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Diadenosine 5′,5‴-P^1,P^4-tetraphosphate, a ligand of the 57-kilodalton subunit of DNA polymerase α

(replication/proliferation control/signal molecule/neuronal development/affinity labeling)

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ABSTRACT By equilibrium dialysis a diadenosine 5′,5‴-P^1,P^2-tetraphosphate (Ap_4A) binding activity is shown to be present in mammalian cells. The Ap_4A binding activity copurifies with DNA polymerase α during the isolation procedure, which includes chromatography on phospho-, DEAE-, and DNA-cellulose; gel filtration; sucrose gradient centrifugation; and electrophoresis in nondenaturing polyacrylamide gels. After these purification steps, DNA polymerase α appears to be homogeneous in nondenaturing polyacrylamide gels. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of such a purified DNA polymerase α preparation reveals seven distinct protein bands with apparent M_rs of 64,000, 63,000, 62,000, 60,000, 57,000, 55,000, and 52,000. By affinity labeling, the protein with M_r 57,000 has been shown to be the Ap_4A-binding constituent of DNA polymerase α. The binding activity of DNA polymerase α for Ap_4A is highly specific because neither structural analogs nor several other adenine nucleotides compete effectively with Ap_4A for its binding site. The Ap_4A binding site is lost in neuronal cells during maturation of rat brains concomitantly with the loss of DNA polymerase α and mitotic activity in those cells. From these results, DNA polymerase seems to be the intracellular target of Ap_4A. This is discussed in respect to the recently reported capability of Ap_4A to trigger DNA replication in quiescent mammalian cells [Grummt, F. (1978) *Proc. Natl. Acad. Sci. USA* 75, 371–375].

Diadenosine 5′,5‴-P^1,P^2-tetraphosphate (Ap_4A) was found to occur in various eukaryotic and prokaryotic cells (1–3). In mammalian cells the intracellular concentration of this compound fluctuates drastically in response to the proliferation rate—i.e., between 0.01 μM in resting or slowly growing cells and 1 μM in rapidly growing cells (2). Also, in the ciliate Tetrahymena, a significant increase of cellular Ap_4A content is observed after mitosis while cells prepare for entrance into the S phase of a subsequent division cycle (3). We demonstrated (4; 5) that Ap_4A is capable of stimulating DNA synthesis *in vitro* in baby hamster kidney cells. Ap_4A stimulates [^{3}H]dTTP incorporation only in G_1-arrested cells, not in cells from exponentially growing cultures.

Both the growth rate-dependent fluctuation in the intracellular concentration of Ap_4A as well as the induction of DNA replication in quiescent cells by Ap_4A support the hypothesis that Ap_4A is an intracellular (positive) signal molecule for the control of the transition from the resting to the growing state in animal cells (2).

To further substantiate this hypothesis and to approach a molecular understanding of the action of Ap_4A in DNA replication, we carried out experiments to characterize the intracellular target of Ap_4A. We describe here equilibrium dialysis experiments that demonstrate that DNA polymerase α specifically binds Ap_4A.

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MATERIALS AND METHODS

Materials. [*methyl*-^{3}H]dTTP (30 Ci/mmol) and [^{3}H]ATP (20 Ci/mmol) were from Amersham; nucleotides were from Boehringer Mannheim; Ap_2A, Ap_3A, Ap_5A, Ap_6A, and Gp_4G were from P-L Biochemicals. Ap_4A, Ap_4T, and [^{3}H]Ap_4A (20 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) were synthesized as described (4).

Equilibrium Dialysis. Equilibrium dialysis was carried out in chambers made from a pair of Plexiglas acryl plastic disks with a shallow cylindrical depression cut into each. A membrane cut from dialysis tubing was placed between the disks to form two chambers, each having a volume of 45 μl. A narrow hole at the top of each disk allowed an access to each side. Experiments were carried out by loading a solution of protein on one side and a solution of [^{3}H]Ap_4A (50 nM, 20 Ci/mmol) on the other and allowing the system to come to equilibrium at 4°C. In 50 mM Tris·HCl, pH 8.0/3 mM $MgCl_2$/7 mM 2-mercaptoethanol, the buffer used in all these experiments, equilibrium was reached in 24 hr. After equilibrium, 5-μl samples were withdrawn in triplicate from each side and assayed for radioactivity.

Preparation of Calf Thymus DNA Polymerase α. Calf thymus (1.5 kg) was thawed slowly and blended with 3 vol of 0.25 M sucrose/20 mM Tris·HCl, pH 8.0/7 mM 2-mercaptoethanol in a Waring Blendor at 2000 rpm for 2 min. The suspension filtered through cheesecloth was centrifuged twice at 15,000 rpm for 15 min. The final supernatant, after removal of fat, was absorbed on phosphocellulose in a 1-liter column that was washed with 4 liters of 50 mM potassium phosphate, pH 7.2/7 mM 2-mercaptoethanol and then with 2 liters of 100 mM potassium phosphate, pH 7.2. DNA polymerase α activity was eluted with 250 mM potassium phosphate (pH 7.2), dialyzed overnight against 10 liters of 50 mM potassium phosphate, pH 8.0/7 mM 2-mercaptoethanol/20% glycerol, and adsorbed on DEAE-cellulose in a 400-ml column. The DEAE-cellulose column was washed with 2 liters of 50 mM potassium phosphate, pH 8.0/7 mM 2-mercaptoethanol/20% glycerol and the DNA polymerase α activity was eluted with 300 mM potassium chloride/7 mM 2-mercaptoethanol/20% glycerol, concentrated 20-fold by vacuum dialysis, and dialyzed overnight against 20 mM Tris·HCl, pH 8.0/7 mM 2-mercaptoethanol/20% glycerol. This enzyme was applied on a 15 × 1.5 cm column of native calf thymus DNA-cellulose. The column was washed with 200 ml of 20 mM Tris·HCl, pH 8.0/7 mM 2-mercaptoethanol/20% glycerol and the enzyme was eluted with a 500-ml salt gradient (0–200 mM potassium chloride).

The polymerase α-containing fractions were concentrated by vacuum dialysis and further purified by gel filtration. The enzyme solution (4 ml) was applied on a Bio-Gel A 0.5 column

Abbreviation: Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate.

(2.5 × 80 cm) equilibrated with 20 mM Tris·HCl, pH 8.0/7 mM 2-mercaptoethanol/20% glycerol. The eluated fractions were concentrated by vacuum dialysis and tested for catalytic and Ap_4A binding activity. DNA polymerase α had an apparent M_r of about 404,000. The enzyme was then purified by electrophoresis in nondenaturing polyacrylamide gels as described by Fisher and Korn (6). The gels contained 5% acrylamide, 0.05% bisacrylamide, 20% glycerol, 125 mM Tris·H_3PO_4 (pH 8.9), 0.8 mg of sodium persulfate per ml, and 0.2% *N,N,N′,N′*-tetramethylethylenediamine (TEMED). Preelectrophoresis of the gels (10 × 0.5 cm) was carried out for 4 hr at 2.5 mA/cm^2 and 4°C in 125 mM Tris·H_3PO_4, pH 8.9/7 mM 2-mercaptoethanol/20% glycerol. A portion (0.2 ml) of the concentrated enzyme solution of the gel filtration step was electrophoretically separated overnight under the same conditions but with a running buffer containing 12.5 mM Tris·HCl, pH 8.9. For recovery of DNA polymerase, unstained gels were sectioned into 1-mm slices and eluted with 50 μl of 20 mM Tris·HCl, pH 8.0/7 mM 2-mercaptoethanol/20% glycerol/0.1% Nonidet P-40. Reference gels were stained and destained by the method of Laemmli (7). Sodium dodecyl sulfate/polyacrylamide gels (consisting of a 3% stacking and a 12% running gel) were run as described by Laemmli (7).

Protein determination was carried out according to Heil and Zillig (8).

Preparation of Neuronal Perikarya from Cerebral Cortices of Rats. Perikarya and nuclei were isolated from either the entire cerebral hemispheres (fetuses and newborns) or from cortices (postnatal ages of 7–60 days) by a described method (9, 10). For the preparation of neuronal perikarya the cerebral hemispheres or cortices were dissected and rinsed in ice-cold buffer A (0.32 M sucrose/2 mM $MgCl_2$/1 mM potassium phosphate, pH 6.5) before chopping in 7.5% polyvinylpyrrolidone/1% bovine serum albumin/10 mM $CaCl_2$. The minced brains were filtered through four mesh sizes of nylon bolting cloth (670, 335, 115, and 75 μm). The isolated perikarya were washed three times with buffer A by centrifugation at 5000 × *g* for 5 min. The perikarya pellets were frozen and later used for equilibrium dialysis experiments after thawing and lysis in 0.2% Nonidet P-40/50 mM Tris·HCl, pH 8.0/3 mM $MgCl_2$/7 mM 2-mercaptoethanol.

DNA Polymerase Assay. The reaction mixture contained in 100 μl: 50 mM Tris·HCl (pH 8.0); 5 mM $MgCl_2$; dATP, dGTP, and dCTP at 0.1 mM each; 0.01 mM dTTP (0.2 μCi of [^{3}H]dTTP), 1 mM dithiothreitol, 20 μg of calf thymus DNA activated according to Kornberg and Gefter (11), and enzyme fraction. After incubation for 30 min at 37°C, the samples were collected on glass fiber filters (Whatman GF/C) as described (4).

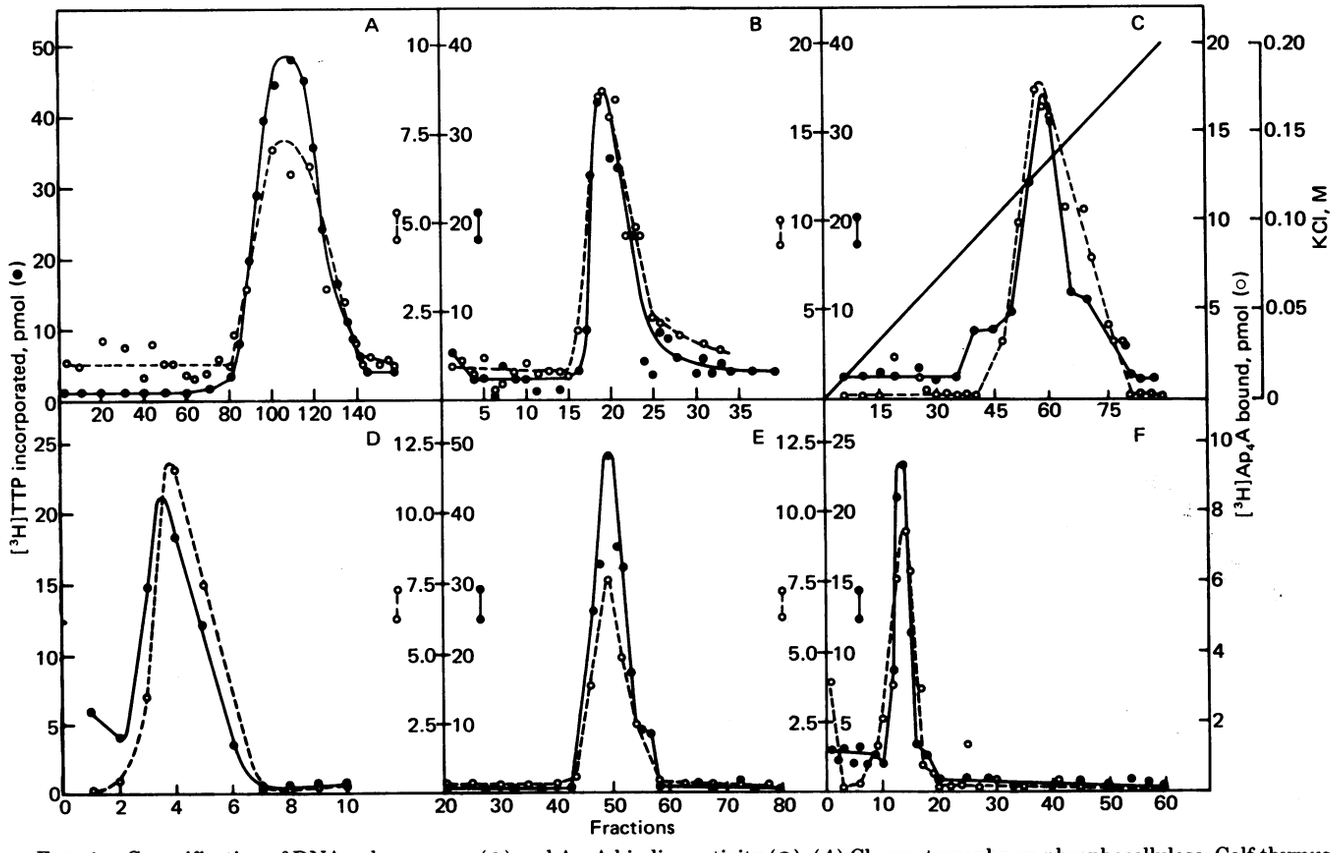

FIG. 1. Copurification of DNA polymerase α (●) and Ap_4A binding activity (○). (*A*) Chromatography on phosphocellulose. Calf thymus extract was adsorbed and finally eluted with 250 mM potassium phosphate; 10 μl of the eluate was tested in the [^{3}H]dTTP incorporation assay and 20 μl was assayed in equilibrium dialysis for Ap_4A binding activity. (*B*) Chromatography on DEAE-cellulose. The dialyzed phosphocellulose eluate was adsorbed and eluted with 250 mM KCl. Ten and 20 μl, respectively, of the eluate were assayed for [^{3}H]dTTP incorporation and Ap_4A binding. (*C*) Chromatography on DNA cellulose. (*D*) Sucrose gradient centrifugation. A portion (100 μl) of the enzyme from the DEAE-cellulose purification step was centrifuged in a 5-ml sucrose gradient (5–20%) in 10 mM Tris·HCl, pH 8.0/100 mM KCl/5 mM magnesium chloride/7 mM 2-mercaptoethanol for 17 hr at 42,000 rpm and 4°C in a Spinco rotor SW 50.1. Aliquots (20 μl) were assayed for both catalytic and Ap_4A binding activities. (*E*) Gel filtration. The DNA cellulose eluate was concentrated by vacuum dialysis and applied to a Bio-Gel A-0.5 column. Aliquots (20 μl) of the eluate were assayed for [^{3}H]dTTP incorporation and Ap_4A binding. (*F*) Polyacrylamide gel electrophoresis. Concentrated fractions of the gel-filtrated enzyme were electrophoresed, eluted, and assayed.

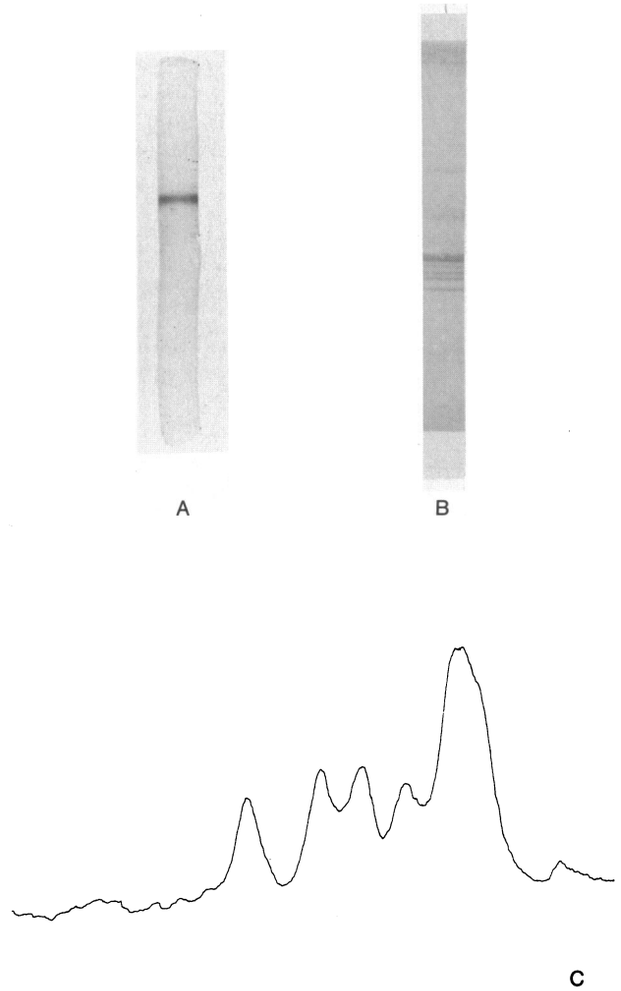

FIG. 2. (*A*) Electrophoresis of DNA polymerase α in polyacrylamide gels under nondenaturing conditions. A portion (50 μl) of the concentrated gel-filtrated enzyme was applied on a tube gel (0.5 cm diameter). (*B*) Electrophoretic separation of DNA polymerase α purified by gel filtration in a sodium dodecyl sulfate/12% polyacrylamide gel. Molecular weights of the components are given in *Results*. (*C*) Densitometric scanning of the electropherogram shown in *B*. The 52,000-dalton peak is at the left.

RESULTS

Ap_4A Binding Activity Is Copurified with DNA Polymerase α. DNA polymerase α was shown to be involved in the replication of nuclear and viral DNA in mammalian cells (12–18). Therefore, this enzyme might represent the intracellular target of Ap_4A, an odd purine nucleotide that was shown (4) to induce DNA replication in quiescent baby hamster kidney cells. This was approached by purifying DNA polymerase α from calf thymus to apparent homogeneity and studying at all purification steps both the catalytic and the Ap_4A binding activity of the enzyme fractions by measuring the [^{3}H]dTTP incorporation into acid-precipitable DNA and the binding of [^{3}H]Ap_4A in equilibrium dialysis, respectively. DNA polymerase α was purified from calf thymus extracts by means of chromatography on phospho-, DEAE-, and DNA-cellulose; sucrose gradient centrifugation; gel filtration; and electrophoresis in nondenaturing polyacrylamide gels.

A comparison of catalytic activity and the Ap_4A binding activity in equilibrium dialysis studies revealed a copurification of both [^{3}H]dTTP incorporation activity and a Ap_4A binding activity during all the isolation procedures applied (Fig. 1). DNA polymerase α of apparent homogeneity was isolated by this purification scheme, as can be seen after staining of the enzyme separated in a polyacrylamide gel under nondenaturing conditions (Fig. 2*A*). This DNA polymerase α preparation reveals seven distinct protein components (64, 63, 62, 60, 57, 55, and 52 kilodaltons) if separated under denaturing conditions in sodium dodecyl sulfate/polyacrylamide gels (Fig. 2*B*).

The 64-, 63-, and 62-kilodalton components were not well separated from each other under the conditions applied, but densitometric scanning revealed that the broad band with the lowest electrophoretic mobility consists of three distinct components (Fig. 2*C*). All seven subunits were present in the holoenzyme at a stoichiometry of 1. These seven protein components become visible and disappear, respectively, together with the peak of the catalytic and the Ap_4A binding activities. This was observed both at gel filtration and at electrophoresis in native polyacrylamide gels. After gel filtration in Bio-Gel A-0.5 the enzyme still contains several other protein components, but at strongly substoichiometric amounts in comparison with the 52- to 64-kilodalton proteins (Fig. 2*B*). These minor components are not observed after electrophoresis in native gels. The total M_r of the holoenzyme as determined by polyacrylamide gel electrophoresis under denaturing conditions is 413,000. This corresponds with the apparent M_r of 404,000 determined for the holoenzyme by gel filtration.

In order to decide which of those constituents of DNA polymerase α represents the Ap_4A binding subunit, we carried out affinity labeling experiments with periodate-oxidized [^{3}H]Ap_4A. The result showed that predominantly the M_r-57,000 subunit of DNA polymerase α can be labeled with periodate-oxidized [^{3}H]Ap_4A (Fig. 3).

Furthermore, we studied the inactivation of both the catalytic and the Ap_4A binding sites of DNA polymerase α by heat and

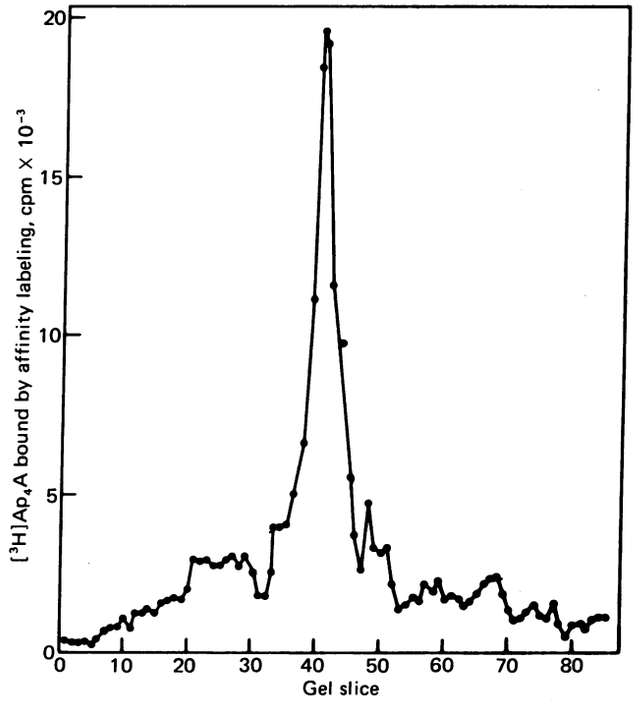

FIG. 3. Electrophoresis of DNA polymerase α after affinity labeling with [^{3}H]Ap_4A in a sodium dodecyl sulfate/polyacrylamide gel. A portion (50 μl) of the gel-filtrated enzyme were incubated for 6 hr with 1 nmol (20 Ci/mmol) of [^{3}H]Ap_4A that was oxidized with 5 mM sodium periodate. The gel was sliced into 1-mm sections and the radioactivity was assayed in a toluene-based scintillation fluid with 10% (vol/vol) Lumasolv.

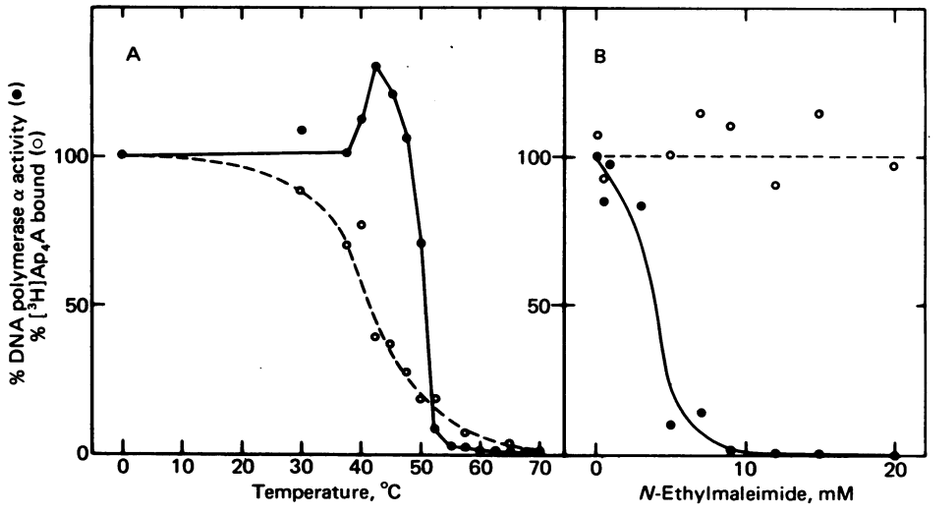

FIG. 4. Inactivation of catalytic (●) and Ap_4A binding (○) activities by heat (*A*) or *N*-ethylmaleimide (*B*). (*A*) DNA polymerase α from the DEAE-cellulose purification step was preincubated for 15 min at the temperatures given; 20-μl portions were assayed for [^{3}H]dTTP incorporation and Ap_4A binding. (*B*) The enzyme was preincubated with 10 mM *N*-ethylmaleimide for 10 min at 0°C; 70 mM 2-mercaptoethanol was then added and 20-μl aliquots were assayed for catalytic and binding activities.

by the sulfhydryl reagent *N*-ethylmaleimide. Fig. 4*A* shows the effect of preincubation at different temperatures for 15 min. Fifty percent of the Ap_4A binding activity was lost after preincubation of the enzyme at 41°C, whereas the catalytic activity was diminished to the same extent only after heating at more than 50°C. *N*-Ethylmaleimide, which completely destroys the catalytic activity of DNA polymerase α, does not influence the Ap_4A binding capacity of this enzyme (Fig. 4*B*). From these results it is highly probable that the catalytic and the Ap_4A binding sites are not identical, but whether they are located on the same or on different polymerase subunits cannot be decided.

Specificity of Ap_4A Binding to DNA Polymerase α. Control experiments revealed that the binding of [^{3}H]Ap_4A to DNA polymerase α is highly specific. The presence of a 2000-fold excess of structural analogs of Ap_4A leads to an inhibition of binding of [^{3}H]Ap_4A to DNA polymerase α by only 60% or less (Table 1). Similar results were obtained in competition experiments with excess amounts (1000-fold) of unlabeled adenosine, AMP, ADP, dATP, or GTP (Table 2). ATP inhibited binding of [^{3}H]Ap_4A to DNA polymerase α at a 100-fold excess by 50%, whereas dATP did not inhibit at all (not shown). These results demonstrate that the binding of Ap_4A is very specific not only for the target molecule DNA polymerase α but also for the ligand.

Table 1. Effects of unlabeled dinucleoside polyphosphates on the binding of [^{3}H]Ap_4A to DNA polymerase α

Additions	[^{3}H]Ap_4A bound	
	pmol	%
None	124.3	100
Ap_2A	83.2	67
Ap_3A	59.6	48
Ap_4A	0.0	0
Ap_5A	47.1	38
Ap_6A	67.1	54
Ap_4T	113.0	91
Gp_4G	109.3	88

Calf thymus DNA polymerase α (0.04 units) was used in equilibrium dialysis experiments. The concentration of [^{3}H]Ap_4A was 50 nM and that of unlabeled competitor dinucleoside polyphosphates was 0.1 mM.

Ap_4A Binding Activity Is Lost Concomitantly with DNA Polymerase α in Cerebral Neurons of Rats. If DNA polymerase α represents the intracellular target for Ap_4A in animal cells, then the Ap_4A binding activity should be diminished in cells that have lost their polymerase α activity. We have studied this by using neuronal cells at different developmental stages. Cerebral rat neurons develop from actively proliferating precursor cells at late fetal stages via nondividing immature neurons at birth to terminally postmitotic neurons thereafter. These changes are accompanied by a specific and eventually complete loss of DNA polymerase α, correlating temporally with the decline of the *in vivo* rate of mitotic activity (19). In order to find out whether the cellular Ap_4A binding capacity declines concomitantly with the DNA polymerase α activity, the binding of [^{3}H]Ap_4A by lysates of rat neuronal cells was analyzed. Fig. 5 demonstrates that neurons from rat embryos at day 5 before birth have a significant Ap_4A binding activity. The capability to bind [^{3}H]Ap_4A decreased sharply at the end of the fetal period, resulting eventually in a complete loss in the early postnatal period.

Fig. 5 also shows the decrease in DNA polymerase α activity reflecting the mitotic rate (12) during the development of rat neurons. The results clearly show that the capacity of neuronal lysates to bind [^{3}H]Ap_4A declines at a similar rate as the activity of the replicating enzyme and the mitotic activity of neuronal cells. In control experiments the binding of [^{3}H]ATP by rat neuronal lysates was studied. In contrast to the Ap_4A binding

Table 2. Effects of nucleosides and nucleotides on the binding of Ap_4A to DNA polymerase α

Additions	[^{3}H]Ap_4A bound	
	pmol	%
None	118.8	100
Adenosine	77.2	65
AMP	64.2	54
ADP	59.4	50
ATP	14.2	12
dATP	109.3	92
GTP	67.7	57

Calf thymus DNA polymerase α (0.04 units) was used in equilibrium dialysis experiments. The concentration of [^{3}H]Ap_4A was 50 nM and that of unlabeled competitor compounds was 50 μM.

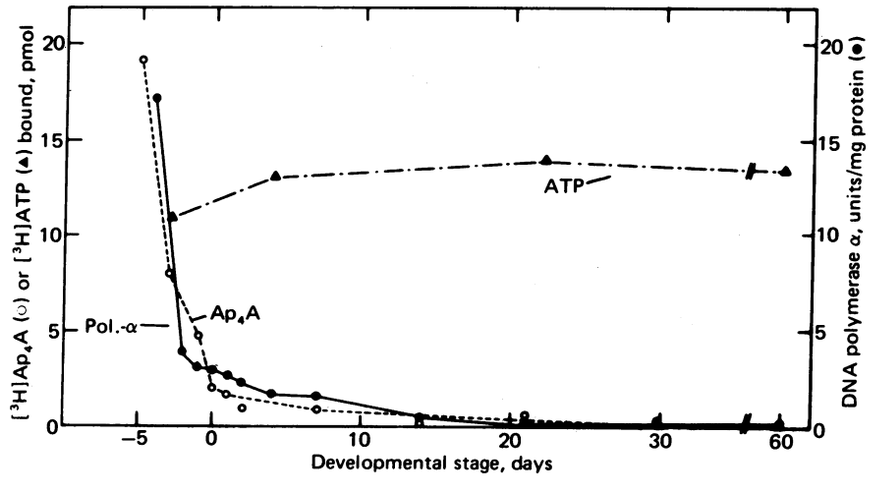

FIG. 5. Loss of Ap_4A binding activity during development of rat brain neurons. ○, [³H]Ap_4A binding activity; x, [³H]ATP binding activity in neuronal extracts; ●, DNA polymerase α activity in neuronal extracts, according to ref. 19.

capacity, no loss of ATP-binding activity was observed in rat neuronal cells during pre- and postnatal development (Fig. 5). Therefore, a specific correlation seems to exist between the level of replicating activity and the Ap_4A binding capacity in neuronal cells.

DISCUSSION

A previous study (4) has suggested that Ap_4A triggers *in vitro* DNA replication in baby hamster kidney cells that are arrested in the G_1 phase of the cell division cycle. The present studies extend these observations and show that Ap_4A binds to DNA polymerase α. There is accumulating evidence that DNA polymerase α is responsible for replication of both chromosomal and viral DNA in the nucleus of eukaryotic cells (12–18). Therefore, the replicating enzyme by itself seems to be the direct cellular target for the signal molecule Ap_4A. This assumption is strongly supported by the observation that the cellular binding site is lost in neuronal cells in which DNA polymerase α activity is reduced during the late embryonic development and eventually completely lost in the early postnatal period concomitantly with the decline in the mitotic rate in cortical neurons.

So far, the identification of DNA polymerase α as the Ap_4A-binding component was hampered by the fact that this enzyme could not yet be purified to absolute molecular homogeneity. Recent reports on the purification of DNA polymerase α do not yet give unequivocal data on the molecular weight of the catalytically active component of the enzyme (6, 20–22). Highly purified preparations of DNA polymerase α from various animal sources still contain several different components with M_rs between 50,000 and 150,000 (6, 20–22). Our purification procedure yielded a DNA polymerase α preparation that appears to be homogenous in nondenaturing polyacrylamide gels. Both the polymerizing and the Ap_4A binding activity are found in the eluate of this single band.

Because electrophoresis in sodium dodecyl sulfate/polyacrylamide gels revealed seven distinct protein bands, DNA polymerase α from calf thymus seems to consist of subunits. By affinity labeling, the 57,000-dalton constituent was identified as the subunit that binds periodate-oxidized Ap_4A. Whether this subunit also contains the catalytic center is not known. The different inactivation patterns after heat and *N*-ethylmaleimide treatment suggest that different sites are responsible for both activities.

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