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A PROTEIN STIMULATORY FACTOR FOR DNA POLYMERASE α IN RAT GIANT TROPHOBLAST CELLS[1]

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SUMMARY - Rat giant trophoblast cells contain a factor which stimulates DNA polymerase α derived from trophoblast cells as well as from calf thymus. The factor, which has been partially purified on a glycerol gradient, has an approximate M_r of 85,000 and is non-dialyzable, heat-labile, sensitive to trypsin and insensitive to N-ethylmaleimide. With activated DNA as the preferred template/primer, the factor stimulates both the initial rate and the extent of DNA synthesis as a linear function of the concentration of the factor. The stimulation is abolished by aphidicolin but is unaffected by 2', 3' dideoxythymidine triphosphate.

INTRODUCTION - The basal zone of the chorioallantoic placenta and the parietal yolk sac in rodents is composed in part of giant trophoblast cells which contain many times the haploid amount of DNA (1-4). During the process of implantation, the trophectoderm layer is transformed into trophoblast cells which gradually cease to divide (5-8); however, endoreduplication of the entire genome continues to occur by repetitive replication cycles up to Day 16 of pregnancy (7,8) and leads to polyploidy and the formation of giant nuclei (4). We have recently reported that giant nuclei from rat trophoblast cells at Day 13 of gestation carry out DNA endoreduplication *in vitro* at chromosomal sites that were previously active *in*

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3. Abbreviations used: ddTTP, 2', 3'-dideoxythymidine triphosphate; NEM, N-ethylmaleimide; SF, stimulatory factor; DTE, dithioerythritol; Pol, polymerase.

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vivo (9). DNA polymerases α, β and γ are present in these cells; however, the amount of Pol α activity present in an extract of these trophoblast cells appeared to be low after cell division had ceased, and any residual Pol α present in isolated nuclei appeared to be inactive on the basis of insensitivity of [^{3}H]-dTTP incorporation to the specific Pol α inhibitor, aphidicolin (9). Subsequent studies on the Pol α activity in extracts of trophoblast cells from Day 11 to Day 14 of pregnancy indicated that the level of Pol α activity dropped sharply from a high level on Day 11 to 10 percent of that level on Day 12, and remained at a constant level thereafter (10). The sharp drop in Pol α activity from Day 11 to Day 12 appeared to result from the loss of a protein stimulatory factor specific for Pol α (10). The factor appears to be present in dividing trophoblast cells, but absent from trophoblast cells at 12 days of gestation which do not divide but do carry out DNA endoreduplication. We report here on preliminary studies on the characterization of the Pol α stimulatory factor.

MATERIALS AND METHODS

Materials: Timed pregnant rats of the Wistar strain were obtained from Marland Breeding Farms, Inc., Hewitt, N.J. The method of timing the pregnancy was as previously reported (9). Fetal calf liver DNA Pol α (E.C.2.7.7.7) was obtained from Worthington Biochemical Corporation, Freehold, N.J. Calf thymus DNA polymerase α was either purchased from Worthington Biochemicals or prepared in our laboratory; in either case, the enzyme was prepared according to the method of Bollum and Chang (13) and had a specific activity of 322 units/mg protein. Poly d(AT) and 2', 3'-dideoxythymidine triphosphate (ddTTP) were purchased from P.L. Biochemicals, Inc., Milwaukee, WI. Poly d(T)·oligo $r(A)_{12-18}$ was obtained from Collaborative Research, Inc., Waltham, MA. Aphidicolin was gift from Dr. B. Hesp of Imperial Chemicals, Inc., Maccesfield, England. All other chemicals and reagents were obtained as previously described (9).

METHODS

Preparation of Trophoblasts: The pregnant rat was killed, the parietal yolk sac isolated and the trophoblast layer separated from endodermal cells and Reichert's membrane as described previously (9).

Extraction of DNA Polymerases from Trophoblast Cells: Thawed trophoblast tissue from parietal yolk sacs on the appropriate day of pregnancy was homogenized in 4 volumes of 20 mM Tris, pH 7.6 (4°C), 2 mM DTT, 0.5 M KCl, 0.5% Triton X-100 and 8% (v/v) glycerol in an all glass Potter-Elvejhem

homogenizer with 20 passes of the pestle. Generally, approximately 500 mg of trophoblast tissue was extracted except in the case of the tissue from 11-day parietal yolk sac where 200 mg was extracted. At Day 12 this amount of trophoblast tissue represented the pooled tissue from 40-60 feto-placental units dissected from 6 animals whereas at Day 11 it represented the pooled tissue from 100-200 feto-placental units from 12 animals. The homogenate was centrifuged in a Spinco No. 40 rotor at 105,000 xg for 60 min after which the supernatant fluid was carefully drawn off so as to avoid contamination with the lipid layer and rapidly dialyzed against 20 mM Tris, pH 7.6 (4°C), 2 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 M KCl and 8% glycerol. After determination of the protein content (Lowry method), the extract was assayed for Pol activity and then frozen at -70°C. Each of the trophoblast DNA polymerases was partially purified by velocity sedimentation on a 10-30% glycerol gradient.

Preparation of Stimulatory Factor: Stimulatory factor (SF) was present in an extract of 11-day trophoblast tissue prepared as described for the extraction of the DNA polymerases. Any Pol α or Pol γ activity present was inactivated by treatment of the extract with NEM at a final concentration of 3 mM at 4°C for 30 min. (9). The residual NEM was inactivated by the addition of DTE to a final concentration of 6 mM. The treated extract generally contained between 4 and 5 mg protein per ml and showed no Pol α or γ activity and about 80 percent of the original Pol β activity.

Glycerol Gradient Centrifugation: The appropriate dialyzed extract was overlayered on a linear 4.6 ml, 10-30% glycerol gradient made up in 50 mM Tris, pH 7.4 (4°C), 2 mM DTE, 1 mM EDTA and 300 mM KCl. The gradients were formed using a Buchler gradient mixer and linearity was checked by monitoring the refractive index of each gradient fraction with a Bausch and Lomb Abbe 3-L refractometer. Centrifugation was carried out at 37,500 rpm in a Spinco SW 50.1 rotor for 18 h at 4°C. Fractions (12 drops) were collected from the bottom of the tube and 25 µl alliquots were assayed for enzyme activity. Sedimentation coefficients were estimated from the co-sedimenting protein markers, E. coli alkaline phosphatase (S_w^{20}= 6.3) and immunoglobulin G (S_w^{20}=7) using the method of Martin and Ames (14). Alkaline phosphatase in the gradient fractions was assayed by the method of Garen and Levinthal (12) and IgG by measuring the A_{280} nm of the fractions.

Assay of DNA Polymerases α, β and γ: Each reaction was carried out for 60 min at 37°C or as stated in the appropriate Table or Figure legend. The Pol α reaction was carried out in a mixture containing 20 mM phosphate buffer, pH 7.2, 1 mM DTE, 8 mM $MgCl_2$, 2 mM EDTA, activated calf thymus DNA, 200 µg/ml, 50 µM each of dATP, dCTP, dGTP, 50 µM[^{3}H]-dTTP, (specific activity 500 cpm/pmole) in a final volume of 100 µl. DNA Pol β responds with a 20% effeciency under condition of the Pol α assay; therefore, 0.25 mM ddTTP was routinely included as a component of the reaction mixture in order to completely inhibit any Pol β activity present in the 11 day NEM-treated extract used as the source of SF. A unit of activity represented an incorporation of 1 nmol [^{3}H]-dTMP into polynucleotide form hr^{-1}. DNA Pol β was assayed as previously described (9). The gradient fractions were pre-incubated with 3 mM NEM at 4°C for 30 min to inactivate DNA Pol α and γ. Under these conditions the assay was specific for Pol β. DNA Pol γ was assayed according to Knopf et al., (13). The assay is specific for Pol γ since Pol β activity on poly (rA)·$(dT)_{12-18}$ is inhibited by 50 mM sodium phosphate (13) and Pol α is inactive with this template/primer (14). At the end of the polymerase incubations, the tubes were chilled to 4°C, and 75 µl samples were pipeted onto filter paper discs (Whatman 3MM). Discs were washed and prepared for counting as previously described (15). Radioactivity was determined with an Intertechnique SL-30 liquid scintillation system. The counting efficiency for [^{3}H] was 52%.

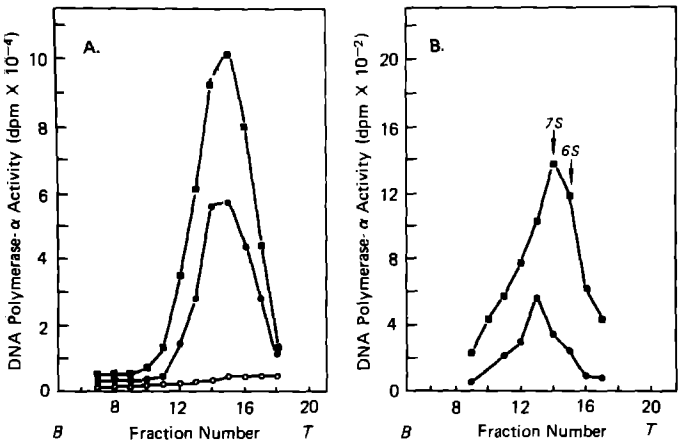

Figure 1. Glycerol gradient profiles of DNA Pol α activity showing the stimulation by an 11-day NEM-treated trophoblast extract. Eleven day extract (60 µl, 660 µg protein) was treated with NEM to inactivate Pol α and γ as described in Methods. (a) 11-day NEM-treated extract and 12-day extract (trophoblast Pol-α) were individually layered on 5 ml 10-30% glycerol gradients as described in Methods. A third gradient received a mixture of 11-day NEM-treated extract and 12-day extract which had been mixed on the basis of equal protein concentrations and incubated at 4°C for 15 min prior to layering on the gradient. The gradients were centrifuged and the fractions collected and assayed for Pol α activity as described in Methods. (■) Pol α activity in a mixture of 11-day NEM-treated extract and 12-day extract (●) Pol α activity in 12-day extract; (○) Pol α activity in 11-day NEM-treated extract. (b) The stimulation of calf thymus Pol α by 11-day NEM-treated extract. 11-day NEM-treated extract or calf thymus Pol α were layered on glycerol gradients and stimulation of Pol α activity by 11-day NEM-treated extract was assayed as described above. (■), 11-day NEM-treated extract plus calf thymus α-Pol; (●) calf thymus Pol α.

RESULTS

When a NEM-treated 11-day trophoblast extract containing stimulatory factor (SF) was mixed with a 12-day trophoblast extract containing Pol α and sedimented on a 10-30% glycerol gradient, Pol α was shown to be stimulated 100%. A representative glycerol gradient profile is presented in Fig. 1A. Only the fractions encompassing the peak of Pol α activity are shown. As can be seen from the gradient profiles, treatment with 3 mM NEM has eliminated all of the Pol α activity in the 11-day extract. However, Pol β is not inhibited by 3 mM NEM and since Pol β is present in the extracts and responds with a 20-30% efficiency in the Pol α assay, ddTTP was routinely added to the assay. Fig. 1B presents a similar glycerol gradient profile which shows a 2.8-fold stimulation of calf thymus Pol α by NEM-treated 11-day extract.

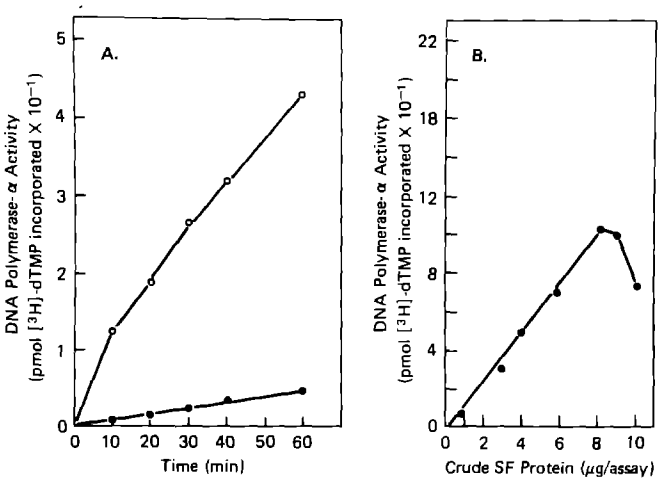

Figure 2. The stimulation of Pol α by SF protein as a function of time and concentration of SF. (a) Pol α (0.02 unit) was preincubated with SF (6 μg protein) for 15 min at 4°C after which time the mixture was assayed in the Pol α assay as described in Methods. 75 microliter samples were removed at the appropriate time, placed on paper discs and washed and counted as described in Methods, (○) Pol α plus SF; (●) Pol α. (b) These incubations were carried out as just described with the exception that the amount of SF protein incubated varied as is indicated in Fig. 2B. All points represent the average of duplicate determinations which generally agreed within 5 percent.

Effect of Stimulatory Factor on the Polymerizing Activity of DNA Pol α.

The addition of SF to Pol α assays resulted in an increase in both the initial rate and final extent of DNA polymerization when compared to control reactions without SF. As can be seen in Fig. 2A, after 60 min of incubation approximately 0.02 unit calf thymus Pol α was stimulated 8.6-fold by 6 μg of SF protein when activated DNA served as template/primer. The amount of DNA synthesis increased in a linear manner with the amount of SF protein added up to 8 μg per assay after which an inhibition of synthesis occurred (Fig. 2B). That the observed increase in [^{3}H]-dTMP incorporation in the presence of SF results from the stimulation of Pol α is shown by the fact that the stimulation is completely abolished by the specific inhibitor of Pol α, aphidicolin (Table 1), and is unaffected by ddTTP which is routinely included in the incubation mixture and which inhibits Pol β and γ.

TABLE I

The Effect of Aphidicolin on the Stimulation of Pol α by SF

Conditions	[^{3}H]-dTMP Incorporated pmol hr^{-1}
Pol α	9.4
Pol α plus SF	76.6
Pol α plus SF plus Aphidicolin	3.8

Calf thymus Pol α (0.02 unit) was incubated in the presence or absence of SF (6 μg per tube) under Pol α assay conditions as described in Methods. Aphidicolin was added at a concentration of 41 μg per ml.

Some Properties of SF. SF is non-dialyzable, heat-labile (90°C, 3 min), and insensitive to 3 mM NEM. Trypsin completely eliminates the stimulatory activity of NEM-treated 11-day trophoblast extract. Addition of soybean trypsin inhibitor concomitantly with the trypsin prevented the inactivation of SF (10). These results indicate that the stimulatory activity resides in a protein. SF was analyzed by velocity sedimentation through a 10-30% glycerol gradient containing 0.3 M KCl. SF appeared to sediment in a fairly broad peak of molecular weight range of 65,000 - 85,000 (Fig.3). The greatest amount of SF activity sedimented at M_r 85,000. A small peak of activity was also observed at an S value of 9 which may represent aggregation of the 65-85,000 species in 0.3 M KCl. Chromatography of SF on a Sephadex G-100 column resulted in a peak of stimulatory activity, indicative of a molecular weight range of 66,000 - 78,000 (data not shown).

The stimulation of Polymerase α by SF with Various Template/Primers. The stimulatory effect of SF on calf thymus Pol α was examined with activated and heat-denatured DNA as well as with several synthetic deoxynucleotide polymers primed with both oligo (dT) and oligo (rA) primers. The amount of template/primer was 8 μg per assay in all cases.

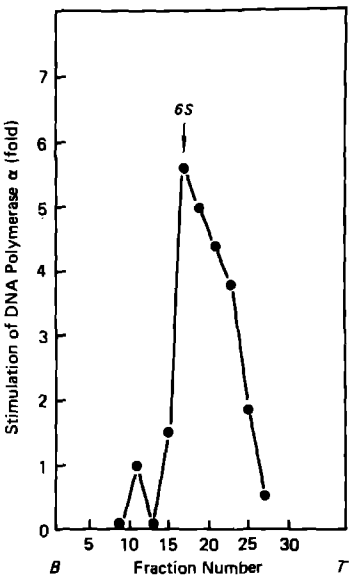

Figure 3. Glycerol gradient sedimentation of trophoblast SF protein. 11-Day NEM-treated extract (400 µg protein) was applied to a 10-30% glycerol gradient containing 300 mM KCl. The gradient was prepared, centrifuged and fractions collected as described in Methods. The location of SF activity in the gradient was analyzed by assaying each fraction (25 µl) in the standard Pol α assay containing 0.02 unit of Pol α as described in Methods. Stimulation of Pol α activity by SF protein in the various gradient fractions was expressed as multiples of the activity of a control incubation which contained an identical concentration of Pol α but lacked SF protein. The control assay showed an incorporation of 16.8 pmoles (16,800 dpm) total deoxynucleoside monophosphate incorporated per hour under the standard α-Pol assay conditions. The arrow indicates the position of the alkaline phosphatase marker.

As can be seen from the data presented in Table II, SF showed some specificity with regard to its ability to stimulate calf thymus Pol α with different template/primers. The largest stimulation was observed with either activated calf thymus DNA or with poly d(T)·oligo $r(A)_{12-18}$ (template to primer ratio, 1:1). Poly d(AT) and heat-denatured calf thymus DNA were used to a lesser extent. The deoxynucleotide primed template, poly d(A)·oligo $d(T)_{10}$ (template to primer ratio, 1:1) was not used by Pol α in the presence of SF as effectively as the ribonucleotide primed template poly d(T)·oligo $r(A)_{12-18}$.

Specificity of SF for DNA Polymerase α. The ability of SF to stimulate DNA synthesis on activated calf thymus DNA by several polymerases was investi-

TABLE II

The Effect of Template/Primer on the Stimulation of Polymerase α by SF

Template	Total Nucleotide Incorporated pmol hr^{-1}		Stimulation Fold
	-SF	+SF	
Calf thymus DNA (activated)	49.0	259.5	5.3
Calf thymus DNA (denatured)	84.6	368.7	4.4
Poly d(AT)	17.0	67.1	3.9
Poly d(A)·$d(pT)_{10}$	33.2	126.0	3.8
Poly d(T)·oligo $r(A)_{12-18}$	47.0	235.0	5.0

Pol α (0.04 unit) was incubated with the appropriate template/primer (8 μg) with or without SF (12 μg per tube) under Pol α assay conditions as described in Methods.

gated. Each polymerase was assayed under optimal conditions and each of the assays contained comparable units of polymerase activity. Under these conditions, SF was shown to specifically stimulate synthesis by either calf thymus Pol α or rat trophoblast Pol α 5 to 7-fold, whereas little or no stimulation of trophoblast Pol β, trophoblast Pol γ or fetal calf liver Pol γ was observed. However, E. coli Pol I was stimulated 1 to 2-fold (data not shown).

Binding of SF to DNA. There have been several reports in the recent literature (16,17) on the characterization of proteins which stimulate DNA polymerase α and which possess single-stranded DNA-binding properties. Addition of increasing amounts of such proteins to heat-denatured DNA results in the retention of radioactively labeled denatured DNA on the filter (16,17). Therefore, SF was tested at several protein concentrations for its ability to increase the binding of both native and heat-denatured [^{3}H]-labeled rat trophoblast DNA and [^{3}H]-labeled single-stranded circular fd phage DNA to millipore filters (0.45 μ) according to the method of Otto, et al. (16). The binding studies were carried out under two sets of

conditions; conditions described for single stranded DNA-binding proteins which stimulate Pol α (16,17) and also under conditions of the Pol α assay where SF stimulates Pol α. SF did not effect the binding of more than 1 or 2% of the [^{3}H]-labeled single-stranded DNA which could potentially be bound to the filter when incubated with the DNA (1 µg) over a range of SF concentration from 3 to 48 µg (data not shown).

DISCUSSION

Rat giant trophoblast cells contain a protein factor which stimulates DNA synthesis by Pol α *in vitro*. The stimulatory factor has been partially purified by velocity sedimentation on a 10-30% glycerol gradient and has been shown to have an approximate M_r of 65 - 85,000. SF is devoid of DNA polymerase activity and does not act as a nuclease in altering the DNA template by further activation. Stimulation of the same magnitude is seen when the synthetic template/primer poly d(T)·oligo $r(A)_{12-18}$ is used in place of activated DNA. This template cannot be activated by DNases and any endonucleolytic activity on the polymer should result in a decrease in template activity. Furthermore, no stimulation by SF is observed in the Pol β assay which uses the same preparation of activated DNA as template/primer. SF stimulates both the initial rate and the final extent of the polymerization reaction by Pol α as a linear function of SF concentration. SF appears to be specific for Pol α, however, it was shown to stimulate *E. coli* Pol I about 2-fold as do other Pol α stimulatory factors (16,18). Although SF shares certain properties with other recently reported stimulatory factors from mouse ascites cells (16) Hela cells (18) and *Tetrahymena thermophila* (17), it does not appear to have single-stranded DNA-binding capability as do the factors from mouse cells (16) and *Tetrahymena* (17).

The molecular mechanism by which SF stimulates Pol α remains to be elucidated; stimulation of both the initiation and elongation phases of

DNA synthesis might be mediated by an interaction between SF, Pol α and the template/primer. Purification of SF and the elucidation of its molecular mechanism of action are currently under study in our laboratory.

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