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Diadenosine 5′,5‴-P^1,P^4-tetraphosphate triggers initiation of *in vitro* DNA replication in baby hamster kidney cells

(growth regulation/cell cycle/signal nucleotides/pleiotypic control/electron microscopy)

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ABSTRACT **Addition of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) to permeabilized G_1-arrested baby hamster kidney cells resulted in the stimulation of DNA synthesis. No stimulation was observed in cells from exponentially growing cultures. The Ap_4A-stimulated [^{3}H]dTTP incorporation was inhibited by nalidixic acid, daunomycin, chloroquine diphosphate, EDTA, and *N*-ethylmaleimide. It was dose-dependent in regard to the amount of permeabilized cells and of Ap_4A. Numerous replication eyes were formed in the DNA molecules of stimulated cells. Pulse-chase experiments showed that the synthesis of DNA was discontinuous, resulting in the appearance of approximately 4S Okazaki fragments and their ligation to high molecular weight DNA. These results strongly suggest that Ap_4A stimulated the initiation of DNA synthesis in baby hamster kidney cells that had been arrested in G_1 by serum deprival.**

Transition from the resting to the growing state in eukaryotic cells is characterized by progression through the G_1 period of the cell cycle and initiation of DNA synthesis. Although the complex metabolic events that lead to the start of chromosomal DNA replication are not yet understood, it is generally accepted that initiation of DNA synthesis in the cell nucleus is under the control of cytoplasmic components (1, 2). The cytoplasmic factors seem to act as positive regulatory signals (3). For investigation of the control mechanisms *in vitro* systems have been described (4–6) in which the DNA synthetic activity reflects the physiological state of the cells. Several reports describe cytoplasmic fractions active in stimulating DNA synthesis in these systems (4, 6–20). They have been characterized either as low molecular weight heat-stable components (7, 8) or as proteins of high molecular weight (6, 15, 17–19).

Previous studies on growth control in animal cells have suggested that purine nucleotides play a predominant role in the regulation of some cellular activities that are described to be part of the pleiotypic program by which cells regulate in an orderly fashion their progression from one generation to the next (21–25). These studies revealed that changes in the cellular purine nucleoside triphosphate pools are tightly correlated with the growth state as well as with the rate of ribosomal RNA synthesis in the cell. But, although high intracellular ATP levels and a high adenylate energy charge are prerequisites for initiation of DNA replication, increased ATP pools alone do not suffice to induce DNA synthesis unless further metabolic events are triggered by serum growth factors (24).

Recently, the purine nucleotide diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) has been discovered in mammalian cells (26). The intracellular concentration of this purine nucleotide has been described to be high in rapidly proliferating (about 1 μM) and low (10 nM) in resting or slowly growing cells. The synthesis of Ap_4A seems to depend on high intracellular ATP levels. Therefore, this nucleotide could play the role of a signal molecule that accumulates during the G_1 phase and triggers initiation of DNA synthesis when a critical threshold concentration is reached at the G_1/S phase boundary.

The experiments described in this paper show that Ap_4A is capable of inducing initiation of DNA replication in resting baby hamster kidney (BHK) cells (cell line 21/13) when incubated in an *in vitro* DNA-synthesizing system.

MATERIALS AND METHODS

[*methyl*-^{3}H]dTTP (30 Ci/mmol) was from Amersham; nucleotides, pyruvate kinase, snake venome phosphodiesterase, alkaline phosphatase, daunomycin, nalidixic acid, cycloheximide, and chloroquine diphosphate were from Boehringer, Mannheim.

Preparation of Permeabilized Cells. BHK cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in 15-cm φ dishes. G_1-arrested cells were obtained by culturing cells with 0.3% serum for 48 hr. Cells were trypsinized, washed twice with medium containing 10% calf serum, suspended in an ice-cold hypotonic buffer [20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate, pH 8.0/5 mM KCl/0.5 mM $MgCl_2$/0.1 mM (ethylenedinitrilo)tetraacetate (EDTA)/0.5 mM dithiothreitol] at a concentration of 5×10^6 cells per ml, and allowed to stand for 30 min at 0°. The change in permeability of cells after hypotonic treatment was tested by the loss of their ability to exclude trypan blue and by their ability to incorporate [^{3}H]dTTP into DNA (97% of untreated, viable cells exclude trypan blue, compared to 23% after hypotonic treatment; viable cells without hypotonic treatment are able to incorporate a significant amount of label from [^{3}H]thymidine into DNA but not from [^{3}H]dTTP, and hypotonic treatment renders cells capable of incorporating [^{3}H]-dTTP into DNA].

Preparation of Ap_4A. Ap_4A was prepared by a modification of the reported procedure (27). Disodium ATP was converted to the tributylammonium salt and allowed to react with an equivalent of the 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt of adenosine 5′-phosphoromorpholidate in dry pyridine for 3 days at room temperature. For preparation of ^{3}H-labeled Ap_4A, 0.1 mmol of [^{3}H]ATP (10 mCi/mmol) was treated with an equimolar amount of adenosine 5′-phosphoromorpholidate.

Assay for DNA Synthesis. The reaction medium contained in 100 μl: 40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate (pH 8.0), 5 mM $MgCl_2$, 100 mM KCl, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate (EGTA),

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Abbreviations: Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; BHK, baby hamster kidney 21/13 cell line.

Table 1. Effects of Ap_4A on [^{3}H]dTTP incorporation in G_1-arrested BHK cells

Cells, phase	[^{3}H]dTTP incorporated, pmol/10^6 cells
Logarithmic	22.8
G_1	3.8
G_1 + Ap_4A	13.2

Permeabilized BHK cells (1.5×10^5) were incubated in 100-μl samples for 60 min. Ap_4A was added at 0.1 mM. The blank values have been substracted.

dATP, dGTP, dCTP, GTP, CTP, and UTP at 0.1 mM each 2 mM ATP, 0.01 mM dTTP (1 μCi of [^{3}H]dTTP), 5 mM phospho*enol*pyruvate, 1 unit of pyruvate kinase, 1 mM dithiothreitol, and 1.5×10^5 permeabilized cells. After incubation for 60 min at 37°, 0.2 ml of a saturated neutralized $Na_4P_2O_7$ solution and 2 ml of 5% trichloroacetic acid were added, and the samples were filtered on Whatman GF/C glass fiber filters (presoaked with saturated neutralized $Na_4P_2O_7$) and washed three times with 10-ml portions of 5% trichloroacetic acid and once with 5 ml of ethanol. The samples were dried at 100°, and 0.2 ml of Hyamine hydroxide was added. After cooling at room temperature, 10 ml of a toluene-base scintillation fluid was added and radioactivity was measured.

Preparation of DNA for Electron Microscopy. Cells were incubated with or without 0.1 mM Ap_4A in a 500-μl reaction mixture for 60 min at 37° in the assay for DNA synthesis, but without [^{3}H]dTTP. Cells were collected by centrifugation for 5 min at 10,000 × *g* and were resuspended in 200 μl of 20 mM EDTA/10 mM 3-cyclohexylaminopropane sulfonic acid, pH 10.4; 20 μl of 10% Sarkosyl was added and the lysate was gently agitated for 5 min at room temperature. The lysate was centrifuged in a preformed CsCl gradient for 20 h at 2° as described by Benbow and Ford (15). Fractions were collected after monitoring of absorbance in a flow cell-equipped Guilford photometer and the three peak fractions were dialyzed overnight against 0.05 M Tris·HCl pH 7.5/5 mM EDTA/0.1 M NaCl and were spread and visualized by the procedures of Davis *et al.* (28).

Discontinuous DNA Synthesis. Permeabilized G_1-arrested cells (7.5×10^5) were incubated with or without 0.1 mM Ap_4A in a 0.5-ml reaction mixture as described above for *in vitro* DNA synthesis except that [^{3}H]dTTP was present at 100 μCi/ml and 0.001 mM, during the pulse for 1 min at 37°. For the chase experiment, dTTP was added after 1 min to a final concentration of 1 mM and the sample was incubated for another 15 min. After incubation, the samples were cooled quickly in a dry ice bath and centrifuged for 1 min at 10,000 x *g* and 0°. The cell pellets were treated with 0.3 M NaOH/1 mM EDTA, applied to 5–20% linear sucrose gradients containing 0.3 M NaOH/0.7 M NaCl/1 mM EDTA formed over a cushion of 25% sucrose/6.3 M CsCl, and centrifuged for 18 hr at 20,000 rpm (2°) in a Spinco SW56 rotor. Fractions were collected and the radioactivity of the fractions was determined.

RESULTS

G_1-Arrested Permeabilized Cells Incorporated [^{3}H]dTTP during Incubation with Ap_4A. When permeabilized BHK cells from growing cultures were incubated *in vitro* in the presence of Mg^{2+} and the four ribo- and deoxyribonucleotides, incorporation of [^{3}H]dTTP into acid-precipitable material was observed (Table 1). However, if the cells were from cultures arrested in G_1 by serum deprival, [^{3}H]dTTP incorporation was low. Addition of Ap_4A at 0.1 mM to G_1-arrested cells significantly stimulated [^{3}H]dTTP incorporation into acid-precipitable material, reaching about half of that observed with cells from exponentially growing cultures.

Table 2. Effects of inhibitors and antibiotics on [^{3}H]dTTP incorporation in G_1-arrested cells

Additions		[^{3}H]dTTP incorporated, pmol/10^6 cells
None		0.5
Ap_4A		6.4
Ap_4A plus:		
	Nalidixic acid	1.0
	Daunomycin	1.4
	Chloroquine diphosphate	1.3
	Cycloheximide	7.7
	EDTA	0
	N-Ethylmaleimide	2.7

G_1-arrested permeabilized BHK cells (1.5×10^5) were incubated in 100-μl samples for 60 min. Ap_4A was added at 0.1 mM; nalidixic acid, daunomycin, chloroquine diphosphate, and cycloheximide were added at 50 μg/ml; EDTA and *N*-ethylmaleimide were added at 2 mM. The blank values have been substracted.

In order to find out whether the [^{3}H]dTTP incorporation measured was really due to DNA replication, the effect of specific inhibitors of cellular DNA replication as well as *in vitro* DNA polymerase activity was studied (Table 2). When specific inhibitors of DNA replication (nalidixic acid, daunomycin, or chloroquine diphosphate) were added to the reaction mixture, incorporation of [^{3}H]dTTP was strongly inhibited. Cycloheximide, however, had no inhibitory effect. In the presence of an excess of EDTA over Mg^{2+}, [^{3}H]dTTP incorporation was not observed, indicating a requirement for Mg^{2+}. *In vitro*, [^{3}H]-dTTP incorporation was also sensitive to thiol reagents: the addition of *N*-ethylmaleimide inhibited the incorporation of the label. These results strongly suggest that the observed incorporation of [^{3}H]dTTP reflects *in vitro* DNA replication.

In control experiments the specificity of Ap_4A as a stimulating agent of *in vitro* DNA replication was proved (Table 3).

Table 3. Effects of phosphodiesterase- and alkaline phosphatase-treated Ap_4A, ADP, AMP, adenosine, and pyrophosphate on [^{3}H]dTTP incorporation in G_1-arrested cells

Additions	[^{3}H]dTTP incorporated, pmol/10^6 cells
None	3.8
Ap_4A	13.2
PDE-Ap_4A	5.4
Alk. *P*ase-Ap_4A	12.3
ADP	4.3
AMP	4.1
Adenosine	3.6
Sodium pyrophosphate	2.2

G_1-arrested cells (1.5×10^5) were incubated in 100-μl samples for 60 min. Additions indicated were made at concentrations of 0.1 mM. PDE-Ap_4A, Ap_4A after treatment with snake venom phosphodiesterase; alk. *P*ase-Ap_4A, after treatment with alkaline phosphatase from *Escherichia coli*. For enzymatic digestion, 0.1 μmol of Ap_4A was incubated with 50 μg of phosphodiesterase or alkaline phosphatase, respectively, in 100 μl of 50 mM triethanolamine/bicarbonate buffer, pH 8.0, at 37°. After 24 hr, 2 μl of 88% formic acid was added, the samples were centrifuged at 12,000 rpm, the supernatant was lyophilized, and the compounds were redissolved in 100 μl of H_2O.

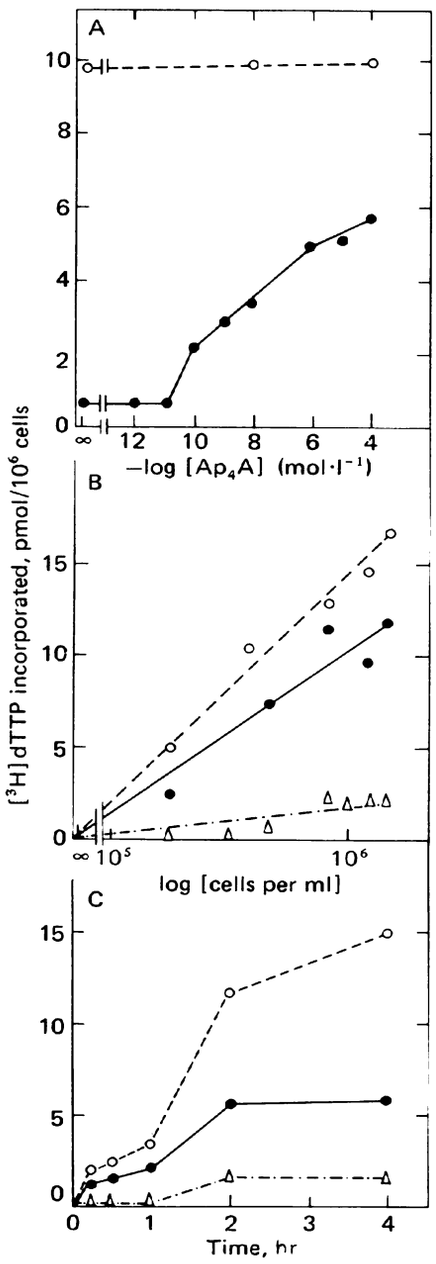

FIG. 1. Kinetics of [^{3}H]dTTP incorporation and the dependence on added Ap_4A in permeabilized cells. O- - -O, Exponentially growing cells; Δ - - - Δ, G_1-arrested cells; ●—●, G_1-arrested cells plus 0.1 mM Ap_4A (except in *A*). (*A*) Ap_4A dependence. Varying concentrations of Ap_4A were added to 100 μl-samples containing 1.5×10^5 G_1-arrested or exponentially growing cells. (*B*) Cell dependence. Varying amounts of cells were added to 1-ml samples and incubated for 60 min. The values for blanks have been substrated. (*C*) Time course. Cells (1.5×10^5) were incubated in 100-μl samples for times indicated.

Digestion of Ap_4A with snake venom phosphodiesterase, which creates AMP (not shown), abolished the stimulatory effect on DNA synthesis; however, treatment with alkaline phosphatase, which does not affect the structural integrity of Ap_4A, did not. ADP, AMP, adenosine, and sodium pyrophosphate could not substitute for Ap_4A in the stimulatory response on DNA synthesis.

Fig. 1*A* shows the effect of increasing concentration of Ap_4A on DNA synthesis in permeabilized BHK cells. Whereas DNA synthesis was stimulated by Ap_4A in a dose-dependent manner in resting cells, no influence of this compound was observed when permeabilized cells from exponentially growing cultures were tested. The extent of [^{3}H]dTTP incorporation in growing or Ap_4A-stimulated resting cells depended on the amount of cells added to the reaction mixture (Fig. 1*B*). Kinetic analysis

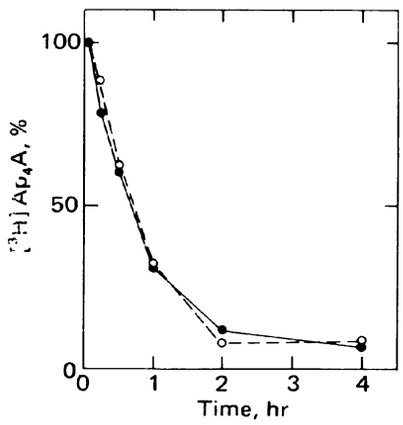

FIG. 2. Kinetics of degradation of [^{3}H]Ap_4A by permeabilized BHK cells *in vitro*. Logarithmic (O) or G_1-arrested (●) (5×10^4) cells were incubated in 25-μl samples. The concentration of [^{3}H]Ap_4A was 0.1 mM (specific activity 10 mCi/mmol). After various incubation periods, 1 μl of concentrated formic acid was added, the samples were centrifuged, and 5 μl of the supernatant was spotted on polyethyleneimine thin-layer sheets and developed with 1 M LiCl. The plates were cut into 10 × 15 mm-pieces and the radioactivity was measured.

indicated that the reaction proceeded for at least 2 hr (Fig. 1*C*). The stimulatory effect of Ap_4A was most pronounced during the first 60 min of the reaction period. This might be due to decomposition of the compound in the reaction mixture, which seems to be highly probable because Rapaport and Zamecnik (26) described Ap_4A as very labile *in vivo*. Therefore, the stability of Ap_4A in this *in vitro* system was analyzed. For this purpose, ^{3}H-labeled Ap_4A was incubated under the assay conditions. Fig. 2 demonstrates that [^{3}H]Ap_4A was degraded under the condition of the *in vitro* DNA synthesis system with a half-life of approximately 40 min. This proved true in the presence of both resting and growing cells. Thus, the relative instability of Ap_4A under the condition of *in vitro* DNA synthesis may explain why DNA replication is markedly stimulated only during a relative short period of time.

Replication Eyes Appear in the DNA Molecules After Incubation of Permeabilized G_1-Arrested Cells with Ap_4A. Characteristic structures called "replication eyes" (29, 30) can be observed by electron microscopy in nuclear DNA molecules from replicating cells. These structures arise in the DNA molecules by progression of replication forks from the origin of replication toward the replicon terminator. To obtain evidence for initiation of DNA replication, DNA molecules from the reaction mixture were isolated after a 60-min incubation of G_1-arrested permeabilized BHK cells in the presence and absence of 0.1 mM Ap_4A. These molecules were analyzed in the electron microscope and were scored for the presence of replication eyes (Fig. 3). In the control, the frequency of molecules possessing replication eyes was 0.14% (2 in 1407 molecules). In contrast, in the DNA of cells exposed to Ap_4A *in vitro* the frequency was 20-fold higher, 2.6% (47 in 1685 molecules). The total length of DNA in the control and Ap_4A-stimulated reaction was similar. The mean (±SD) size of the eyes was 0.07 ± 0.03 μm. This indicates that most replication eyes formed during 1 hr of incubation were considerably smaller than those observed previously in DNA molecules isolated from rapidly replicating embryonic cells (31, 32) or from resting *Xenopus* cell nuclei after prolonged (15 hr) incubation *in vitro* with extracts of rapidly proliferating cells (15). The eyes observed in these experiments were of the small type, postulated to be "initiation" eyes (30).

Discontinuous DNA Replication in Ap_4A-Treated Cells.

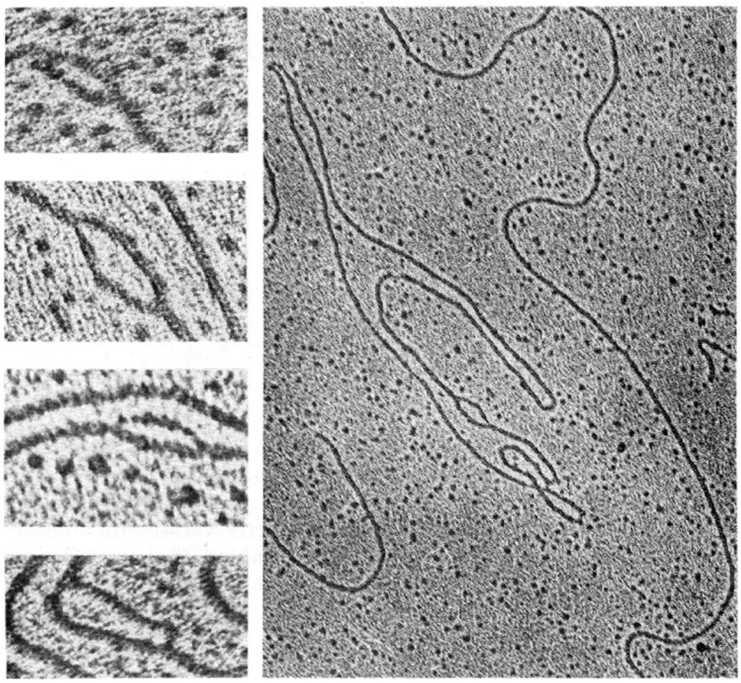

FIG. 3. Replication eyes in DNA molecules from G_1-arrested cells after *in vitro* incubation with Ap_4A.

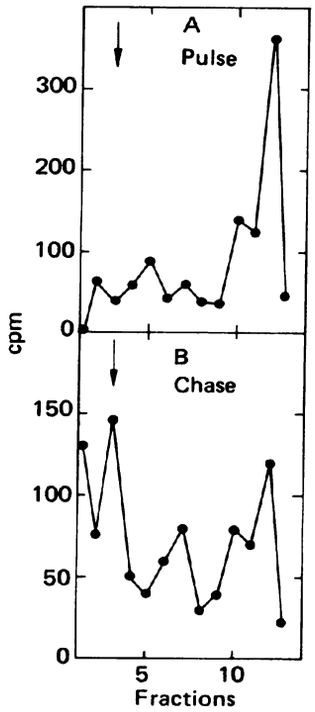

FIG. 4. Discontinuous DNA synthesis. (*A*) Pulse, 1 min with [^{3}H]dTTP; (*B*) chase, 1 min with [^{3}H]dTTP followed by 15 min with unlabeled dTTP. G_1-arrested permeabilized BHK cells (7.5×10^5) were incubated with 0.1 mM Ap_4A, and the DNA was analyzed by alkaline sucrose gradient centrifugation. The arrows indicate the position of 24S marker DNA in parallel gradients in which RF2-form DNA from phage M-13 was sedimented. Sedimentation was from right to left.

In order to find out whether Ap_4A-induced DNA synthesis in G_1-arrested cells occurs discontinuously, permeabilized cells were incubated *in vitro* for 1 min with [^{3}H]dTTP and the reaction product was analyzed by sedimentation in an alkaline sucrose gradient. Fig. 4*A* demonstrates that the reaction product had a sedimentation coefficient of about 4 S, as measured with respect to M-13 phage DNA. Most of these nascent fragments were chased into rapidly sedimenting DNA molecules when the incubation was continued for an additional 15 min with an excess (1 mM) of unlabeled dTTP (Fig. 4*B*). No further gain in label was observed during the chase period. Furthermore, there was only an insignificant, if any, loss of label during the chase period. This result demonstrates that discontinuous DNA synthesis occurs in Ap_4A-exposed cells from G_1-arrested cultures.

DISCUSSION

This paper provides evidence for the role of Ap_4A as a signal molecule that triggers initiation of DNA replication in BHK cells. The formation of this compound in the back-reaction of the amino acid activation process (33) and its presence at relatively high concentrations in rapidly proliferating cells and at low levels in resting cells (26) has been established. In the present experiments, carried out in an *in vitro* system for studying DNA synthesis with permeabilized mammalian cells, Ap_4A stimulated DNA synthesis in G_1-arrested BHK cells. The reaction was dependent on the amount of cells, the concentration of Ap_4A, and the presence of Mg^{2+}, and it could by inhibited specifically by various inhibitors of DNA replication. The appearance of DNA replication eyes and the fact that DNA synthesis was discontinuous suggest that initiation and continuation of DNA synthesis occured in this system. The stimulatory activity was specific for structurally intact Ap_4A: neither phosphodiesterase-treated Ap_4A nor its degradation products (ADP, AMP, adenosine, pyrophosphate) could substitute for Ap_4A in the reaction.

On the basis of these experimental results, it appears that Ap_4A acts as an intracellular trigger molecule for the onset of DNA replication at the G_1/S phase boundary of the cell division cycle. After growth stimulation by mitogens, Ap_4A could accumulate in the cell, reaching a critical threshold concentration at the G_1/S boundary which would allow initiation of DNA synthesis. Such an accumulation of Ap_4A during the traverse of the G_1 period seems to be highly probable because (*i*) significant differences in intracellular concentrations of this compound between resting and growing cells have been observed (26), (*ii*) ATP, the substrate used for Ap_4A formation, derives from an ATP compartment that has an especially high turnover rate (26), and (*iii*) the overall cellular ATP content increases about 3-fold during the progression through G_1 (24, 26).

It is tempting to speculate that the cytoplasmic factors from dividing cells that have been described to lead to *in vitro* DNA synthesis in nuclei from nondividing cells (4, 6, 9–20) are proteins involved in Ap_4A formation.

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