A$_{260}$ 266 nm, $R_f$ with respect to d(T)G = 0.79) were recovered after final purification.

**Assay of Ribonucleotide Incorporation**—The reaction mixture contained 40 mM potassium cacodylate (pH 6.8), 8 mM MgCl$_2$, 200 $\mu$M dithiothreitol, d(pT)$_2$, and [4-$^{3}$C]rATP (4,400 cpm per nmole) at concentrations indicated in the figures, and 2 to 4 $\mu$g of terminal transferase in final volumes of 25 to 50 $\mu$l. The incubation period was 240 min at 37°C. The reaction mixture was subjected to paper (Schleicher and Schüll, 2043b) chromatography in Solvent System I for 50 to 70 hours (see details in Fig. 1). There is always some radioactivity associated with the origins of chromatograms. The nature of this radioactivity has been discussed in a previous communication (2). The radioactivity in the chromatogram was detected by using a Berthold chromatogram scanner model LB-280 (Berthold Laboratory, Wildbad, West Germany).

**Polymerization Reaction**—The incubation mixture contained 40 mM potassium cacodylate (pH 7.2), 8 mM MgCl$_2$, 200 $\mu$M dithiothreitol, d(pT)$_2$, and [4-$^{3}$C]rATP (4,400 cpm per nmole) at concentrations indicated in the figures, and 2 to 4 $\mu$g of terminal transferase in final volumes of 25 to 50 $\mu$l. The incubation period was 240 min at 37°C. The reaction mixture was subjected to paper (Schleicher and Schüll, 2043b) chromatography in Solvent System I for 50 to 70 hours (see details in Fig. 1). There is always some radioactivity associated with the origins of chromatograms. The nature of this radioactivity has been discussed in a previous communication (2). The radioactivity in the chromatogram was detected by using a Berthold chromatogram scanner model LB-280 (Berthold Laboratory, Wildbad, West Germany).
RESULTS AND DISCUSSION

Nature of Incorporation of Ribonucleotides at Different Primer to Substrate Ratios—In the case of polymerization of deoxynucleoside triphosphates at the 3' end of an oligodeoxynucleotide catalyzed by terminal transferase, several hundred labeled residues are incorporated per molecule of primer. Thus, a very low concentration of primer (1 to 10 μM) is sufficient for the detection of a significant amount of radioactivity in the highly polymerized product which is quantitatively acid-insoluble. In contrast, since only 1 or 2 labeled residues are added per molecule of primer (2), the incorporation of ribonucleotide is not so easily detectable at a very low concentration of primer, using measurement of acid-insoluble radioactivity. Moreover, small oligonucleotides (chain length less than 10) are not quantitatively precipitated. Therefore, paper chromatographic procedure has been employed. As shown below, this simple procedure enables one to determine how heavily the incorporation of ribonucleotide is dependent upon primer concentration.

When the products of reaction are separated by paper chromatography in Solvent System I (see Fig. 1), the synthesis of the diaddition product (shown in Fig. 2) is readily detectable at 1-, 5-, 10-, 50- and 100-μM concentrations of primer. The use of low concentrations of primer (1 to 50 μM) in the presence of an excess of substrate (1000 μM) thus results in the exclusive synthesis of the diaddition product. In this range, up to a primer to substrate ratio of 1:10, the monoaddition product is not detectable. When the primer to substrate ratio reaches 1:10 and higher, the monoaddition product gradually becomes detectable (Fig. 2).

If, however, the concentration of primer is varied from 0.1 mM up to 2 mM (thereby increasing the ratio from 1:10 to 20:10), it is observed that the incorporation of ribonucleotide into the individual products varies as the ratio changes towards higher values. An increase followed by levelling in the formation of the monoaddition product is associated with a gradual decrease in the formation of the diaddition product (Fig. 34). The radioactivity tracings of the chromatograms show that more of the monoaddition product is formed with increasing concentrations.
FIG. 2. Incorporation of [$^{14}$C]rAMP at low concentrations of primer. The incubation conditions and assay were those described in Fig. 1, and d(pT)$_6$ concentrations were varied as indicated. [$^{14}$C]rATP concentration was kept constant at 1 mM.

of primer, while the diaddition product almost disappears (Fig. 3, B, C, and D).

Utilization of Primer—A salient point of interest emerges from these studies on incorporation at different primer to substrate ratios. The presence of an excess of rATP not only favors the formation of the diaddition product but also causes a very efficient utilization of primer. At a primer to substrate ratio of 1:10, more than 80% of the primer is utilized. The utilization of primer plotted against different ratios is an asymptote, tending towards 100% utilization at a very small concentration and towards 0% utilization at unlimited concentration of primer (Fig. 4).

Utilization of Substrate—At a 10-fold decreased level of rATP (kept constant at 0.1 mM), other conditions being similar to that described in Fig. 3, the incorporation pattern appears apparently similar (Fig. 5). There are, however, certain major differences.

The saturation points for the formation of the di- and monoaddition products, observed earlier to be at 2:10 and 10:10 ratios, respectively, are now being noticed at 10:10 and 40:10 ratios, respectively. The substrate utilization, as reflected by the total incorporation curve, which previously did not exceed 20% (Fig. 3), has now reached a value of 66% indicating a very efficient utilization of substrate (Fig. 5).

In certain instances, where a very precious substrate is used (e.g. chemically modified, isotopically labeled) for the study of end terminal addition, it may be desirable to obtain an efficient utilization of substrate. The conditions described in Fig. 5 would fulfill such a requirement.

Changing Concentrations of Substrate—From the results outlined above, the conditions for optimal utilization of primer or substrate can be deduced at a constant level of rATP concentration. When, however, the level of rATP is varied with the primer concentration maintained at a constant level, a different picture emerges.

As shown in Fig. 6, with the primer kept constant at 0.1 mM, the utilization of rATP decreases hyperbolically while the utilization of primer increases linearly.

For the specific labeling of 3' ends of deoxyoligonucleotides it may sometimes be desirable to obtain a quantitative conversion of primer into a ribonucleotide-terminated product. This may be necessary for sequence analysis of some specific fragment of DNA (6). The results outlined above indicate that such a requirement can be fulfilled by using a 10-fold or larger excess of substrate in the presence of enzyme.
Xynthesis of Polynucleotides

FIG. 5. Substrate utilization in the presence of a constant low amount of substrate and increasingly higher concentrations of primer. The incubation and assay conditions were those described in Fig. 1, except that $[^{14}C]ATP$ was kept constant at 100 nM and the primer concentrations were varied as indicated.

FIG. 6. Primer and substrate utilization with changing concentrations of substrate. The incubation and assay conditions were those described in Fig. 1, except that d(pT) concentration was kept constant at 0.3 mM and [3H]rAMP concentrations were varied as indicated.

Terminal Addition of rGMP, rCMP, and rUMP—In all experiments described above, the incubation period was kept constant (4 hours) in order to obtain comparable pictures. With prolonged incubation, however, a very large proportion of substrates can be incorporated into the products, even though the primer to substrate ratio is not ideal for an efficient substrate utilization.

As shown in Fig. 7, a chromatographic separation of products from incubations containing rUTP, rGTP, and rCTP reveals the formation of mono- and diaddition products in each case. After 8 hours of incubation with rUTP as substrate, more than 20% of the radioactivity appears in the products (Fig. 7A). As much as 60 to 80% of the radioactivity from rGTP and rCTP appears in the products after 24 hours of incubation (Fig. 7, B and C).

Since all four common ribonucleotides can be added at the 3' end of a given deoxyoligonucleotide (Figs. 2 and 7), it thus appears possible to add two ribonucleotides in specific sequence in a stepwise fashion. After obtaining first a single addition product with rXTP as substrate, another ribonucleotide can subsequently be added with rYTP as substrate, where X and Y are two different types of bases. Because the upper limit of

4 H. Kössel and R. Roychoudhury, unpublished data.
this polymerization is up to two ribonucleotide units (2), the addition of more than two nucleotides is not possible by this method. However, such end additions may prove useful in increasing the hybridization efficiency of short deoxyligohomocotides of specific base sequences. By adding at the 3' end of a given short (chain length 8 or 9) oligodeoxynucleotide two specific ribonucleotides complementary to the template strand, the effective length of the hybridizable stretch may be increased.

Priming Activity of rAMP-Terminated Hexathymidylate for Polymerization of Deoxynucleoside Triphosphates—Determina
tion of acid-insoluble radioactivity indicates that all four common deoxynucleoside triphosphates are polymerized when d(pT-T-T-T-T-T)-rA is used as primer. Polymerization with standard
primer d(pT-T-T-T-T-T) is also presented for comparison (Fig. 8). In the presence of magnesium, best polymerization is obtained with dATP as substrate (Fig. 8A). The polymerization of dGTP proceeds very rapidly and reaches a saturation level already after 4 min of incubation with standard primer and somewhat later when a ribonucleotide-terminated primer is used (Fig. 8B). In agreement with results of Lefler and Bollum (13), the enzymic synthesis of poly(dG) ceases abruptly after an initial rapid but limited reaction. Prolongation of the incubation period for more than 1 hour (not shown) exhibits the same amount of incorporation as that obtained after 10 min (Fig. 8B). The results presented by Hayes et al. (14) indicate that in 24 hours of incubation the upper limit of polymerization was up to 15 dGMP residues tested with different oligothymidylates as primer. With d(pT)₄ as primer, it has been shown (13) that the saturation point for dGTP polymerization is reached within 15 min of incubation. The use of dNacGTP in the presence of dGTP has not improved the reaction, although copolymerization with other triphosphates yielded better results (13, 15). Our results with ribonucleotide-terminated primer indicate that the extent of polymerization of dGMP residues is the same as that obtained with standard primer (Fig. 8B).

The polymerization of deoxynucleoside triphosphates on a ribonucleotide-terminated primer, however, shows a lag period which is distinctly evident when purine deoxynucleoside triphosphates are used as substrates (Fig. 8, A and B). In the case of pyrimidine deoxynucleoside triphosphates as substrates this is not so distinctly evident. But similar to purine triphosphates the rate of polymerization of dCTP and dTTP on a ribonucleo-
tide-terminated primer is much less than that observed with the standard primer (Fig. 8, C and D). Also, the polymerization of pyrimidine triphosphates has been found to be much lower than that of dATP in the presence of magnesium and confirms earlier observations made by Yoneda and Bollum (7) and by Kato et al. (8) tested with oligothymidylate as primer. Our observations with rAMP-terminated oligothymidylate also show similar results (Fig. 8 C and D).

When magnesium is replaced by cobalt according to Kato et al. (8), an efficient rate of polymerization is noticed with pyrimidine triphosphates as substrates and d(pT)₄ as primer. Almost complete utilization of dCTP is evident after 30 min of incubation with d(pT-T-T-T-T-T). During this period, the utilization of dCTP with d(pT-T-T-T-T-T)-rA as primer, is more than 60% (Fig. 9A). The rate of dTTP polymerization is also improved (Fig. 9B) as compared to that obtained in the presence of magnesium (Fig. 8D).

It is interesting to note that the differences in the rate of polymerization observed using a standard primer and a ribonucleotide-terminated primer is much narrower in the presence of cobalt (Fig. 9) than that observed in the presence of magnesium (Fig. 8). The results presented above show that all four common deoxynucleoside triphosphates can be polymerized at the ribo-3' ter-

---

Footnotes:
ties in the primer are not available, one would expect considerable comparative studies concerning ribo- and deoxyribo-sugar moieties not to be possible. Although primer binding to the enzyme is influenced not only by the chain length but also by the nature of bases in the oligomer. Although extensive data presented by Kate et al. (8) indicate that the thymidylate primer never exceeded more than 2 nucleotide units. Such polymerization can be carried out, although presumably base specific differences in rates and yields analogous to the results observed in the case of a deoxynucleotide at the 3' end when the chain growth is caused by deoxynucleotide additions (Fig. 10). This is further evident from the fact that the diaddition product (two ribonucleotides at the 3' end) as well as an oligoribonucleotide are capable of acting as primers (18), although at a slower rate. The rate obtained with r(A A A A A A) is at least 50 times less than that obtained with d(pT)_4 as primer (18), and it is not detectable under normal conditions of assay unless a very high concentration (25- to 50-fold higher than d(pT)_4 concentration) of primer is used (18). However, if one or two deoxynucleotides are added at the 3' end of an oligoribonucleotide, such primers show an activity almost similar to that obtained with d(pT)_4 as primer (19).

In contrast, when the chain growth is caused by the addition of ribonucleotides only, the enzyme shows a stringent requirement for deoxynucleotide residues in the primer, at least in the second position from the 3' end. When one ribonucleotide is already present at the 3' end, such a primer is capable of accepting another ribonucleotide, presumably without the chain movement. When two ribonucleotides have been added, the enzyme now has to recognize a ribo-residue in the second position from the 3' end in order to put the third ribonucleotide in the chain. According to the results, the enzyme fails to do so. Therefore, it appears that more than two additions are not possible.

Acknowledgments—This work was carried out in the laboratory of Dr. Hans Kössel, to whom I am grateful for encouragement and support. I am also indebted to Drs. Günter Feix, Eckhart Häle, and Hans Kössel of this Institute for repeated readings and painstaking revisions of the manuscript.

REFERENCES
Enzymic Synthesis of Polynucleotides: OLIGODEOXYNUCLEOTIDES WITH ONE 3' ‐ TERMINAL RIBONUCLEOTIDE AS PRIMERS FOR POLYDEOXYNUCLEOTIDE SYNTHESIS
Ranajit Roychoudhury


Access the most updated version of this article at http://www.jbc.org/content/247/12/3910

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/247/12/3910.full.html#ref-list-1