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# Elongation mechanism and substrate specificity of 2′,5′-oligoadenylate synthetase

(2′,5′-co-oligonucleotides/2′,5′-oligonucleotidyltransferases/synchronous mechanism/thin-layer chromatography)

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**ABSTRACT** **2′,5′-Oligoadenylate synthetase has been purified from a rabbit reticulocyte lysate to a high degree of purity. The enzyme contained no detectable interfering activities that could degrade the nucleoside triphosphate substrate or the oligomeric products. Two basic properties of this enzyme have been examined: the elongation mechanism for the synthesis of oligoadenylates and the substrate specificity for nucleotides. Kinetic studies on the formation of different oligomeric intermediates show that the dimer pppA2′p5′A is the first product to accumulate in predominant proportion during the first period of reaction; the trimer and other longer oligomers appear after a lag phase. The amount of the trimer increases at the expense of the dimer. Preformed dimers and trimers added to the incubation mixture were readily incorporated into higher oligomers, suggesting the free access of these dimers and trimers to the active center after the onset of polymerization of ATP. The results indicate clearly that the enzyme catalyzes the *de novo* synthesis of the oligonucleotide from ATP and that the mechanism of elongation of the 2′,5′-oligonucleotides catalyzed by the enzyme is not processive. Polymerization of a mixture of ATP and another nucleoside triphosphate shows that the enzyme is not only an ATP polymerase. The 2′,5′-oligoadenylate synthetase is in fact a 2′,5′-nucleotidyltransferase that catalyzes the formation of co-oligonucleotides. However, the purified reticulocyte enzyme catalyzed only the addition of one unit of GMP, UMP, CMP, 2′-dAMP, 3′-dAMP, dCMP, dGMP, or TMP to the 2′-OH end of a preformed oligoadenylate. A procedure for the separation of 2′,5′-oligonucleotides with or without the 5′-triphosphate end also is described.**

The 2′,5′-oligoadenylate [oligo(A)] synthetase (1) catalyzes the synthesis of a natural nucleic acid with a 2′,5′-phosphodiester linkage. Discovered in mouse cells treated with interferon, its presence was demonstrated in other cell lines treated with homologous interferon (2–5) or supposedly exposed to endogenous interferon (6). However, a low level of this enzyme has been detected in cells and tissues (7) not known to have been in contact with interferon. In particular, in rabbit reticulocytes the synthetase activity is at a level comparable to that found in interferon-treated cells (8).

A possible function of this enzyme in interferon-treated cells may be the synthesis of the oligo(A)s that activate a latent cellular endonuclease to cleave preferentially the viral mRNA(9–17). However, there is no established evidence for the direct implication of this enzyme in the viral resistance state of the cell induced by interferon. There is one example of dissociation of the phenomenon of virus resistance and the activation of the synthetase by interferon (18), suggesting the need for a multiple process for blocking virus replication. The wide occurrence of the oligo(A) synthetase (7) indicates that this enzyme also has a function independent of interferon.

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In this paper, we describe the reaction mechanism of the enzyme purified from rabbit reticulocytes and the synthesis of 2′,5′-co-oligonucleotides.

## MATERIALS AND METHODS

**Materials.** All radioactive nucleotides were obtained from Amersham. All chemicals were the purest obtainable. Poly(I)·poly(C) and poly(I)·poly(C)-agarose were from Choay Institute (Paris, France). Calf intestine alkaline phosphatase was from Boehringer. Polyethyleneimine (Polymin P) was a gift from BASF. Cellulose MN 300 was from Macherey Nagel (Düren, Germany). Nuclease P1 was from Yamasa Shoyo Co (Tokyo, Japan).

**Purification of Oligo(A) Synthetase.** Phenylhydrazine was injected into two rabbits for 5 days, according to Borsook *et al.* (19), and the blood was collected 2 days later, and the reticulocytes were isolated. The lysate (85 ml; 160 mg of protein per ml) obtained by osmotic shock was passed through a DEAE-cellulose (DE-52) column (50 ml) equilibrated in 20 mM Tris·HCl, pH 8.0/5 mM $Mg(OAc)_2$/25 mM KCl/1 mM dithiothreitol/10% (vol/vol) glycerol (buffer A). The oligo(A) synthetase activity was recovered with the proteins not retained on the column (105 ml, 32 mg of protein per ml), and this filtrate was applied to a poly(I)·poly(C)-agarose column (3 ml). After washing with buffer A and buffer A plus 0.1 M KCl, the oligo(A) synthetase activity was eluted by 0.2 M KCl in buffer A. To the active fractions (9.7 ml, 0.35 mg of protein per ml) was added $(NH_4)_2SO_4$ to 1.8 M. After centrifugation, poly(I)·poly(C) (50 μg/ml) was added to the supernatant and the oligo(A) synthetase precipitated out. The precipitate was redissolved in buffer A and dialyzed aginst buffer A and stored at −80°C. This preparation (0.9 ml, 2.7 mg of protein per ml), corresponding to 1000-fold purification, contained no interfering activities with respect to the ATP substrate, such as phosphatases, active under the assay conditions used. Its specific activity was 32 units/mg of protein; 1 unit is defined as 1 nmol of AMP-residue incorporated into oligo(A) per min at 37°C.

**Assay of Oligo(A) Synthetase.** The incubation mixture for oligo(A) synthetase was: 20 mM Tris·HCl (pH 8.0), 20 mM $Mg(OAc)_2$, 0.1 mg of bovine serum albumin per ml, 4 μg of poly(I)·poly(C) per ml, 1 mM dithiothreitol, 1 mM ATP, and 1 μCi (1 Ci = $3.7 \times 10^{10}$ becquerels) of [α-$^{32}$P]ATP in a volume of 20 μl except where otherwise stated. The reaction was stopped normally after 2 hr, or at times indicated, by heating to 72°C for 2 min.

A sample (2–8 μl) of the mixture was applied directly onto a 18 × 18 cm polyethyleneimine-cellulose thin-layer plate prepared according to Randerath and Randerath (20). (These

Abbreviation: oligo(A), 2′,5′-oligoadenylate.

plates were much better for the following chromatographic systems than were commercially obtainable plates.) After application of the samples, the plate was dried and washed in water (10 min) and methanol (5 min) with drying in between. A 4-cm broad paper wick (Desaga Paperbridge, Heidelberg, Germany) was attached to the top of the plate. The chromatogram was developed in methanol to 1 cm above the application line and transferred directly to 2 M Tris·HCl (pH 8.6). Audoradiography was performed by exposure of the dried plate to x-ray film overnight. The spots containing oligo(A)s and ATP were cut out and their radioactivity was determined by liquid scintillation counting. The reference standards were oligo(A)s purified by DEAE-Sepharose chromatography (21).

**Composition of Oligomers A(pN)$_n$.** The composition of oligo(A)s or co-oligonucleotides was determined by analysis of the core oligomers after the removal of the 5′-triphosphate by alkaline phosphatase treatment. The incubation mixture was heated to 72°C and treated with alkaline phosphatase (25 μg/ml) at 37°C for 2 hr. Samples (5–10 μl) were applied to polyethyleneimine-cellulose plates that were dried and washed in methanol. The chromatogram was developed in a gradient of Tris·HCl, pH 8.0/7.2 M urea according to a modified procedure of Gupta and Randerath (22).

Products labeled with $^{14}C$ and $^{3}H$ were revealed by fluorography. The plate was treated with 1 g of 2,5-diphenyloxazol (scintillation grade) dissolved in ether. After evaporation, the chromatogram was exposed at −80°C to a Kodak Royal X-Omat film.

## RESULTS

**Elongation Mechanism.** The reaction catalyzed by oligo(A) synthetase,

$$(n + 1)\text{pppA} \rightleftarrows \text{pppA2}'(\text{p5}'\text{A})_n + n\text{PP}_i \quad n \geq 1$$

can be visualized as a sequence of successive reactions in which an adenosine monophosphate is added to the 2′-OH end of the growing oligonucleotide chain (or to the first residue of the chain).

Two elongation mechanisms can be considered for this reaction. One, called the processive mechanism, implies that the intermediates of the polymerization leading to the synthesis of the longest products do not dissociate from the enzyme during the elongation process (23)—i.e., the time of residence of the growing chain on the enzyme is long enough for the addition of the next nucleotide (24). The other, the nonprocessive mechanism, requires the dissociation of each intermediate during the elongation.

We analyzed the composition of products during the reaction until 90% conversion. The kinetics were studied by using radioactive ATP as substrate: [γ-$^{32}$P]ATP or [α-$^{32}$P]ATP. The oligonucleotides synthesized were analyzed by thin-layer chromatography. Early in the incubation, the dinucleotide pppApA was predominant (Fig. 1*A*). The trimer and then longer oligonucleotides appeared subsequently. Patterns (not shown) obtained by autoradiography of products made with [γ-$^{32}$P]ATP showed that the 5′-end triphosphate was present.

The amounts of dimer, trimer, tetramer, and pentamer formed are plotted as a function of time of incubation in Fig. 1*B*. The first observation from this plot is the high yield of the reaction: the ATP was used almost completely. The disappearance of ATP was quantitatively related to the appearance of oligonucleotides. The dinucleotide represented the major product during the first period of reaction, when up to 50% of the ATP was used. After this the amount of dinucleotide leveled off and then decreased. After a lag that corresponded to the period of accumulation of the dimer, the formation of the trimer increased with reaction time up to 90% conversion of ATP, apparently at the expense of the dimer. The formation of the tetramer and the pentamer, nearly absent for the first 30 min in the experiment shown, proceeded at a very low rate.

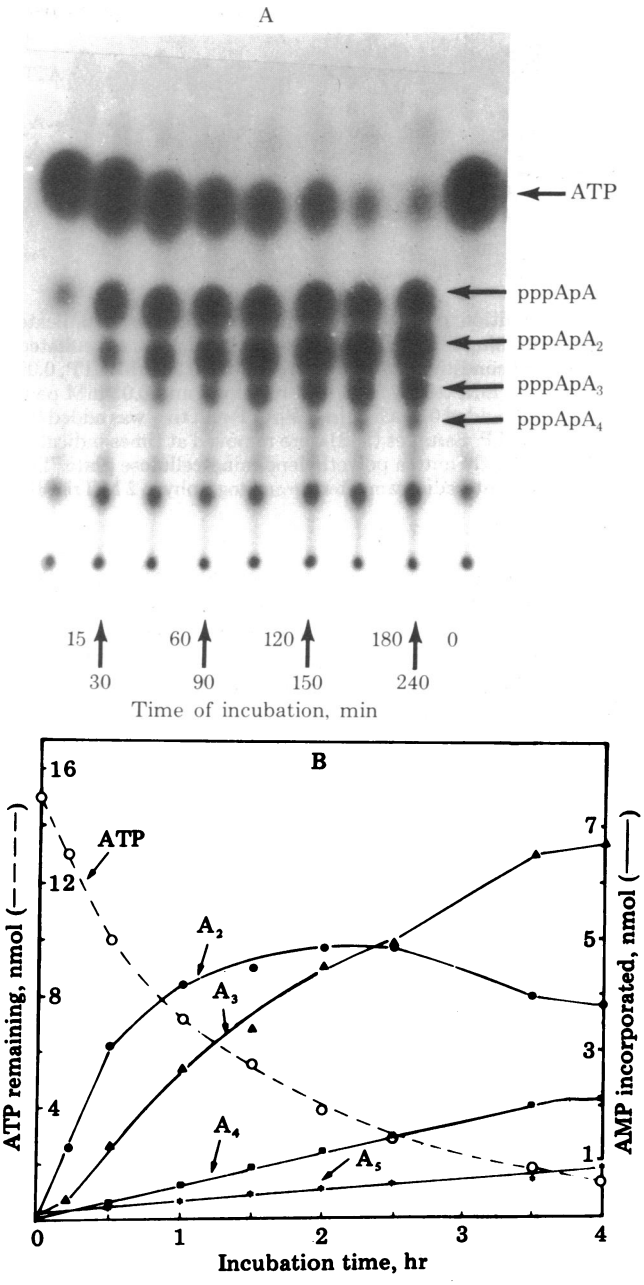


FIG. 1. Kinetics of oligo(A) formation. Time course for incubation, at 37°C, of 1.6 units (50 μg) of the purest oligo(A) synthetase with 1.5 mM ATP and 0.4 μCi of [α-$^{32}$P]ATP in a total volume of 100 μl under conditions otherwise as indicated under standard assay. At the times indicated, 10 μl was withdrawn and 3 μl was spotted directly onto a polyethyleneimine-cellulose plate and chromatographed with 2 M Tris·HCl (pH 8.6). (*A*) Autoradiogram of the chromatogram. The oligo(A)s are indicated by $A_2$, $A_3$, etc. (*B*) Quantitative representation. The spots containing ATP and the individual 2′5′ pppA(pA)$_n$ ($1 \leq n \leq 4$) were cut out and assayed. The amounts of oligo(A) formed per 10 μl are expressed as AMP equivalents.

Direct evidence for the addition of AMP residue(s) to free (dissociated) oligomers was obtained by the use of preformed oligo(A)s as primers in a reaction mixture containing either

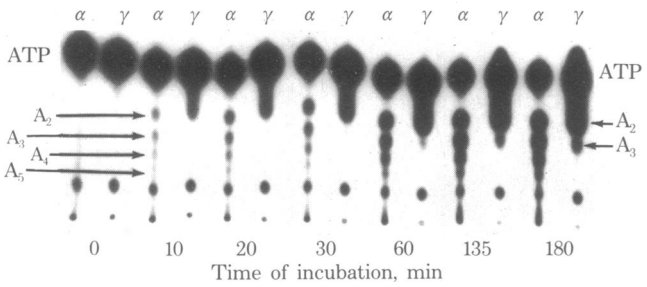


FIG. 2. Addition of AMP to preformed oligo(A)s. Duplicate mixtures containing oligo(A) synthetase (0.1 unit) were incubated under standard conditions in 30 μl at 37°C with 0.22 mM ATP, 0.09 mM dimer, 0.12 mM trimer, 0.04 mM tetramer, and 0.01 mM pentamer. To α was added 0.2 μCi of [α-$^{32}$P]ATP and to γ was added 0.5 μCi of [γ-$^{32}$P]ATP. Samples (3 μl) were removed at times indicated and spotted directly onto a polyethyleneimine-cellulose plate. The figure shows the autoradiogram after chromatography in 2 M Tris·HCl (pH 8.6).

[γ-$^{32}$P]ATP or [α-$^{32}$P]ATP. The radioactive products synthesized as a function of time of reaction were analyzed directly on polyethyleneimine-cellulose plate as described in Fig. 1*A*. The patterns of the oligonucleotides synthesized with the label originating either from γ-$^{32}$P or from α-$^{32}$P were strikingly different (Fig. 2). The pattern of γ-$^{32}$P-labeled products was similar to that shown in Fig. 1—i.e., appearance of dinucleotide at an early time and formation of trimer later on. These radioactive oligonucleotides represent molecules synthesized *de novo* and having the labeling only at the 5′-end triphosphate. On the contrary, in the serie of α-$^{32}$P-labeled products, the di-, tri-, tetra-, and pentanucleotides appeared together early in the incubation, indicating the presence of molecules formed by incorporation of [α-$^{32}$P]AMP onto the preformed unlabeled oligomers, in addition to those synthesized *de novo*.

Quantitative measurements of each oligonucleotide formed allowed the determination of the rate of its synthesis. Because the experimental conditions were identical for the γ-$^{32}$P and α-$^{32}$P labeling series, it is reasonable to subtract the concentration of molecules synthesized *de novo* (represented by γ-$^{32}$P incorporation) from that of molecules labeled with α-$^{32}$P to give the concentrations of molecules formed by addition of AMP residue(s) to a primer. The rates of formation of the dimer, trimer, and tetramer by addition of AMP to ATP and to the preformed oligomers were not very different (Table 1). It appears that the short intermediates of the reactions catalyzed by the enzyme dissociate readily from and reassociate to the enzyme.

Moreover, additions of ATP into preformed oligomers have also been demonstrated by another way. Addition of radioactive dimer to a mixture containing unlabeled ATP prior or after the onset of polymerization led to the formation of radioactive tri-, tetra-, and pentanucleotides.

All the results presented in this section indicate clearly that the elongation mechanism of the oligo(A) synthetase is not processive.

**Substrate Specificity.** Until now the oligo(A) synthetase has been considered to be an ATP polymerase. With the purified enzyme, we reexamined its substrate specificity with other nucleoside triphosphates. Under various conditions, the enzyme could not synthesize any homo-oligonucleotide in the presence of GTP, UTP, or CTP. However, when ATP was added to the reaction mixture containing these nucleotides, they were incorporated into the oligomeric products.

As shown in Fig. 3 (lanes 2 and 3) extra spots, in addition to the serie of oligo(A) (lane 1), formed when the reaction mixture

Table 1. Rates of AMP addition to pppA(pA)$_n$ ($0 \leq n \leq 2$)

| Substrate* 2 | Product | Rate of incorporation |
|---|---|---|
| 0.22 mM ATP | 2′,5′-pppApA | 0.19 |
| 0.09 mM 2′,5′-pppApA | 2′,5′-pppApApA | 0.12 |
| 0.12 mM 2′,5′-pppApApA | 2′,5′-pppApApApA | 0.13 |

From the experiment illustrated in Fig. 2 the spots containing ATP, dimer, trimer, and tetramer were cut out, and their radioactivities were measured. The total incorporation of AMP residues with ATP into dimer, with dimer into trimer, and with trimer into tetramer is represented by the labeling from [α-$^{32}$P]ATP (concentration $C_\alpha$), while the dimer, trimer, and tetramer derived from ATP alone and not from the oligomers existing at time 0 are represented by the labeling from [γ-$^{32}$P]ATP (concentration $C_\gamma$). Each $C_\gamma$ represents 2 AMP residues in the dimer, 3 AMP residues in the trimer, and 4 AMP residues in the tetramer. The concentration of AMP residues added to the preformed dimer is equal to $C_\alpha$(trimer) − 3 × $C_\gamma$(trimer) and to the trimer is $C_\alpha$(tetramer) − 4 × $C_\gamma$(tetramer). The dimer concentration is equal to 0.5 × $C_\alpha$(dimer) as well as $C_\gamma$(dimer). The initial rates of AMP addition to a primer are determined by plotting the calculated concentrations of added AMP residue as a function of time. The values are expressed as nmol/min per ml.
* Substrate 1 was 0.22 mM ATP.

contained [α-$^{32}$P]ATP and unlabeled GTP or UTP. These spots are the co-oligonucleotides (A,G) or (A,U) as demonstrated by the analysis of products formed in the presence of radioactive GTP or UTP. When [$^{14}$C]GTP was used in the presence of unlabeled ATP, there only were spots corresponding to these additional compounds (guanosine, ApG, ApApG, and ApApApG). The same result was obtained with [$^{3}$H]UTP and unlabeled ATP. The tetramer ApApApU migrated slower than ApApApA, whereas ApApU was in the same position as ApApA. This explains why there is no clear separation of

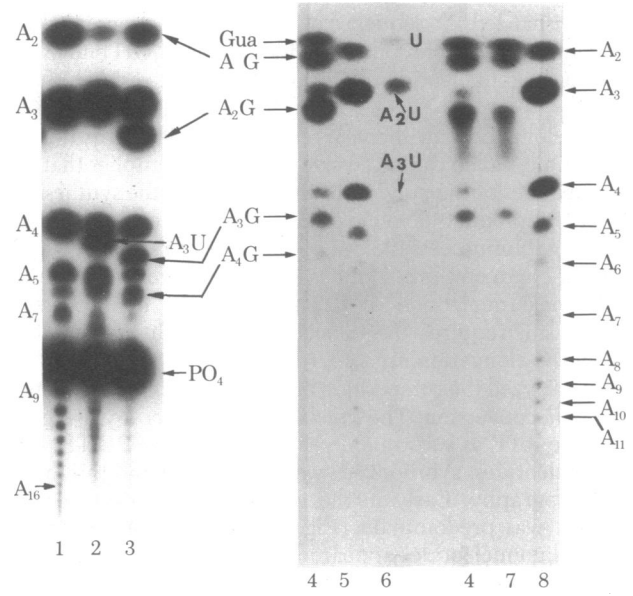


FIG. 3. Incorporation of UTP and GTP into the co-oligonucleotides. Oligo(A) synthetase (0.3 unit/100 μl) was incubated under standard conditions for 14 hr with ATP and a mixture of ATP and GTP or UTP, all at 1 mM. Samples (10 μl) of the incubation mixture were treated with alkaline phosphatase and were chromatographed with a Tris gradient in urea. The lanes represent the following mixtures: 1, [α-$^{32}$P]ATP alone; 2, [α-$^{32}$P]ATP + UTP; 3, [α-$^{32}$P]ATP + [$^{14}$C]GTP; 4, [$^{3}$H]ATP + [$^{14}$C]GTP; 5, [$^{3}$H]ATP; 6, ATP + [$^{3}$H]UTP; 7, ATP + [$^{14}$C]GTP; 8, [$^{14}$C]ATP alone. Lanes 1–3 were visualized by autoradiography; lanes 4–8 were visualized by fluorography. Gua, guanosine; U, uridine; $A_3$, ApApA; $A_2G$, for ApApG; etc.

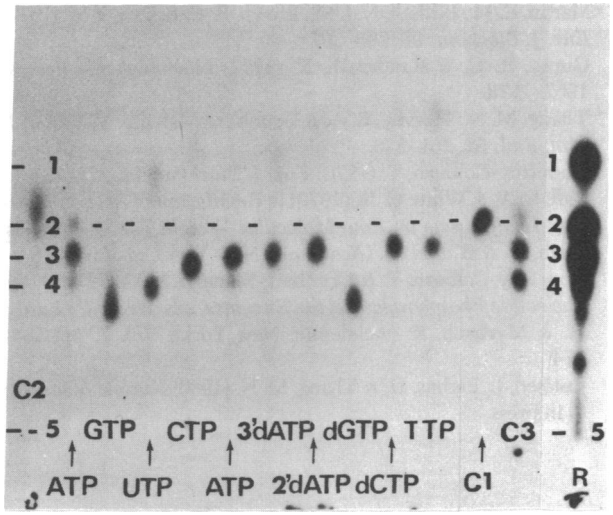


FIG. 4. Incorporation of 1 unit of NTP onto 2'5'pppApA. Purified oligo(A) synthetase (0.16 unit/20 μl) was incubated under standard conditions with γ-$^{32}$P-labeled 2',5'-pppApA at 0.06 mM plus NTP at 1.0 mM for 3 hr at 37°C; 5 μl was applied directly onto polyethyleneimine-cellulose plates and chromatographed with 2 M Tris·HCl at pH 8.6. The NTP is noted for each test. The controls were (C1) with no enzyme present and (C2) with no NTP present. In an extra test the dimer was replaced by the trimer and incubated with ATP (C3). R, reference mixture of $^{32}$P-labeled ATP and 2',5'-pppA(pA)$_n$. The positions of nucleotides are: 1, ATP; 2, 2',5'-pppApA; 3, 2',5'-pppApApA; 4, 2',5'-pppApApApA; 5, origin.

ApApA and ApApU and why ApApApA and ApApApU appear as two adjacent spots.

Similarly, addition of radioactive pppApA or pppApApA (with label in γ or α position) to a reaction mixture containing GTP, UTP, CTP, dATP, cordycepin triphosphate, dCTP, dGTP, or TTP gave rise to the formation of a radioactive trimer or tetramer (Fig. 4).

Because the label of γ-$^{32}$P of the preformed primer is preserved in the product, the direction of elongation is from 5' to 2' as already suggested by Ball and White (25). Moreover, the formation of the trimer by a single addition of one of these nucleotides to the preformed dimer (or formation of a tetramer from the trimer) indicates that all nucleoside triphosphates except ATP are chain terminators in this reaction under the experimental conditions used. Such results are different from those obtained in the case of elongation of a primer to several higher polymeric products when ATP is substrate as mentioned above.

## DISCUSSION

The oligo(A) synthetase catalyzes the *de novo* formation of pppA2'(p5'A)$_n$ oligonucleotides without template. The reaction model can be compared to the reaction catalyzed by polynucleotide phosphorylase (26) as far as the *de novo* character of the activity and the independence of template are concerned. On the contrary, the mechanism of elongation of these enzymes is different. Polynucleotide phosphorylase catalyzes the polymerization of NDP in a processive fashion whereas the polymerization of ATP by the synthetase studied here is not processive. The nonprocessive mechanism is clearly indicated by the following results: predominant formation of dinucleotide during early periods of incubation, and subsequent accumulation of the trimer and tetramer after a lag phase; increase in the amount of trimer at the expense of the dimer; incorporation of preformed oligo(A)s into longer oligomers when these primers are added to the reaction mixture prior to or after the onset of polymerization. This mechanism suggests that short-chain oligonucleotides could be the main products of the enzyme in the cell.

The enzyme is not solely an ATP polymerase. It also incorporates other nucleoside triphosphates into co-oligonucleotides. However, this incorporation of nucleotide other than ATP takes place only in the presence of ATP or a primer such as 2',5'-pppA(pA)$_n$. Under the experimental conditions described, the incorporation of extra nucleotide seems to be a one-unit addition which terminates the chain elongation. The single addition is shown by the fact that the incorporation of NTP by the enzyme in the presence of a primer such as pppApA or pppApApA leads to the formation of a single product, pppA(pA)$_n$pN; all of these oligomer products are resistant to nuclease P1 degradation, indicating the 2',5' linkage (1).

Recently, Ball and White (25) have shown that diribonucleoside monophosphates, linked either 3'–5' or 2'–5', can serve as primer for oligo(A) synthetase, but only if they contain an adenosine residue at their 3' terminus (NpA). These results are in good agreement with our observations that the requirement for ATP or an oligo(A) primer for the incorporation of other nucleotides which strongly suggests that only the AMP moiety can fit the acceptor site of the enzyme.

**Note Added in Proof.** The method for separation of 5'-phosphorylated 2'5'-oligonucleotides and additional procedures using different solvent systems for the separation of the core 2'5'-oligonucleotides [2'5'-A(pA)pN] containing AMP residues entirely and those with another ribonucleotide or deoxyribonucleotide at the 2' end are described in ref. 27.

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