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DETECTION OF A DNA POLYMERASE β STIMULATING PROTEIN IN HELA CELL NUCLEAR EXTRACTS

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SUMMARY:

HeLa cell nuclei contain a protein which stimulates the in vitro activity of HeLa cell DNA polymerase β, but does not affect the activity of DNA polymerase α and γ. The protein, which binds to both single- and double-stranded DNA, does not possess nuclease activity and is heat stable, surviving 100 degrees C for 10 min. The molecular weight of the protein is approximately 85,000 and evidence is presented that it may exert its stimulatory effect by direct interaction with β-polymerase.

INTRODUCTION:

It is evident that DNA replication in prokaryotic systems is a complex process requiring not only DNA polymerases but other accessory proteins as well. Among these accessory proteins are initiation and elongation factors (1, 2, 3, 4), DNA unwinding (DNA binding) proteins (5, 6) and other proteins acting on DNA (7, 8, 9, 10, 11). Proteins with DNA unwinding properties have been isolated from eukaryotic cells such as calf thymus (12, 13, 14) and mouse ascites cells (15), and have been shown to stimulate the in vitro activity of their homologous DNA polymerase α (14, 15). We report here the detection of a DNA binding protein in extracts of HeLa cell nuclei which specifically stimulates HeLa cell DNA polymerase β. It appears to differ significantly from two β-polymerase stimulating proteins recently described from rat cells (16) and sea urchin nuclei (17), and from other known eukaryotic DNA unwinding proteins.

MATERIALS AND METHODS:

Growth of Cells.

Monolayer cultures of HeLa F cells (Flow Laboratories) were grown in roller bottles in F-11 medium (Gibco, Grand Island, New York) supplemented with 10% fetal calf serum, 4 mM glutamine 50 units per ml of penicillin and 50 μg per ml of streptomycin.

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Isolation of Nuclei and Salt Extraction.

Cells were harvested with scraping into phosphate buffered saline (PBS). They were then swollen in reticulocyte standard buffer (RSB - 0.01 M NaCl, 0.01 M Tris buffer, pH 7.5, 0.0015 M $MgCl_2$, 0.003 M EDTA) for 20 min. on ice, and broken with a dounce homogenizer. Nuclei were separated from cytoplasm by centrifugation through a cushion of 25% sucrose in RSB at 3500 rpm for 30 min. Pelleted nuclei were washed several times in RSB and then extracted with 2.0 M NaCl - 0.001 M Tris (pH 7.5) by dounce homogenization. The extract was centrifuged at 130,000 X g for 15 h to pellet cell debris and DNA, and the supernatant was dialyzed against 0.1 M Tris (pH 7.5) and then fractionated by DNA cellulose chromatography.

DNA Cellulose Chromatography.

The nuclear extract (35 ml, 10 mg protein) was adsorbed to a column of native DNA cellulose (10 ml bed volume containing 10 mg DNA) previously equilibrated with 0.1 M NaCl - 0.01 M Tris (pH 7.5). Following a five column volume wash with the equilibration buffer, elution was carried out with a stepwise salt gradient of three column volumes each of 0.25 M, 0.5 M, 0.75 M, 1.0 M, 1.5 M, and 2.0 M NaCl in 0.01 M Tris (pH 7.5).

DNA Binding Protein Assay.

DNA cellulose fractions (2.0 ml) were assayed for DNA binding protein activity by the method of Tsai and Green (18), utilizing sheared, denatured [^{3}H] E. coli DNA. Peaks containing DNA binding activity were dialyzed against 0.01 M Tris (pH 7.5), and made 30% with respect to glycerol, 1.0 mg/ml bovine serum albumin (BSA) and 0.5 mM dithiothreitol (DTT).

DNA Polymerase and Stimulatory Activity Assays.

The reaction mixtures for α, β, γ, and HSV-induced DNA polymerases have been previously described (19, 20). Assays were carried out in a final volume of 100 µl, containing 3.0 µg/ml of template $(dG)_{12-18}$·poly (dC), or $(dT)_{17}$·poly (dA), or activated salmon sperm DNA, or activated HeLa DNA or exonuclease III activated HeLa DNA and [^{3}H] labeled deoxyribonucleoside triphosphate with a specific activity of 300 or 390 cpm per picomole. Incubations were for 30 min at 37 degrees C, and the incorporation of [^{3}H] labeled deoxynucleoside triphosphate into a trichloroacetic acid insoluble product was measured by collection onto glass fiber discs (GF/C, Whatman Ltd., England), which were counted in LSC (Yorktown, Hackensack, N.J.) in a Beckman liquid scintillation counter. A unit of DNA polymerase is defined as the amount of enzyme catalyzing the incorporation of 1 nanomole of deoxynucleotide in 60 min at 37 degrees C with activated salmon sperm DNA as a template. To assay for stimulatory activity, aliquots of DNA binding proteins were added to the standard polymerase assay mixtures. A unit of stimulatory activity is defined as the amount which causes a five-fold stimulation of the in vitro activity of a DNA polymerase, in 30 min at 37 degrees C.

RESULTS:

HeLa cell nuclei were extracted with 2.0 M NaCl, and the extract was dialyzed and chromatographed on a double-stranded calf thymus DNA cellulose column. As shown in Figure 1, the DNA binding protein peak eluting at 1.5 M NaCl contained an activity which specifically stimulated the in vitro activity of HeLa cell DNA polymerase β, measured under the optimum in vitro conditions for the enzyme. At the same levels of protein which stimulated

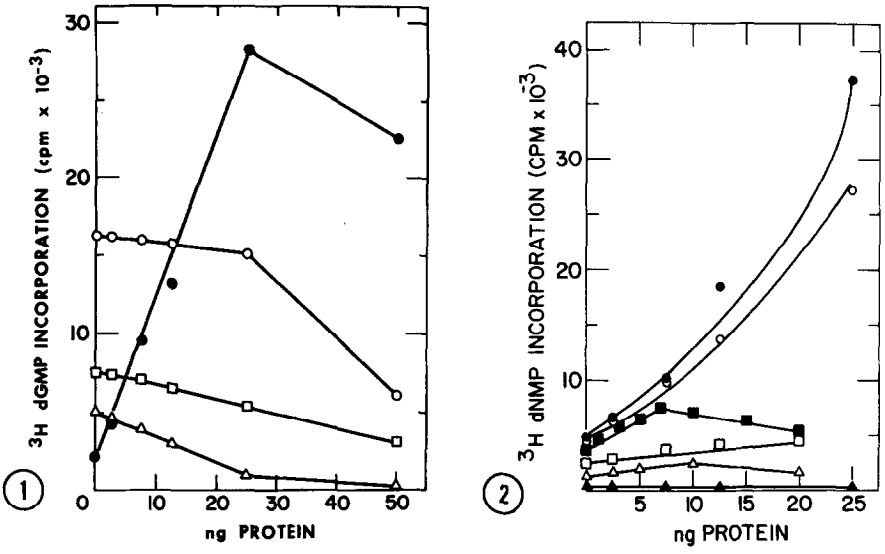

Figure 1. Stimulation of DNA polymerase β by the DNA binding protein. Aliquots of the peak material eluted from DNA cellulose at 1.5 M NaCl were added to DNA polymerase assay mixtures containing 0.3 μg $(dG)_{12-18}$·poly(dC), and either 0.4 units of DNA polymerase β (●——●), 0.4 units of DNA polymerase α (□——□), 0.08 units of DNA polymerase γ (○——○) or 0.4 units of the Herpes Simplex Virus-induced DNA polymerase (△——△), under the optimum conditions for each enzyme. After incubation for 30 min at 37 degrees C in the incorporation of [^{3}H]dGTP (specific activity 300 cpm per picomole) into acid precipitable material was measured.

Figure 2. Stimulation of DNA polymerase β with various templates. DNA polymerase β assays were carried out using 0.3 μg per assay of $(dG)_{12-18}$·poly(dC) (●——●), $(dT)_{17}$·poly(dA) (○——○), exonuclease III treated (to 25% acid solubility) HeLa DNA (■——■), pancreatic DNase I "activated" salmon sperm DNA (△——△), pancreatic DNase "activated" HeLa DNA (□——□), or single-stranded or double-stranded HeLa DNA (▲——▲). Various amounts of the 1.5 M NaCl DNA cellulose peak and 0.4 units of DNA polymerase β were added to each assay. [^{3}H]TTP (specific activity 390 cpm per picomole) in a mixture containing all four deoxyribonuclease triphosphates was substituted for [^{3}H]GTP where appropriate. The incorporation of [^{3}H] deoxyribonucleotides (dNMP) into an acid insoluble form was measured.

the β -polymerase, the activities of the α, γ and Herpes Simplex Virus-induced DNA polymerases were slightly inhibited. Stimulations could be achieved utilizing either the synthetic DNA templates $(dG)_{12-18}$·poly (dC) or $(dT)_{17}$·poly (dA), or with "activated" salmon sperm or HeLa DNA, but not with single-stranded or double-stranded native HeLa DNA, as is shown in Figure 2.

Further purification of the β-polymerase stimulating activity was carried out employing phosphocellulose and DEAE-cellulose chromatography. Although the activity did not bind to either ion-exchange resin, at pH 7.5, and in an 0.02 M KPO_4 ionic strength buffer, significant amounts of extraneous protein were removed by these procedures, resulting in an estimated final 4000-fold purification.

The partially purified stimulating activity is stable at 100 degrees C for 10 min, with little or no loss in stimulatory activity. The stimulating activity is, however, completely destroyed by trypsin pretreatment, suggesting that it is a protein. In our most purified preparations, no nuclease activity could be detected, utilizing either [^{3}H] thymidine labeled single-stranded or double-stranded E. coli DNA as the substrate. In these tests, which can easily detect as little as 1.0 ng of either micrococcal nuclease or pancreatic DNase I, 50 ng of the purified stimulatory protein showed no nuclease activity. The specificity of the stimulating protein for the β-polymerase would also tend to rule out possible nuclease involvement.

From both sedimentation analysis in glycerol gradients, and SDS gel electrophoresis, the molecular weight of the stimulating protein was estimated to be 85,000.

DNA unwinding proteins, which also stimulate the in vitro activities of homologous DNA polymerases, bind selectively to single-stranded DNA (5, 6, 12). To further determine the DNA binding characteristics of the DNA polymerase β stimulating protein, it was preincubated at 37 degrees C for 10 min with either [^{3}H] thymidine labeled single-stranded or double-stranded HeLa DNA, then sedimented through 20% glycerol at 45,000 rpm. The DNA polymerase β stimulating protein cosedimented with both single-stranded and double-stranded DNA (data not shown). Unwinding proteins, due to their selective affinity for single-stranded DNA, facilitate the denaturation of double-stranded DNA and thus lower their thermal melting temperatures (5, 6, 13). We have attempted to determine if the β-polymerase stimulating protein

also lowers the melting point of DNA, in this case the synthetic copolymer poly(dA)·poly(dT). Aliquots of the purified stimulating protein were incubated at various protein:DNA ratios with poly(dA)·poly(dT) and the melting of the duplex was monitored by measuring the absorbance at 260 nm while the temperature was slowly increased (10 degrees C per h). The E. coli unwinding protein, (a gift from Dr. Jerard Hurwitz) at a protein to DNA ratio of 10:1, was included as a positive control (21). The β-polymerase stimulating protein had no effect on the melting temperature of poly(dA)·poly(dT), at protein to synthetic DNA ratios of 1:1, 1:5, or 1:30 (not shown). In contrast, a control with the E. coli unwinding protein depressed the melting temperature by approximately 10 degrees C. Because of lack of material, we were unable to use higher ratios of β-stimulating protein to synthetic DNA than 1:1.

To determine if a direct interaction between the stimulatory protein and the β-polymerase occurred, we determined whether the stimulating protein could confer heat stability to the β-polymerase. Aliquots of β-polymerase were incubated at 50 degrees C in the presence or absence of the stimulating protein in a final concentration of 0.5 mg/ml of BSA. At various times the residual β-polymerase activity was determined, and the results are plotted in Figure 3. By four min, in the absence of stimulating protein, 90% of the β-polymerase had been inactivated at 50 degrees C. In contrast, the presence of the stimulating protein, less than 10% inactivation had occurred. We also determined whether the addition of template $(dG)_{12-18}$·poly(dC) to the β-polymerase stimulating protein mixture would confer additional heat stability to the enzyme, i.e., if a ternary complex might be formed between the enzyme, the stimulating protein and the template. While additional heat stability was conferred, it did not exceed the additive effects of the template and the β-polymerase stimulating protein when added alone.

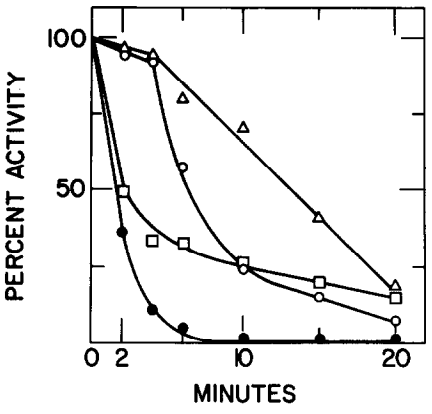

Figure 3. Effect of the β-polymerase stimulating protein on the heat inactivation of DNA polymerase β. Aliquots of DNA polymerase β (1.6 units, 228 ng protein) were incubated with (O———O) or without (●———●) 20 ng of β-polymerase stimulating protein, or with 0.1 μg $(dG)_{12-18}$·poly(dC) (□———□), or with 20 ng of the stimulating protein plus 0.1 μg $(dG)_{12-18}$·poly(dC) (Δ———Δ) in a total volume of 60 μl. Incubation was at 50 degrees C and 30 μl samples were removed and measured for residual β-polymerase activity at the indicated times. BSA was included in each mixture at a final concentration of 0.5 mg/ml.

DISCUSSION:

We have isolated a HeLa cell nuclear protein which specifically stimulates the *in vitro* activity of the HeLa DNA polymerase β. Although not conclusive, several lines of evidence indicate that the protein exerts part of its stimulatory effect by interacting with the β-polymerase. First, the stimulating protein is specific only for the β-polymerase. Second, it does not recognize specific base sequences, since stimulation is achieved with almost equal proficiency when $(dT)_{17}$·poly(dA) or $(dG)_{12-18}$·poly(dC) is used as a template. Although "activated" native DNA templates show less stimulation than synthetic templates, they also allow the stimulatory effect of the protein on the β-polymerase to be expressed. Third, unlike unwinding proteins, the stimulating protein does not bind specifically to single-stranded DNA, and it does not promote the denaturation of a synthetic duplex DNA at levels up to 1:1 (protein/DNA). We assume this to indicate the β-polymerase stimulatory protein is not an unwinding protein and its action

is therefore different from the E. coli or calf thymus unwinding proteins (13, 14, 21, 22). Finally, evidence for a direct interaction between the stimulating protein and β-polymerase would appear to be indicated by its protection of the β-polymerase from heat inactivation.

The in vivo function of the β-polymerase stimulating protein remains obscure, as does its relationship to two recently identified β-polymerase stimulating proteins from rat cells and sea urchin nuclei (16, 17). All three stimulating proteins appear to have significantly different characteristics, including their molecular weights, their binding to and elution from DNA cellulose, their heat stabilities and their cofactor requirements. This may reflect the fact that they have been isolated from three widely separated eukaryotic species, or they may be three distinct entities carrying out separate functions which relate to the function of the β-polymerase. The HeLa stimulating protein may in itself be a cofactor which works in concert with the enzyme, and may participate in vivo in whatever DNA replication or repair synthesis is catalyzed by the β-polymerase.

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