Bot. Bull. Academia Sinica 17: 95-105 (1976)

DNA SYNTHESIS OF WALKER-256 CARCINOSARCOMA NUCLEI *IN VITRO*:

III. Possible Regulating Factors in DNA Synthesis and Their Responses to Adenosine Triphosphate[1,2]

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(Accepted for publication November 25, 1975)

Abstract

ATP at 2 mM inhibits TTP incorporation into DNA by acidic protein fraction I (AP-I) when either native DNA or denatured DNA is used as template. However, ATP stimulates TTP incorporation into DNA by acidic protein fraction II (AP-II) under similar conditions. ATP also stimulates TTP incorporation into DNA when AP-I is used as enzyme source and DNA-Histone complex (or DNP) fraction as template. A heat-sensitive factor which can be isolated from DNP fraction seems to be responsible for the positive effect of ATP. DNA polymerase activity of AP-I (or AP) prefers denatured DNA to native DNA as template. Time course of ^{3}H-TTP incorporation suggests that this may be due to the difference in initiation sites (free 3′-OH sites) between two templates. ATP may exert its inhibitory effect on DNA synthesis through a similar mechanism by inhibiting an endonuclease activity of AP fraction.

Introduction

Isolated nuclei from Walker-256 carcinosarcoma can be fractionated into nucleoplasmic, acidic protein (AP), and DNA-Histone complex (or Deoxyribonucleoprotein=DNP) fractions according to Wang (1967). Ballal *et al.* (1967)

(1) This work was done at the Department of Chemistry, UCLA, Los Angeles, CA 90024 USA.

(2) Paper No. 170 of the Scientific Journal Series, Institute of Botany, Academia Sinica.

(3) The following abbreviations are used: ATP, adenosine 5′-triphosphate; TTP, thymidine 5′-triphosphate; TDP, thymidine 5′-diphosphate; TMP, thymidine 5′-monophosphate; d-ATP, deoxyadenosine 5′-triphosphate; d-GTP, deoxyguanosine 5′-triphosphate; d-CTP, deoxycytidine 5′-triphosphate.

showed that ATP at high concentration (2 mM) inhibits TTP incorporation into DNA by acidic protein fraction when either native DNA or denatured DNA is used as template. However, ATP stimulates TTP incorporation into DNA by using either acidic protein fraction or partially purified DNA polymerase as enzyme source and DNP as template. Other nucleoside triphosphates can not replace ATP for this stimulating effect. They also showed that ^{3}H-TTP can be hydrolyzed to thymidine by crude nuclear extract. ATP as well as other nucleoside triphosphates inhibits this hydrolysis. This suggests that the stimulating effect of ATP for TTP incorporation into DNA may be partially explained in terms of its inhibitory effect on the hydrolysis of TTP by dephosphorylating enzyme(s). In previous papers of this series (Lin and Smith 1975a; Lin and Smith 1975b), the existence of both TTPase and TDPase activities and their responses to ATP have been described.

In this work active stimulating factor of DNA synthesis was separated from DNP fraction. However, its properties except heat-sensitive nature are not known. An endonuclease activity was found in AP fraction. Responses of these two factors to ATP and their effects on DNA synthesis are discussed.

Materials and Methods

Materials

Unlabeled ribonucleoside and deoxyribonucleoside 5'-mono, -di, and triphosphates were purchased from Calbiochem. U. S. A. ^{3}H-TTP was purchased from New England Nuclear. Enzyme grade Tris-base, ATP, and bovine serum albumin (fraction V) were purchased from Sigma. Enzyme grade ammonium sulfate was obtained from Mann. Dialysis tubing was purchased from Arthur H. Thomas Co. and was treated by boiling in 2.5×10^{-2} M EDTA, pH 7 (10 min), boiling in deionized water (10 min), soaking in 95% ethanol (15 min), and finally extensively washing with deionized water.

Animals and preparation of tissues

Female Sprague-Dawley rats were used. The techniques of transplantation of the tumors, anesthesia, exsanguination, and initial treatment of the tissues followed those described by Busch *et al.* (1958).

Isolation of Walker-256 carcinosarcoma cell nuclei

This was done according to Lin and Smith (1975a).

Preparation of different nuclear fractions

This was also done by the procedure of Lin and Smith (1975a). The

nuclear pellet after 0.14 M NaCl extraction was stirred in 20 volumes of a solution containing 1 mM 2-mercaptoethanol, 0.05 M imidazole buffer (pH 7.4), and 1.0 M NaCl for at least 3 hr. The stirred suspension was centrifuged at 12,000 g for 30 min to get supernatant fluid. The pellet was saved for the same treatment. The combined supernatant fluid was dialyzed overnight against exactly 6 volumes of 0.05 M imidazole buffer (pH 7.4) with 1 mM 2-mercaptoethanol. Precipitates formed during dialysis were separated from the supernatant fluid by centrifuging at 12,000 g for 30 min. The supernatant fluid contained acidic proteins (or non-histone proteins) was designated as AP-I, while the precipitate fraction was designated as DNP-I. The pellet from 1.0 M NaCl solution was treated again by 1.0 M NaCl extraction to get AP-II, DNP-II.

For enzyme activity assays of AP-I or AP-II, each fraction was brought to 0.70 ammonium sulfate saturation, allowed to stand for 1 hr, and then centrifuged at 12,000 g for 1 hr to get protein pellet. Protein pellet was dissolved in a suitable volume of solution containing 20% glycerol, 1 mM 2-mercaptoethanol, and 0.05 M imidazole buffer (pH 7.4), and then dialyzed for at least 4 hr against 100 volumes of the same solution twice before use.

For enzyme activity assays of DNP-I or DNP-II, salt was removed by washing with 20% glycerol containing 1 mM 2-mercaptoethanol, and 0.05 M imidazole buffer (pH 7.4) several times before being used.

Preparation of native Walker-256 DNA

Native Walker-256 DNA was prepared by the method of Kay *et al.* (1952).

Preparation of heat denatured Walker-256 DNA

Heat denatured Walker-256 DNA was prepared by heating an aliquot of native DNA in 0.05 M phosphate buffer (pH 7.4) for 10 min in a boiling water bath, followed by rapid cooling in ice.

Preparation of stimulating factor of DNA synthesis from DNP fraction

DNP was suspended in 10 volumes of 30% TGM solution (0.05 M Tris-HCl pH 7.4, 30% glycerol, and 1 mM 2-mercaptoethanol) at 4°C for 2 hrs. The stirred solution was then centrifuged in a Sorvall SS-34 rotor at 10,000 rpm for 15 min. The supernatant fluid contained stimulating factor of DNA synthesis.

Assay of DNA-Polymerase activity

The standard assay mixture for DNA-polymerase activity consisted of 0.15 ml containing the following: 25 nmoles each of d-ATP, d-CTP, d-GTP, and TTP, about 0.2 μCi of ^{3}H-TTP; 1.25 μmoles $MgCl_2$; 0.25 μmoles 2-mercapto-

ethanol; 10 μmoles Tris-HCl, pH 8.0. In addition, 0.05 ml of native DNA or denatured DNA or DNP fraction (as template) and 0.025 to 0.05 ml of enzyme solution were used. The total volume was 0.25 ml. Incubation was set at 37°C for 30 min unless otherwise noted. After incubation, the incorporation of labeled deoxyribonucleotide into acid-insoluble product was measured by pipetting 0.1 ml of the incubation mixtures onto filter paper discs, diameter 2 cm, (Whatman 3 MM). The following batch-washing and counting procedures were the same as those used by Berger and Huang (1971).

Protein determinations

Protein determinations were made by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

DNA determinations

Diphenylamine assay (Burton, 1956) was used for DNA determinations. Deoxyribose was used as the standard.

Preparation of ^{3}H-labeled Walker-256 DNA

^{3}H-labeled Walker-256 DNA was isolated from cells which were maintained in cell culture. Cells (ca. $3\text{–}4\times10^{6}$) being incubated in the presence of 0.1 mCi thymidine [methyl-^{3}H] (specific activity 360 mCi/mmole) were washed 3 times with 0.15 M NaCl containing 0.015 M sodium citrate, pH 7.0, (SSC). The cells were finally suspended in 1.8 ml of SSC and 0.2 ml of 5% SDS (w/v) was added. The DNA was deproteinized with chloroform: isoamylalcohol (9:1, v/v). The aqueous phase was dialyzed against 0.15 M NaCl. This procedure is mainly according to Kay *et al.* (1952).

Nuclease assay

The reaction conditions were mainly the same as used by Burzio and Koide (1973). The reaction mixture contained in 0.25 ml 50 mM Tris-HCl, pH 7.4; 0.5 mM $MnCl_2$ (in some experiments either 5.0 mM $MgCl_2$ or 5.0 mM $MgCl_2$ plus 0.5 mM $CaCl_2$ replaced 0.5 mM $MnCl_2$); 1 μg ^{3}H-Walker-256 DNA (ca. 8.5 $\times10^{3}$ cmp) and suitable amount of AP fraction. After incubation at 37°C, 100 μl of reaction mixture was taken onto filter paper disc which then went through a batch-washing procedure. ^{3}H-counts on paper were measured in a Beckman liquid scintillation spectrometer, Model LS-100. Calculated acid-soluble counts were used to express endonuclease activity.

Results

The inhibitory effect of ATP on the DNA polymerase activity of acidic protein fraction when denatured or native DNA was used as template

When AP-I was used as the enzyme source and the denatured or native Walker-256 DNA was used as the template, 2 mM ATP caused 41% and 58% inhibition respectively (Table 1). The AP-I enzyme showed a preference for heat denatured rather than native Walker-256 DNA.

The stimulating effect of ATP on the DNA polymerase activity of acidic protein fraction when deoxyribonucleoprotein fraction was used as template

From the results presented in Table 1, it was clear that when DNP was used as template 2 mM ATP stimulated TMP incorporation by AP-I fraction about 10 fold. The ATP effect was reproducible in most cases.

It was also found that ATP stimulated TMP incorporation by the AP-II fraction even when denatured DNA was used as template. This raises the possibility that a stimulating factor(s) requiring ATP for activity was extracted from nuclear pellet into AP-II which contained a small amount of DNA polymerase. Since the positive ATP effect was so great it was conceivable that it could conceal a negative ATP effect caused by some factors in the acidic protein fraction. This point of view is further supported by the results in Table 2. Including 50 μl of supernatant from DNP in the reaction mixture changed the ATP effect from 58% inhibition to 38% stimulation when native DNA was used as template. Efforts to solubilize large quantities of proteins from DNP by extraction with 4 N NaCl plus 5 M urea failed to yield active stimulating factor(s).

Table 1. *The effect of ATP on DNA polymerase activity of acidic protein fraction*

The assay mixture contained in μmoles per 0.25 ml: Tris-HCl (pH 8.0), 10; $MgCl_2$, 1.25; 2-mercaptoethanol, 0.25; ATP, 0.5; and in nmoles ^{3}H-TTP (18,200 cpm) 25 and d-ATP, d-GTP, and d-CTP, 25 each; the reaction was started by adding 50 μl (ca. 0.23 mg protein) of enzyme fraction AP-I or AP-II. Control experiments using complete system except AP and template were done. Control values were substracted from all results in the corresponding systems before calculation.

Enzyme source	Template	TMP incorporated in 30 min nmoles per mg protein	
		−ATP	+ATP (2 mM)
AP-I	Native DNA	6.9	2.9
	Denatured DNA	10.4	6.1
	DNP-I	0.2	1.9
AP-II	Native DNA	—	—
	Denatured DNA	0.7	1.7
	DNP-I	0	0.3

Table 2. *Effect of the supernatant from DNP on the DNA polymerase activity of acidic protein fraction*

Experimental conditions were similar to those described under Table 1. Protein concentration in the supernatant was not determined. The observation was repeated 3 times. The values shown were the observed ones minus background counts of one experiment.

Additions	TMP incorporated in 30 min cpm	
	−ATP	+ATP (2 mM)
A. 50 μg native DNA, 50 μl of supernatant from DNP	85	103
B. 50 μg native DNA, 50 μl of supernatant from DNP, 50 μl of AP (0.12 mg)	225	296
B-A	140	193

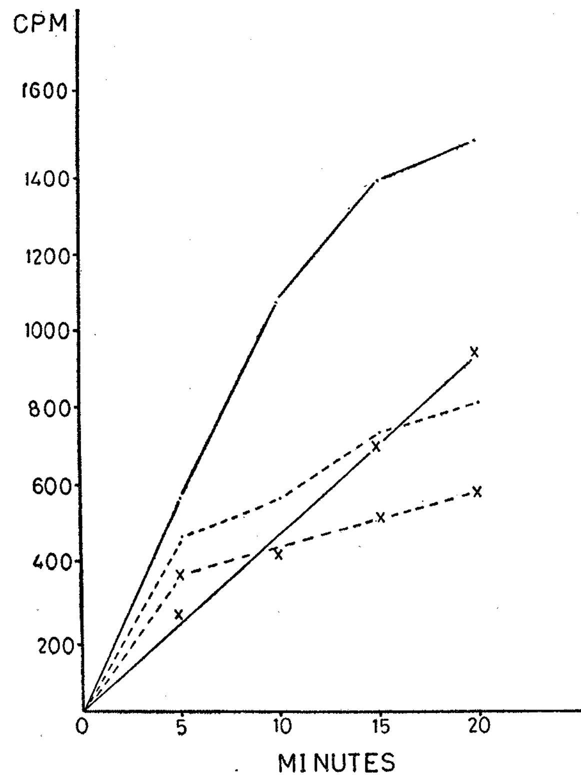

Fig. 1. Time course of ^{3}H-TMP incorporation into acid-insoluble material. The assay mixture contained in μmoles per 0.25 ml: Tris-HCl (pH 8.0), 10; $MgCl_2$, 1.25; 2-mercaptoethanol, 0.25; ATP (if included) 0.5; in nmoles ^{3}H-TTP (18,200 cpm) 25 and dATP, dGTP, and dCTP, 25 each; and 50 μg of native or denatured DNA; the reaction was started by adding 50 μl (ca. 0.23 mg protein) of enzyme fraction AP-I. •——•——•, denatured DNA without ATP; ×——×——×, denatured DNA with ATP, •……•……•, native DNA without ATP; ×……×……×, native DNA with ATP.

Time course of ^{3}H-TMP cncorporation

Fig. 1 represents the time course of ^{3}H-TMP incorporation. The initial rate of the system using native DNA as template was not much lower than that of the system using denatured DNA. But the difference became quite obvious at later times. It appears that this was due to the difference in initiation sites (free 3′-OH sites) between two templates (Aposhian and Kornberg, 1961). ATP might exert its inhibitory effect through a similar mechanism. This explanation is in aggreement with the finding that 2 mM ATP inhibited the Ca^{++}, Mg^{++} -dependent (or Mn^{++} -dependent) endonuclease activity in the acidic protein fraction to a substantial extent (Table 3).

The effect of ATP on endonuclease activity

Table 3 indicates that nuclease activity requires a divalent cation for activity. In the presence of Mg^{++} plus Ca^{++} or just Mn^{++} alone, 2 mM ATP inhibits nuclease activity by 43% or 50% respectively. A time course study of the nuclease assay and analysis by PEI-cellulose chromatography of radioactive products formed in nuclease assay at various times (unpublished data) suggest that the Ca^{++}, Mg^{++} -dependent nuclease activity is of the endonucleolytic type.

Table 3. *Effects of divalent cations and ATP on nuclease activity of the acidic protein fraction*

The common components of the reaction mixture (0.25 ml) were: 50 mM Tris-HCl, pH 7.4; 1 μg of native ^{3}H-Walker-256 DNA (ca. 8.5×10^3 cpm) and suitable amount of AP fraction. Concentrations of metal ions and ATP were shown whenever they were included. The reaction was carried out for 1 hr. Each value shown was the average of duplicate trials. The error limits were less than 5%.

Additions	5% TCA-insoluble counts (cpm)	Nuclease activity: 5% TCA-soluble counts (Total cpm minus acid-insoluble cpm)
A. Control (buffer+native ^{3}H-Walker DNA+5.0 mM $MgCl_2$)	45,368	—
B. Buffer+native ^{3}H-Walker DNA+AP	45,301	67
C. B+5.0 mM $MgCl_2$+0.5 mM $CaCl_2$+2 mM ATP	32,497	12,871
B+5.0 mM $MgCl_2$+0.5 mM $CaCl_2$	22,868	22,500
D. B+0.5 mM $MnCl_2$+2 mM ATP	36,896	8,472
B+0.5 mM $MnCl_2$	28,400	16,968

Discussion

Possible nuclear enzyme activities and factors affecting DNA synthesis and their responses to ATP *in vitro* are summaried in Table 4.

Table 4. *Possible Walker-256 nuclear enzyme activities and factors affecting* ***in vitro*** *DNA synthesis and their responses to ATP*

Enzyme activity or other factor	Response to 2 mM ATP	Apparent effect on *in vitro* DNA synthesis in presence of 2 mM ATP	Source of information
Exonuclease	inhibited by 2 mM ATP	increasing TMP incorporation	Ballal *et al.* (unpublished)
TTP phosphatase	inhibited by 2 mM ATP	increasing TMP incorporation	this work
Factor (*s*) from DNP (activating DNA polymerase)	ATP is necessary for activation to occur	increasing TMP incorporation	Ballal *et al.* (unpublished)
DNA polymerase	?	?	—
Endonuclease	inhibited by 2 mM ATP	decreasing TMP incorporation	this work
Factor(*s*) (inhibiting DNA polymerase)	?	?	—

Both TTP and TDP phosphatase activities were found in AP fraction (Lin and Smith 1975a, 1975b).

In DNA polymerase activity assays, 25 nmoles of TTP and other deoxynucleoside triphosphates were used. If acidic protein fraction containing 230 μg protein is added per tube (Table 1), about 10 nmoles of TTP would be hydrolyzed after 15 min (Lin and Smith 1975a) and this might cause an apparent effect on DNA synthesis. However the conditions used for TTPase activity assays were not exactly as, though similar to, those used for DNA polymerase activity assay. For example, pHs were close but buffers were different (the reason being already stated, Lin and Smith 1975b) in two systems; other deoxynucleoside triphosphates, DNA and DNP were not included in TTPase activity assays. To understand exactly the apparent effect of the TTPase on DNA synthesis *in vitro*, the phosphatase activity assays might be done under the conditions used for DNA polymerase assays.

Hewish and Burgoyne (1973) reported that the template capacity for DNA synthesis of isolated nuclei from normal mouse liver, normal and regenerating rat liver, rat thymus, rabbit thymus, and calf thymus was dependent upon the extent of nicks in the DNA. They demonstrated that the activation process was related to a Ca^{++}, Mg^{++} -dependent endonucleolytic activity located in the nucleus. Burzio and Koide (1973) also observed the same phenomenon using isolated rat liver nuclei. Hence, endonuclease activity in the AP fraction increased the total TMP incorporation by creating nicks on template DNA.

Therefore it is possible that inhibition of the endonuclease activity by ATP decreases the apparent TMP incorporation.

It was found that an exonuclease degraded native or denatured DNA in the assay mixture into small 5% TCA-soluble mono- or di-nucleotides (Ballal *et al.*, unpublished data). In doing so, the exonuclease reduced the total TMP incorporation by rendering less templates available. Therefore the inhibition of the exonuclease activity by ATP increased apparent TMP incorporation.

Since only a negative ATP response was observed when native or denatured DNA was used as template, one might assum that a negative ATP response of the endonuclease overcame the positive ATP responses of both the exonuclease and the TTPase, causing a net negative ATP response to be observed. Alternatively, one might assume that in addition to the endonuclease, a DNA polymerase inhibitor or inhibitors which require ATP for activity overcame the positive ATP responses of both the exonuclease and the TTPase. Here, postulation of the existence of such a DNA polymerase inhibitor(s) is quite indirect.

On the other hand, a positive ATP response could be observed only when DNP, in place of native or denatured DNA, was used as template. The positive ATP response was quite remarkable causing about a 10 fold increase in TMP incorporation. Since the stimulating effect of DNP factor(s) on TMP incorporation in the presence of ATP was so great it was easy to explain why the negative ATP response was overcome by it. Recently, Murakami and Mano (1973) reported the stimulation of sea urchin DNA polymerase by two protein factors. DNA polymerase and protein factors stimulating DNA polymerase activity were isolated from nuclei of sea urchin embryos by DNA affinity chromatography. These factors, which were heat-labile and trypsin sensitive, stimulated the enzyme activity by 3 to 4 fold. ATP was essential for the stimulation by these factors. Our findings agree with their results. Incubation of a DNP fraction at 60°C for 5 min completely destroyed its positive ATP response.

In order to check the validity of the proposal concerning the inhibitory and stimulating ATP effects on DNA synthesis shown in Table 4, some reconstitution experiments should be done now. TTPase, exonuclease, endonuclease, and factors in AP fraction might be purified by procedures using different combinations of ammonium sulfate precipitation, DEAE-cellulose column, phosphocellulose column, Sephadex column, hydroxyapatite column, DNA affinity column, and other enzyme purification methods. ATP effects might be examined accurately by adding the above enzymes (or factors) separately and in various combinations to the DNA synthesis assay mixtures containing isolated Walker-256 DNA as template and purified DNA polymerase as enzyme source.

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Walker-256 惡性瘤細胞核之去氧核糖核酸的合成：

III. 可能調節去氧核糖核酸合成的若干因子以及它們對腺嘌呤核苷 5′-三磷酸的反應

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使用自然狀態的去氧核糖核酸（雙條的）或者變性狀態的去氧核糖核酸（單條的）爲模板而以酸性蛋白質第一部分（AP-I）爲酵素來源時，腺嘌呤核苷5′-三磷酸（ATP, 2 mM）抑制胸腺核苷 5′-三磷酸（TTP）結合成去氧核糖核酸之酵素反應。可是在類似的條件下，以酸性蛋白質第二部分（AP-II）爲酵素來源時同一濃度（2 mM）的腺嘌呤核苷 5′-三磷酸却可促進胸腺核苷 5′-三磷酸結合成去氧核糖核酸之酵素反應。使用酸性蛋白質第一部分爲酵素來源而把模板換爲 DNA-Histone 複合體時，腺嘌呤核苷 5′-三磷酸也可促進胸腺核苷 5′-三磷酸結合成去氧核糖核酸。從 DNA-Histone 複合體可以分離出一種對熱不穩定的製備液，這似乎含有表現上述對腺嘌呤核苷 5′-三磷酸有正反應現象的因子。酸性蛋白質第一部分的去氧核糖核酸聚合酵素活性，在變性狀態的去氧核糖核酸和自然狀態的去氧核糖核酸兩者之間偏好前者作爲模板。放射性胸腺核苷 5′-三磷酸結合成去氧核糖核酸的時間過程顯示前述之偏好現象可能是兩種模板在啓發部位的數目（也就是 free 3′-OH sites）的差別所導致。腺嘌呤核苷 5′-三磷酸對去氧核糖核酸的抑制作用可以類似的機轉來解釋：腺嘌呤核苷 5′-三磷酸抑制 AP 的一種核酸內水解酵素因而間接導致去氧核糖核酸之合成。