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Priming of DNA synthesis by diadenosine 5′,5‴-P¹,P⁴-tetraphosphate with a double-stranded octadecamer as a template and DNA polymerase α

(DNA replication/synthetic template)

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ABSTRACT **Diadenosine 5′,5‴-P¹,P⁴-tetraphosphate (Ap_4A) primes DNA synthesis in an *in vitro* system containing purified HeLa cell DNA polymerase α, deoxyadenosine triphosphate, and the double-stranded synthetic octadecamer template 5′-d-(G-G-A-G-G-C-T-T-T-T-T-T-G-G-A-G-G-C)**

(C-C-T-C-C-G-A-A-A-A-A-A-C-C-T-C-C-G)-d-5′; this octadecamer sequence is part of the origin region of DNA synthesis in simian virus 40. Ap_4A is shown to be covalently linked to the first residue of the short deoxynucleotide chain synthesized under these experimental conditions. This template-primer system can initiate the new deoxynucleotide chain but cannot extend it beyond the A·T region.

Some years ago, diadenosine 5′,5‴-P¹,P⁴-tetraphosphate (Ap_4A) was discovered in an *in vitro* enzymatic system consisting of ATP, *l*-lysine, and purified *Escherichia coli* lysyl-tRNA synthetase (1, 2). This compound had been identified earlier, in a purely chemical reaction, as a byproduct of the interaction of adenosine 5′-phosphoromorpholidate and tributylammonium pyrophosphate in anhydrous pyridine (3). A search was made for the presence of Ap_4A in living cells, and it was found in various living forms, at concentrations of 10–100 nM (4). It was later noted to increase in concentration from 10 nM in growth-arrested eukaryotic cells in tissue culture to 1 μM in the same cells in the S_1 phase (5). Cells made permeable in the growth-arrested phase of the cell cycle promptly formed replication eyes and initiated DNA synthesis upon addition of AP_4A (6). Furthermore, Ap_4A was observed to bind to a subunit of DNA polymerase α (7, 8). The formation of AP_4A has been found to be greatly enhanced by the addition of Zn^{2+} to a phenylalanyl-tRNA synthetase, amino acid-activating reaction (9).

We recently determined that Ap_4A acts as a primer for DNA synthesis *in vitro* by DNA polymerase α with poly(dT) as template (10). This finding is in contrast to the observation by Ono *et al.* (11), who found no effect of Ap_4A on the catalytic activity of DNA polymerase α, when activated DNA or poly(dT)·oligo$(A)_{12-18}$ was used as template. In pursuit of our above finding, we now show that Ap_4A also serves as a primer for the synthesis of an oligodeoxyribonucleotide chain consisting of a few deoxyadenylate residues when the double-stranded synthetic polymer 5′-d(G-G-A-G-G-C-T-T-T-T-T-T-G-G-A-G-G-C)

(C-C-T-C-C-G-A-A-A-A-A-A-C-C-T-C-C-G)d-5′ is used as a template. This segment of DNA was chosen for synthesis because it occurs within the replication origin region of simian virus 40 (SV40) (12) and contains a sequence of six A·T pairs flanked by two G·C-rich segments.

MATERIALS AND METHODS

DNA polymerase α was prepared from HeLa cells and was purified according to published procedures (13, 14). [α-^{32}P]dATP (specific activity, 408 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), [α-^{32}P]dGTP (473 Ci/mmol), [α-^{32}P]dCTP (422 Ci/mmol), and [α-^{32}P]dTTP (800 Ci/mmol) were purchased from ICN. [^{3}H]Ap_4A (≈50 Ci/mmol) was synthesized enzymatically with highly purified isoleucyl-tRNA synthetase (kindly furnished by R. B. Loftfield), *l*-isoleucine, and [2,8-^{3}H]ATP (25 Ci/mmol) from ICN. The product [^{3}H]Ap_4A was isolated by thin-layer chromatography polyethyleneimine-cellulose (PEI) plates (Brinkmann, Polygram Cel 300, 20 × 20 cm) with elution for 16 hr at 4°C with 1.4 M LiCl, using an attached wick of Whatman no. 1 paper. The radioactive Ap_4A spot was eluted by the Randerath technique (15) from an area cut out of the plate and was estimated to be 98% pure by radioactive assay after rechromatography and isolation from a PEI plate. Beginning with 10 mCi of [2,8-^{3}H]ATP, 0.5 mCi of [^{3}H]AP_4A was prepared in this way and used in these experiments. 5′-d(G-G-A-G-G-C-T-T-T-T-T-T-G-G-A-G-G-C) and 5′-d(G-C-C-T-C-C-A-A-A-A-A-A-C-C-T-C-C) were custom synthesized by Collaborative Research (Waltham, MA). The correctness of sequence was verified by mobility shift analysis (16).

The purity of the polymers was further examined by high-pressure liquid chromatography (17) on a μBondapak C_{18} column with elution by a linear gradient of 8–20% acetonitrile in 0.05 M triethylammonium acetate (pH 7) and was estimated to be 85–95% pure.

DNA Synthesis with Unlabeled Ap_4A as Primer and Single- or Double-Stranded Octadecamers as Template. Assay mixtures were 100 μl and contained [α-^{32}P]dATP, 250 nM dTTP, dGTP, and dCTP; 20 μl (100 μg) of bovine serum albumin previously heated at 55°C for 3 hr, 1 mM ATP, 0.1 mM Ap_4A, 2 μg of the octadecamer template, 0.5 mM complete amino acid mix, and enzyme. Incubations were for 1 hr at 30°C.

After the incubation, 50 μl of reaction mixture was applied to DE-81 discs, followed by one wash with 0.1 M sodium pyrophosphate, two washes with 0.1 M ammonium formate at pH 7.5, and one wash with 95% ethanol. Discs were then dried and assayed for radioactivity in toluene/2,5-diphenyloxazole.

Covalent Linkage of Ap_4A with Product. The details of incubation were as described above. After termination of the incubation, by addition of EDTA to 0.01 M, the incubation mixture was loaded onto a column of Bio-Gel P-2 (0.9 × 15 cm) that was prewashed with 2 ml of native calf thymus DNA (1 mg/ml) and 0.01 M dATP in distilled H_2O. Gel filtration was performed with distilled H_2O at a flow rate of 1 ml/5 min, and 0.5-

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Abbreviations: Ap_4A, diadenosine 5′,5‴-P¹,P⁴-tetraphosphate; PEI, polyethyleneimine-cellulose; SV40, simian virus 40.

ml fractions were collected. Aliquots (10 μl) of the fractions were assayed for radioactivity and the contents of tubes in the excluded fraction containing radioactivity were pooled and lyophilized. The lyophilized product was dissolved in 100 μl of distilled H_2O and an aliquot was measured for ^3H and ^{32}P radioactivities. The major portion of the dissolved product was treated with 0.4 M NH_4OH overnight at room temperature. The alkaline hydrolysate was evaporated to dryness with a stream of nitrogen at 30°C. The dry sample was dissolved in 30 μl of distilled H_2O, spotted as a 1-cm streak on a PEI plate, and eluted with 1.4 M LiCl for 16 hr at 4°C in ascending chromatography. The Ap_4Ap area, which migrates with ATP, was cut out and eluted with 0.4 M NH_4OH (three 100-μl portions). The 300 μl of eluate was evaporated to dryness under a stream of nitrogen in a siliconized, conical tube, and the residue was dissolved in 30 μl of distilled H_2O. This solution was divided into two 15-μl aliquots.

To one 15-μl aliquot, 3 μl of phosphomonoesterase (Worthington, bacterial alkaline phosphatase, EC 3.1.3.1) further purified so as to be free of detectable phosphodiesterase activity, 1 μl of buffer mixture (0.1 M Tris·HCl, pH 8.9/0.05 M $MgCl_2$ containing bovine serum albumin at 100 μg/ml) and 1.5 μl of 1 mM etheno-ATP (which travels slightly more slowly than ATP in 1.4 M LiCl elution and is a more sensitive marker under ultraviolet light due to fluorescence) were added. This mixture was incubated for 6 hr at 37°C in a 1.5-ml microcentrifuge tube. The sample was then treated with 0.4 M NH_4OH for 20 min at room temperature to inactivate the phosphomonesterase (18), neutralized with 2 M HCl, and applied as two 10-μl spots 0.6 cm apart on a PEI plate (5 μl was applied at a time, with partial drying between applications). Then 5 μl of a mixture of 0.7 mM markers (ATP, ADP, AMP, Ap_4A, pAp) was added to each spot.

To the other 15-μl aliquot, 3 μl of distilled H_2O, 1 μl of the buffer mixture described above, and 1.5 μl of 1 mM etheno-ATP were added. This aliquot was similarly incubated and then applied to a PEI plate as above. After elution, the plates were dried, spots were identified and marked under ultraviolet light in a Chromatovue box, and segments were cut from the origin upward for each of the two types of sample, each rectangle of cut plate being roughly 1.3 by 2.5 cm. These segments of plate were transferred to scintillation vials, 0.1 ml of 4 M NH_4OH and 10 ml of Aquasol were added, and radioactivity was measured in a Beckman scintillation counter adjusted to approximately 8% overlap of ^{32}P counts in the ^3H channel but considerably less than 1% of ^3H counts in the ^{32}P channel.

RESULTS

Requirement for Ap_4A as a Primer. Table 1 illustrates the strict requirement for an oligonucleotide primer for DNA synthesis by DNA polymerase α with the double-stranded octadecamer (prehybridized) oligodeoxynucleotide as the template. Oligoribo- and oligodeoxyribonucleotides that are complementary to the internal hexamer dT sequences in this template are able to function as primers for DNA synthesis. However, Ap_4A was the most effective primer among the oligoribo- and oligodeoxynucleotides that were tested.

Table 1. Effect of Ap_4A and other oligo(A) primers on initiation of synthesis of DNA with 5′-d(G-G-A-G-G-C-T-T-T-T-T-T-G-G-A-G-G-C)(C-C-T-C-C-G-A-A-A-A-A-A-C-C-T-C-C-G)d-5′ as template

Primer	dNTP added*	[^{32}P]dNMP incorporated, pmol
Control (no primer)	[^{32}P]dATP	<0.5
Ap_4A	[^{32}P]dATP	17.0
	[^{32}P]dTTP	<0.5
ApA (3′-5′)	[^{32}P]dATP	1.6
Ap_2A (5′-5′)	[^{32}P]dATP	5.4
Ap_3A (5′-5′)	[^{32}P]dATP	4.8
Ap_5A (5′-5′)	[^{32}P]dATP	4.0
Oligo $d(pA)_4$(3′-5′)	[^{32}P]dATP	6.2
Oligo $d(pT)_6$(3′-5′)†	[^{32}P]dATP	5.4

The data shown are averages of two determinations.
* [^{32}P]dATP, 2100 cpm/pmol; [^{32}P]dTTP, 1760 cpm/pmol.
† It is possible that this primer is also acting as a template (10).

The Double-Stranded Structure Is the Preferred Template. The double-stranded structure of the octadecamer (prehybridized) oligodeoxynucleotide was utilized more effectively by DNA polymerase α as a template than were the single-stranded complementary octadecamers with Ap_4A as the primer (Table 2). The incorporation of [^{32}P]dATP was about 20 times greater with the double-stranded template than with the single-stranded form of the octadecamer template that contains an internal hexa(dT) sequence. Synthesis only occurred along this hexa(dT) sequence, which is complementary to the Ap_4A primer.

Only dATP Is Incorporated into Growing Chain. dCTP or dGTP were not incorporated by DNA polymerase α into a growing chain from the AP_4A primer when the single-stranded or double-stranded octadecamer was used as the template (Table 3). Incorporation did not occur even when all four deoxynucleoside triphosphates were present. Only dATP appeared to be incorporated during the extension of the AP_4A primer by DNA polymerase α (Tables 2 and 3).

Covalent Linking of Primer and Growing Chain. We previously presented evidence that Ap_4A is covalently attached to a growing dA chain when poly(dT) is used as the template with DNA polymerase α *in vitro* (10). In the present experiments

Table 2. Preference for double-stranded template

Template	dNTP added	[^{32}P]dNMP incorporated, pmol
d-(G-G-A-G-G-C-T-T-T-T-T-T-G-G-A-G-G-C)	[^{32}P]dATP	2.9
d-(G-C-C-T-C-C-A-A-A-A-A-A-G-C-C-T-C-C)	[^{32}P]dTTP	0
d-(G-G-A-G-G-C-T-T-T-T-T-T-G-G-A-G-G-C)(C-C-T-C-C-G-A-A-A-A-A-A-C-C-T-C-C-G)-d	[^{32}P]dATP	61.2

The double-stranded template was prepared by combining the separate octadecamer chains (each at 0.5 mg/ml) in 10 mM Tris·HCl, pH 7.5/0.1 M KCl, heating to 90°C for 5 min, and cooling slowly to 25°C. The product of the hybridization was desalted by gel filtration on a column (0.9 × 15 cm) of Bio-Gel P-6 in the presence of 10 mM Tris·HCl (pH 7.0). The excluded fraction was concentrated under a stream of N_2, adjusted to a final concentration of 1 mg/ml by addition of 10 mM Tris·HCl (pH 7.0), and stored in small portions at −20°C. Samples thawed for the assays were not refrozen; 0.1 mM AP_4A was present as the primer in all of the assays. The inflection of the melting curve of the double-stranded octadecamer was approximately 50°C, in 0.01 M *N*-morpholinoethanesulfonic acid/0.15 M NaCl/0.01 M $MgCl_2$, pH 6.0.

Table 3. Lack of incorporation of [^{32}P]dCTP and [^{32}P]dGTP with AP_4A as primer and either double-stranded octadecamer or d(G-G-A-G-G-C-T-T-T-T-T-T-G-G-A-G-G-C) as template

Template	dNTP added*	[^{32}P]dNMP incorporated, pmol
Control (no template)	[^{32}P]dCTP	0.6
d-(G-G-A-G-G-C-T-T-T-T-T-T-G-G-A-G-G-C)	[^{32}P]dCTP + dTTP	<0.5
	[^{32}P]dCTP + dTTP + dATP	0.8
	[^{32}P]dCTP + dTTP + dATP + dGTP	<0.5
	[^{32}P]dGTP + dATP	<0.5
	[^{32}P]dGTP + dATP + dCTP	0.7
	[^{32}P]dGTP + dATP + dCTP + dTTP	0.6
d-(G-G-A-G-G-C-T-T-T-T-T-T-G-G-A-G-G-C) (C-C-T-C-C-G-A-A-A-A-A-A-C-C-T-C-C-G)-d	[^{32}P]dCTP + dTTP	0.7
	[^{32}P]dCTP + dTTP + dATP	<0.5
	[^{32}P]dCTP + dTTP + dATP + dGTP	<0.5

The double-stranded template was prepared by the procedure described in the legend to Table 2; 0.1 mM Ap_4A was present as the primer in all of the assays.
* [^{32}P]dCTP, 1520 cpm/pmol; [^{32}P]dGTP, 1230 cpm/pmol.

we looked for covalent attachment of [^{3}H]Ap_4A as primer to [α-^{32}P]dATP as the input monomer unit of the nascent DNA chain. Gel filtration of the template and the labeled DNA product suggested that a deoxynucleotide chain of only a few units was formed in this system. Based on the relative specific activities of the [^{3}H]Ap_4A and the [^{32}P]dATP, it is calculated that one or two nucleotides are added to the primer in the synthetic reaction. The synthetic reaction product was treated with alkali and spotted on a PEI plate. The results are presented in Fig. 1. The major ^{3}H- and ^{32}P-labeled product of the alkaline hydrolysis migrated at an R_F well below that of the [^{3}H]Ap_4A starting material, suggesting additional negative charges. Based on our earlier analysis of this spot (10), we assigned the structure $A^{5'}p_4{}^{5'}A^{2'(3')}p$ to this ^{3}H- and ^{32}P-labeled product. Phosphomonoesterase (alkaline phosphatase) treatment of this spot yielded [^{3}H]Ap_4A and [^{32}P]P_i (Fig. 1, lane 1).

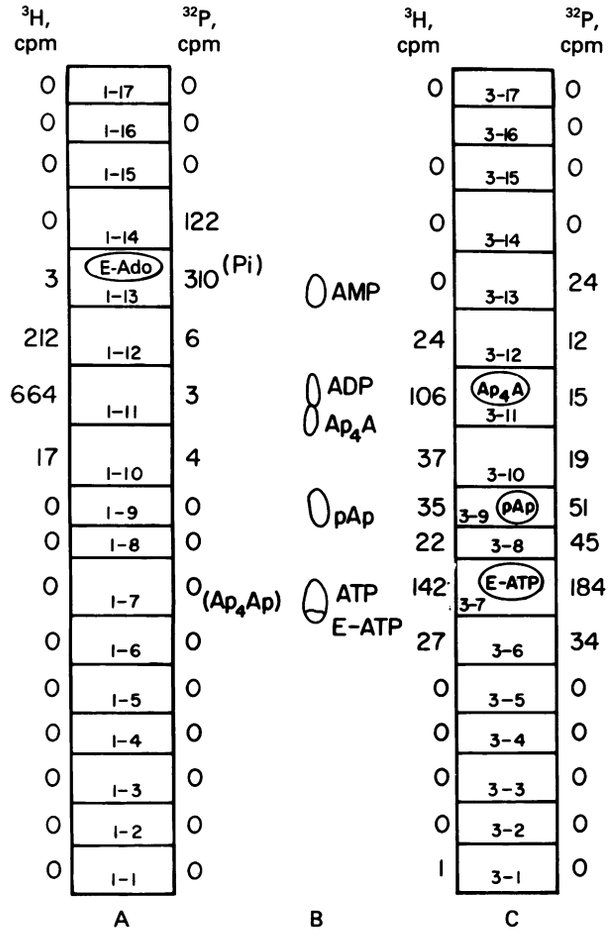

FIG. 1. PEI-cellulose thin-layer chromatography of alkaline hydrolysate of the product obtained from [^{3}H]Ap_4A and double-stranded octadecamer as primer-template, with [α-^{32}P]dATP as label, in a reaction catalyzed by DNA polymerase α. The initial alkaline hydrolysate was digested with phosphomonoesterase. Specific activity of the [α-^{32}P]dATP used was 408 Ci/mmol. Initially, the radioactivity in the ^{32}P channel was greater than that in the ^{3}H channel by a factor of 8 in the ATP/Ap_4Ap area. Counting was repeated at intervals, and the cpm presented represent a decay such that the radioactivities in the two channels are of comparable magnitude, thus eliminating the possibility that only ^{32}P radioactivity was being measured in both channels. Lane B: external standards. Lane C: synthetic product treated with alkali and chromatographed. Lane A: same as lane C but treated subsequently with phosphomonoesterase. E-ATP, pAp, Ap_4A, and E-AMP were used as internal standards. E-ATP, etheno-ATP; E-Ado, ethenoadenosine; pAp, 5'-phosphoadenosine 3'-phosphate. Inorganic phosphate (P_i) travels at the same elution distance as AMP and E-Ado but is spread out more diffusely (data for this statement not shown). (Ap_4Ap), elution spot at which this compound would be expected to travel. In an experiment in which [^{3}H]Ap_4A was incubated with all components except the double-stranded octadecamer, at termination of the incubation the major radioactivity was still present in the Ap_4A spot after PEI chromatography.

DISCUSSION

The special properties of the present system for studying initiation of DNA synthesis are that: (*i*) it preferentially uses intact Ap_4A as a primer; (*ii*) it almost specifically prefers the double-stranded deoxynucleotide octadecamer as a template; (*iii*) it uses the A·T area exclusively for chain initiation, stopping at the G·C-rich region; (*iv*) it is shown to attach growing chain to primer covalently; and (*v*) it requires the presence, in an electrophoretically homogeneous DNA polymerase α complex, of an Ap_4A binding protein (ref. 10; other confirming data not shown).

No cleavage of this template (19, 20) is required for DNA synthesis, presumably because the double-stranded octadecamer is so short as to be partially unwound and sufficiently accessible already for the activity of DNA polymerase α. It may well be that a helix-unwinding protein (19, 20) may be necessary for extension of the growing chain into the G·C-rich region. It is conceivable that the present system may provide a good test for the activity of a helix-unwinding protein. Our present con-

FIG. 2. Only a single nucleotide is shown growing on the Ap_4A primer. The A·T region is depicted as a "breathing area" in which the replication process begins.

ception of the relationship of template, primer, and nascent chain is depicted schematically in Fig. 2.

The present double-stranded octadecamer allows synthesis of DNA on only the G-G-A-G-G-C-T-T-T-T-T-T-G-G-A-G-G-C strand. It is known that SV40 replicates DNA on both parent strands of its circular structure. Whether both new strands begin at the same incipient small replication eye, however, is undetermined. Within the replication origin area of SV40 as defined by sequence determination (21, 22) is another site similar to the double-stranded octadecamer we have used, wherein the position of the A·T region is in the opposite polarity, so that a chain primed by Ap_4A theoretically could run in the opposite direction. It is also possible to hypothesize that initiation of replication of one strand would open a replication eye sufficiently for initiation of replication of the opposite strand with another primer and the aid of a helix-unwinding protein.

As to the basis for preference of AP_4A over other oligonucleotides as a primer in these experiments, AP_4A has a unique base-stacking conformation (23, 24). This structure may play a role in its high binding affinity to DNA polymerase α (7, 8), and the polymerase α-associated Ap_4A specific binding protein (unpublished data). Its polyphosphate character may also contribute to the special priming ability and to stabilization of the dinucleotide association with the template. In order to serve as a primer, hydrogen bonded in Watson–Crick pairing to the template, it would be necessary for an α–α base-stacked Ap_4A to undergo a conformational change. At the present time, we have no firm rationale for the preference of the double-stranded structure as a template.

Finally, the finding that a short double-stranded piece of synthetic DNA whose sequence is taken from the replication origin region of SV40 can function as an initiation sequence for DNA in an *in vitro* system makes it feasible to investigate, in a systematic synthetic way, the base sequence requirements for recognition of the origin site for replication by DNA polymerase α.

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