NUCLEAR PROTEIN FROM CULTURED HEPATOMA CELLS PREFERENTIALLY INHIBITS COPYING OF DENATURED DNA BY ISOLATED CHROMATIN

A. KAFTORY, P. WEISMAN-SHOMER and M. FRY
Unit of Biochemistry, Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel

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1. Introduction

DNA synthesis is conducted in vitro by isolated chromatin from a variety of eukaryotic cells [1–5]. Unlike intact cells or isolated nuclei, the chromatin system is free of permeability barriers which restrict passage of macromolecules to the site of DNA replication. We have thus been able to show that the chromatin-associated replicative system copies exogenous DNA templates at a high efficiency [5]. Of particular interest is the observation that linear DNA, which serves as a poor template for solubilized DNA polymerases is replicated at a high rate by chromatin-associated DNA polymerase α [5]. We describe here a nuclear protein, isolated from cultured rat hepatoma cells which preferentially inhibits copying of single-stranded DNA by chromatin.

2. Materials and methods

2.1. *Cells*

Randomly growing rat hepatoma cultured (HTC) cells were cultivated in suspension in Swim's S-77 medium which contained 10% new-born calf serum [6].

2.2. *Preparation of chromatin and of soluble nuclear proteins*

Chromatin was isolated from washed nuclei [7], as in [4]. The ratio of DNA to protein in the various chromatin preparations was 0.07–0.1. Crude extracts of nuclear proteins were prepared as in [8]. These extracts served as source for soluble DNA polymerase activity which was 90–95% inactivated in the presence of 2 mM *N*-ethyl maleimide, thus representing mostly DNA polymerase α and perhaps γ polymerase activity [9]. A protein which inhibits copying of exogenous DNA templates by chromatin-associated polymerases was partially purified and freed of DNA polymerases by heating the crude nuclear extract at 75°C for 10 min. Denatured proteins were removed by centrifugation at 90 000 × *g* for 30 min and the clear supernatant served as source of inhibitory activity. When stored at –60°C, the inhibitor remained active for at least 2 months.

2.3. *Assay for DNA synthesis by chromatin*

Conditions for maximum chromatin-directed DNA synthesis were as in [5]. Specific activity of the labeled substrate d[^{3}H]TTP was 400 cpm/pmol. Chromatin was added at 8 μg as DNA/assay and exogenous templates were added to saturation at 20 μg/assay. Incorporation d[^{3}H]TMP into acid insoluble material served as measure of DNA synthesis.

2.4. *Other methods*

Amount of protein was determined by the Lowry method [10]. ATP concentration was estimated by the luciferin–luciferase assay adapted to liquid scintillation spectrometer [11]. Chromatin DNA was determined as in [12]. The heat-treated nuclear extract was filtered on 1.5 × 70 cm column of Sephadex G-200 as in [8].

3. Results and discussion

Crude extract of nuclei of HTC cells contain activity which suppresses the in vitro copying of

single-stranded DNA by isolated chromatin. The inhibitory activity is hard to detect in the crude preparation because its effect is masked by that of DNA polymerase which strongly stimulates DNA synthesis in the chromatin system [4]. When heated at 75°C for 10 min, DNA polymerases denature together with 80% of the nuclear proteins and are totally inactivated. By contrast, the inhibitory activity resists the heat treatment and its suppressive effect becomes measurable. Hence, heating the extract and disposing of the denatured proteins (see section 2), expose the inhibitory activity and purify it 5-fold. Figure 1A illustrates the effect of the heated extract on copying of denatured DNA by isolated chromatin. Clearly, replication becomes progressively impeded as increasing amounts of extract are added to the reaction mixture. The inhibitory effect is specific to the nuclear extract and addition of protein such as bovine serum albumin stimulates, rather than hinders DNA synthesis. Note however, that 20–30% of the replicative activity remain resistant to inhibition even in the presence of excess extract.

Preliminary characterization of the inhibitor indicates that it is a protein. Pronase digestion, followed by heat inactivation of the proteolytic enzyme as well as boiling of the inhibitory factor, completely destroy its activity. Full activity is preserved inside a dialysis bag when the extract is dialysed overnight against phosphate buffer (pH 7.5). The macromolecular nature of the inhibitor is evident when the extract is filtered on Sephadex G-200 gels: The activity is eluted as two peaks of approximate molecular sizes 300 000 and 320 000.

Inhibition of DNA synthesis could be trivially explained if the extract contained enzyme(s) which destroy chromatin itself or the template, substrates or product of the DNA synthesis reaction. Copying of denatured DNA by chromatin proceeds at a linear rate for extended periods of time [5]. If addition of extract leads to depletion of an essential component from the system, linearity of the reaction should be interrupted. Figure 1B shows, however, that although DNA synthesis is retarded in the presence of extract, it still advances linearly as in its absence. The follow-

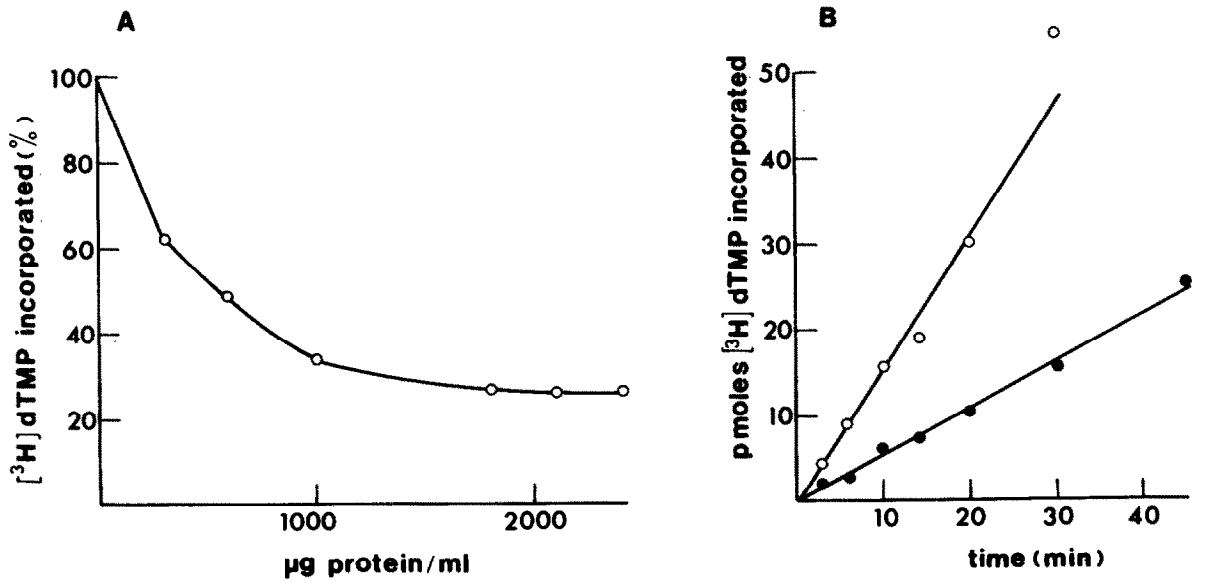

Fig.1. Effect of heated nuclear extract on the copying of denatured DNA by isolated HTC cell chromatin. (A) Titration of the extract. Copying of denatured DNA was conducted for 30 min under standard conditions in the presence of different amounts of heat-treated extract. Incorporation of 100% represents 57.5 pmol acid insoluble d[^{3}H]TMP. (B) Kinetics of extract-inhibited DNA synthesis. Replication of denatured DNA was carried out without (○——○), or in the presence (●——●) of 1500 µg/ml heat-treated extract.

ing series of experiments directly demonstrate that the extract indeed does not deplete the system of any of its essential components. If present in the extract, deoxyribonuclease could decrease net synthesis of DNA by digesting either the denatured DNA template or the reaction product. That the isotopically labeled DNA product of the chromatin-directed reaction is stable in the presence of extract was shown as follows: Copying of denatured DNA was conducted for 30 min and then ATPase, which completely stops DNA synthesis [5], was added with or without heated extract. Fate of the labeled DNA product was followed at various times thereafter. Results shown in fig.2A clearly demonstrate that the labeled DNA remained undegraded in the presence of extract. Parallel experiments showed that the extract did not affect the stability of the DNA template either. Denatured DNA incubated with extract for 30 min and then boiled to destroy the inhibitor, was copied by chromatin as efficiently as DNA not treated with extract (not shown). Reciprocally, addition of excess single-stranded DNA to the reaction mixture during DNA synthesis did not stimulate the reaction (fig.2B). Nuclear extract could also hinder DNA synthesis if it contained activity which degrades deoxyribonucleoside triphosphates (dNTPs). However, neither pre-incubation of all 4 dNTPs with the extract prior to DNA synthesis (not shown) nor addition of excess substrates during the reaction (fig.2B), altered the rate of the inhibited reaction. Arrest of DNA synthesis is also not due to inactivation of the chromatin enzymic system by the nuclear extract: identical retarded rates of DNA synthesis were observed when extract was added to chromatin 60 min prior to addition of dNTPs and outset of DNA synthesis or at time zero of the reaction. Since supply of ATP is essential for chromatin-directed DNA synthesis [4,5], it could also be that inhibition is due to hydrolysis of ATP by the extract. Measurement of ATP levels in reaction mixtures which contained extract, however, failed to demonstrate any decrease in the ATP content. In light of all the above data it should be concluded that the nuclear inhibitor

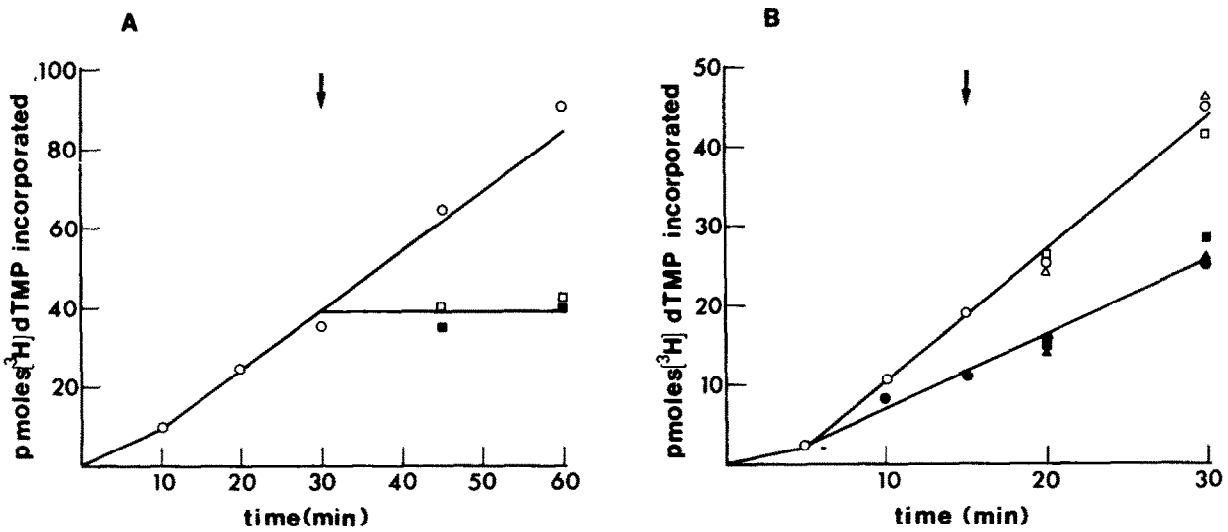

Fig.2.Absence of degradative activities in the heated nuclear extract. (A) Fate of DNA synthesized by chromatin in the presence of extract. Denatured DNA was copied by isolated chromatin under standard conditions. After 30 min (arrow), potato ATPase (100 μg/assay) was added without (□——□), or with (■——■) 1200 μg/ml heat-treated extract. Control samples remained unexposed to either enzyme or extract (○——○). Acid-insoluble radioactivity was determined in sample assays after further incubation. (B) Effect of introduction of excess linear DNA or dNTPs on the rate of DNA synthesis. Replication of linear DNA by chromatin was conducted for 15 min in the absence (○——○) or presence (●——●) of heated extract. At 15 min (arrow), either 20 μg/assay denatured DNA (□——□; ■——■) or 0.1 mM of each dNTP (△——△; ▲——▲) were added to samples devoid of or containing extract, respectively. Control samples remained unexposed to DNA and dNTPs.

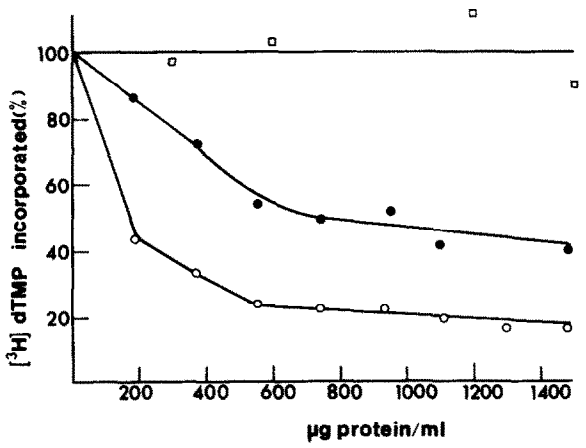

Fig.3. Effect of heated nuclear extract on the copying of different DNA templates by chromatin. Copying of denatured (○——○), activated (●——●) and native DNA (□——□) was conducted for 30 min under standard conditions. Varying concentrations of heat-treated nuclear extract were present in the assay samples. Incorporation of 100% represents 57.5, 190.0 and 4.0 pmol acid insoluble d[^{3}H]TMP determined in the presence of denatured, activated and native templates, respectively.

directly impedes DNA synthesis rather than exerts its effect by depleting the reaction of an essential component.

We have next compared the relative efficiency with which heated extract hinders copying of different DNA templates by isolated chromatin. Results presented in fig.3 show that replication of denatured DNA is arrested by extract to the highest extent. Copying of this template is affected to a larger degree than replication of activated DNA in the presence of 150–500 µg/ml extract. Also, maximum inhibition attained is higher for denatured DNA. Moreover, copying of native DNA is totally unaffected by the inhibitor and endogenous DNA synthesis is slightly stimulated in the presence of heated extract, (not shown). Although the degree of inhibition varied in different nuclear preparations (c.f. fig.1A,3), the relative inhibition exerted on copying of various DNA templates was as detailed for all preparations tested.

Last, we have studied the effect of heated extract on synthesis of DNA by soluble, mostly α-type, DNA polymerases. Table 1 shows that extract hinders the copying of activated and denatured DNA by nuclear DNA polymerases to a similar limited extent. By contrast, copying of denatured DNA by chromatin is impeded to a larger extent than replication of activated DNA and both reactions are retarded to a higher degree in chromatin than in the soluble system. The higher efficiency of inhibition of chromatin-directed copying of denatured DNA might be explained if in addition to its demonstrable effect on α polymerase, the inhibitor interacted with other chromatin-associated components which participate in replication. Such a factor could be the recently described protein which specifically stimulates copying of denatured DNA by α polymerase [13–15]. Another possibility is that the heated extract contains two separate inhibitory activities, one that interacts with DNA polymerase and the other with other chromatin-associated factors.

The apparent effect of the nuclear inhibitor on DNA replication illustrates the advantage in using isolated chromatin for the assay of replication-regulating macromolecules. Copying of exogenous templates by chromatin is related to the cell cycle

Table 1
Inhibition by nuclear extract of DNA synthesis directed by chromatin and soluble polymerases

Polymerizing system	DNA template	d[^{3}H]TMP incorporated (pmol/15 min)		Inhibition (%)
		– heated extract	+ heated[a] extract	
Chromatin	denatured	28.4	8.4	70.6
	activated	100.4	44.6	55.6
Soluble polymerases	denatured	9.9	5.7	42.4
	activated	70.6	39.5	44.0

[a] Heated extract was added at 1250 µg/ml. At this concentration, DNA synthesis was maximally inhibited in both polymerizing systems

distribution of the cells of origin [5]. The possibility that the inhibitory activity described herein participates in the cell cycle-regulated DNA replication is now under investigation.

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