IN VITRO ACTIVATION OF DNA POLYMERASE-α BY A PROTEIN KINASE IN CHICK EMBRYO

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Received 2 December 1980; revised version received 22 December 1980

1. Introduction

Enzyme activity may be regulated by several distinct mechanisms, an example being covalent modification of the enzyme polypeptide, e.g., acetylation, methylation, phosphorylation. A reversible phosphorylation–dephosphorylation mechanism has been shown to regulate or modulate the activity of enzymes such as muscle and liver phosphorylase and glycogen synthetases, and some DNA-dependent RNA polymerases (review [1]).

These findings and the correlation between phosphorylation and rapidly proliferating cells [1], suggest the intervention of a phosphorylation process in the regulation of replication and transcription in eukaryotic cells. Here, we report the effect of phosphorylation by an endogeneous protein kinase of a DNA polymerase fraction from the soluble cytoplasmic fraction of chick embryo. The two enzymatic fractions (DNA polymerase and protein kinase) were pre-isolated by affinity chromatography on DNA-agarose and ion-exchange chromatography on DEAE–Sephacel.

2. Materials and methods

2.1. *Materials*

DNase I from beef pancreas, bovine serum albumin (BSA) and Triton X-100 were obtained from Sigma. Sepharose-2B and DEAE–Sephacel were from Pharmacia. DNA was prepared by a method derived from [2] from solid plasma cell tumours RPC_5 transplantable on Balb/c mice. [^{3}H]TTP (53 Ci/mM) and [γ-^{32}P]ATP (2400 Ci/mM) were from New England Nuclear and deoxyribonucleoside triphosphates from Boehringer. Casein was purchased from Difco and all other products from Merck.

2.2. *Buffer*

The extraction buffer was 50 mM Tris–HCl (pH 8), 5 mM Mg-acetate, 10 mM KCl, 1 mM EDTA, 12 mM 2-mercaptoethanol and 250 mM sucrose (buffer A). Column buffer was buffer A adjusted to 20% glycerol and without sucrose (buffer B). Buffer C was 35 mM Tris–HCl (pH 7.6), 10 mM Mg-acetate and 10 mM 2-mercaptoethanol.

2.3. *Enzyme extraction*

Eleven-day-old chick embryos were used. After removal of eyes and evisceration, embryos were rinsed in cold (4°C) buffer A, then minced in 3 vol. same buffer. All operations were then performed at 4°C. Embryos were ground in a Potter-Elvehjem motor driven homogenizer (clearance 50–100 μm) by 10 strokes at 1000 rev./min and centrifuged for 20 min at 20 000 × *g*. The cytoplasmic soluble fraction (S200) was obtained by centrifugation for 2 h at 200 000 × *g* of the pooled previous supernatants.

2.4. *DNA-agarose*

The DNA covalently bound to agarose (Sepharose-2B) used for affinity chromatography was extracted from RPC_5 tumor [3]. DNA in 40 mM MES (pH 6), 80% formamide buffer at 3 mg/ml was first sonicated for 10 s at 0°C (MSE ultrasonic power unit, 20 Kc/s), then heated at 95°C for 10 min, followed by immediate cooling to 0°C. In such conditions, DNA was converted to single strands 500 nucleotides long [4]. Such DNA is then coupled to CNBr-activated agarose as in [3]. The gel used in the chromatographic experiments contained 50 μg of DNA/ml gel.

2.5. *DNA polymerase assay*

RPC_5 DNA activated with DNase I [5] was used as template. Polymerisation activity was routinely mea-

sured at 37°C for 1 h in 75 μl total vol. medium containing: 30 mM Tris–HCl (pH 8), 6.5 mM 2-mercaptoethanol, 3.7 mM Mg-acetate, 0.5 mM EDTA, 30 mM KCl, 37.5 μg BSA, 1% Triton X-100, 0.08 mM dATP, dCTP and dGTP each, 0.03 mM [^{3}H]TTP (0.1 μCi/mmol) and 30 μg activated DNA. The reaction was stopped by transferring the entire reaction mixture onto 2 × 3 cm pieces of Whatman 3 MM filter paper and immerging them in cold 10% trichloracetic acid as in [6,7]. Radioactivity was determined in an Intertechnique SL 3100 Scintillation Spectrometer.

2.6. *Protein kinase assay*

Protein kinase activity was determined by the incorporation of ^{32}P from [γ-^{32}P]ATP in casein as a protein substrate [8]. The incubation mixture contained, in 75 μl total vol., 35 mM Tris–HCl (pH 7.6), 10 mM Mg-acetate, 10 mM 2-mercaptoethanol, 0.15 mM [^{32}P]ATP (spec. act. 50 000 cpm/nmol), 60 μg casein and 30 μl column fractions. Samples were incubated for 30 min at 35°C and adsorbed onto Whatman 3 MM filters which were immerged into cold 10% trichloracetic acid then treated as in [8].

2.7. *Protein determination*

Determination of the protein concentration was done as in [9] using a solution of BSA of known concentration as standard.

3. Results

3.1. *Separation of DNA polymerase and protein kinase activities*

S200 (200 ml) were adjusted to 20% glycerol before being adsorbed onto a DNA–agarose column equilibrated with buffer B. The column was washed with buffer B, then eluted with a linear gradient from 0–400 mM NaCl in the same buffer (fig.1A). DNA polymerase activity elutes at 100 mM NaCl and protein kinase activity partly at 250 mM and partly with the DNA polymerase. Fractions containing DNA polymerase activity were pooled and diluted with buffer B to 60 mM NaCl final conc., then absorbed onto a DEAE–Sephacel column equilibrated with buffer B (fig.1B). The column was washed, and eluted with a linear gradient from 60–400 mM KCl in the same

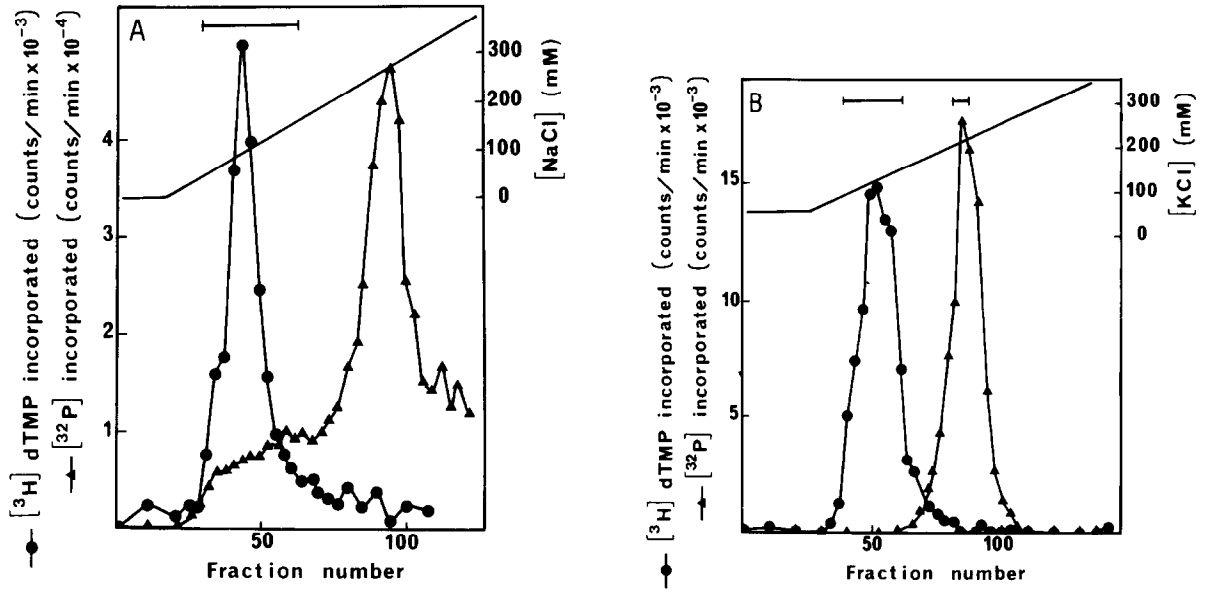

Fig.1. Chromatography of soluble cytoplasmic extract. (A) DNA–agarose chromatography: 200 ml S200 (4.7 mg/ml) were adjusted to 20% glycerol and loaded onto a DNA–agarose (Sepharose-2B) column (2.5 × 24 cm), equilibrated with buffer B. The column was washed with 400 ml buffer B and eluted with a 1l linear gradient from 0–400 mM NaCl in the same buffer. Fractions (8.5 ml) were collected and aliquots (30 μl) were analyzed for enzymatic activities as in section 2. Fractions were pooled as shown (|——|). (B) DEAE–Sepharose chromatography: pooled fractions from the DNA–agarose column (270 ml, 0.1 mg/ml) were diluted with buffer B to a 60 mM NaCl final conc. then loaded onto a DEAE–Sephacel column (1.6 × 35 cm) equilibrated with buffer B. The column was washed with 100 ml buffer B adjusted to 60 mM NaCl, then eluted with 450 ml linear gradient from 60–400 mM KCl in the same buffer. Fractions of 3.3 ml were collected and enzymatic activities determined as for fig.1A. Fractions were pooled as shown (|——|).

buffer. With this second chromatography column, it was possible to separate the two different enzymatic fractions: DNA polymerase and protein kinase activities eluted at 130 mM and 225 mM KCl, respectively.

3.2. *Effect of phosphorylation on DNA polymerase activity*

To observe the possible effect of phosphorylation on the modification of the DNA polymerase activity, the following experiment was performed in two steps after dialysis of the enzymatic fractions against buffer C.

(i) To favor phosphorylation of the DNA polymerase fractions used as substrates, aliquots were incubated for 30 min at 35°C with the DEAE-eluted protein kinase in the presence of 1.15 mM ATP and of the phosphorylation buffer C. These conditions of medium and temperature are more favourable to phosphorylation than the DNA polymerase assay conditions.

(ii) These samples were adjusted to conditions allowing DNA synthesis by addition of the DNA template, the deoxyribonucleoside triphosphates and the appropriate buffer, and incubated 1 h at 37°C.

Table 1 shows that the modification of the DNA polymerase fraction by the protein kinase and ATP notably increases (~2-times) the amount of polynucleotide synthesized, as compared with the assay without protein kinase. In the control experiments, ATP does not influence the DNA polymerase reaction nor the protein kinase without its phosphate donor.

Table 1
Effect of phosphorylation on DNA polymerase activity

Phosphorylation of DNA polymerase			Resulting DNA polymerase activity	
DNA polymerase	Protein kinase	ATP	[^{3}H] cpm incorp.	%
+	–	–	6275	100
+	–	+	6410	102
+	+	–	5925	94
+	+	+	13 435	215

DNA polymerase and protein kinase fractions from DEAE–Sephacel were first dialysed against buffer C. Aliquots of the DNA polymerase fraction (30 μl containing 4 μg protein) were then preincubated 30 min at 35°C with buffer C, with or without ATP (1.15 mM), with or without an aliquot of the protein kinase fraction (30 μl containing 5 μg protein). Thereafter the preincubated mixtures were adjusted to the conditions for the DNA polymerase assay by addition of the DNA template, the deoxyribonucleoside triphosphates and the appropriate buffer and incubated 1 h at 37°C. Resulting DNA polymerase activity was measured as in section 2

Table 2
Substrate specificity of the protein kinase

Substrate	^{32}P cpm incorp.
	μg protein
Casein	335
Histones	4
DNA polymerase fraction	613

Phosphorylation of substrates was conducted as in section 2 with modification of the incubation volume (150 μl). Casein (150 μg) total calf thymus histones (150 μg) and the DNA polymerase fraction (9 μg) were used as phosphorylable substrates

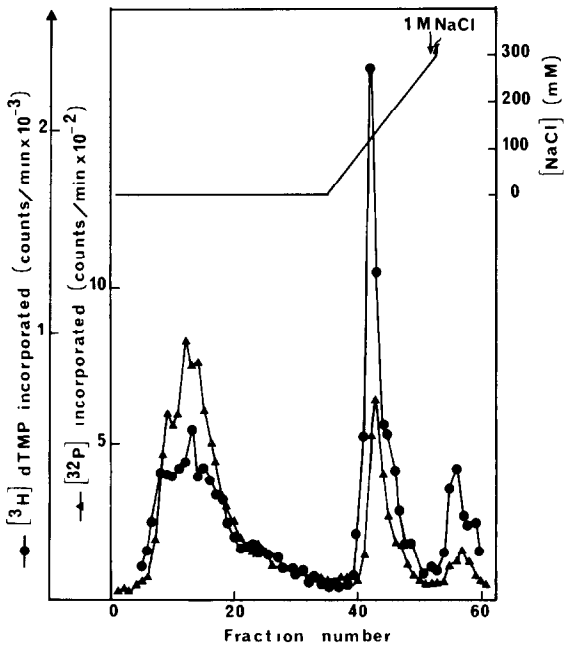

Fig.2. Chromatography of phosphorylated soluble cytoplasmic extract. Phosphorylated S200 (1 ml) was loaded onto a DNA–agarose column (1 × 4.5 cm) equilibrated in buffer B. The column was extensively washed with 70 ml buffer B and eluted with a 40 ml linear gradient from 0–300 mM NaCl followed by a step at 1 M NaCl (→). Fractions (2 ml) were collected and aliquots (60 μl) were analyzed for the DNA polymerase activity as in section 2. ^{32}P radioactivity was counted in 100 μl aliquots.

3.3. *Phosphate fixation on the DNA polymerase fraction*

To check the protein kinase action on the DNA polymerase fraction we performed a protein kinase test with [γ-^{32}P]ATP (table 2). We observed a higher fixation of ^{32}P-label on the DNA polymerase fraction than on casein or on total calf thymus histones as substrates. As a control experiment, we have favored the endogeneous phosphorylation of a S200 extract by addition of [γ-^{32}P]ATP as in section 2. The phosphorylated extract is then chromatographed on a DNA–agarose column as shown in fig.2. The phosphorylated protein peak comigrates with the DNA polymerase activity. These results show a specificity of the protein kinase for its endogeneous substrate. In addition, cyclic AMP does not increase the rate of phosphorylation of the substrates (not shown).

4. Discussion

When the soluble cytoplasmic fraction from chick embryo is chromatographed on a DNA–agarose affinity column, two enzymatic activities are evidenced (fig.1A): a DNA-dependent DNA polymerase and a cyclic AMP-independent protein kinase. However, the protein kinase activity partly coelutes with the DNA polymerase fraction. When the DNA polymerase peak from the DNA–agarose column is rechromatographed on DEAE–Sephacel, an almost complete separation of the enzyme is observed (fig.1B). Other results [10] and the data in [11–16] suggest that the DNA polymerase is of the α-polymerase type. For example, it is sensitive to *N*-ethylmaleimide 1 mM, it binds weakly to DNA–agarose (eluted by 100 mM NaCl), it is inhibited by NaCl or KCl up to 100 mM, it is an acidic protein of isoelectric point 6.1 and it has M_r 100 000.

Since a protein kinase activity was found to be associated with DNA and/or a DNA polymerase enzyme (fig.1A), we have searched if this protein kinase could play a regulatory role towards the DNA polymerase. The protein kinase eluted at 250 mM NaCl from the DNA–agarose column has little or no influence on the DNA polymerase activity (not shown). However, as indicated in table 2, the protein kinase removed from the DNA polymerase on the DEAE–Sephacel column shows a stimulatory effect on the DNA polymerase. This led us suggest that this protein kinase is part of a so-called replication complex [17] that would have a function in the DNA replication. Table 2 led us to suggest the following hypotheses. The increase of the DNA polymerase activity could be the result of:

(i) Either a direct phosphorylation of some specific sites of the enzyme, as suggested in Rous sarcoma virus [18];

(ii) Or a phosphorylation mechanism of an effector: the enzyme could normally be regulated by a factor which would lose its inhibitory effect when phosphorylated, in such a case this factor would be associated with the polymerase fraction.

The enzyme could be regulated by a stimulatory factor associated with the DNA polymerase as found in the case of the avian myeloblastosis virus [19] or with the protein kinase fraction, the factor would have a stimulatory effect only when phosphorylated. Other authors [20] found that the phosphorylation of a DNA-binding protein in mouse cells suppresses its stimulatory effect towards the DNA polymerase α.

The presence of a DNA-dependent ATPase as stimulatory factor in the protein kinase fraction is improbable since it has been shown that when chromatographed on single-stranded DNA or double-stranded DNA–cellulose, the DNA-dependent ATPase from rat liver was excluded at a high salt concentration: 0.7 M (M. Duguet and A. M. de Recondo, personal communication). This activity from prokaryotes is also excluded from DNA-cellulose [21,22] or from DNA–agarose [23,24] at a high salt concentration.

More work is needed in order to verify these hypotheses and identify the possible regulatory factor/mechanism of DNA polymerase enzyme.

Acknowledgements

We thank Mrs N. Pfleger and M. L. Hoffmann for their excellent technical assistance.

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