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A DNA Template Recognition Protein: Partial Purification from Mouse Liver and Stimulation of DNA Polymerase α[†]

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ABSTRACT: A protein that specifically enhances up to 13-fold the rate of copying of poly(dT) template by DNA polymerase α was partially purified from chromatin of regenerating mouse liver cells. This stimulatory protein, designated herein factor D, also increases 2–3-fold the activity of polymerase α with heat-denatured DNA and with primed, circular single-stranded ϕX174 DNA. However, factor D has no detectable effect on the copying by polymerase α of poly(dG), poly(dA), and poly(dC) templates. Activity of mouse DNA polymerase β is not affected by factor D with all the tested templates. In contrast to polymerase α, factor D is resistant to inactivation by *N*-ethylmaleimide and calcium ions, but it is readily heat-inactivated at 46 °C and is inactivated by trypsin digestion. Partially purified factor D is not associated with detectable activities of DNA polymerase, DNA primase, deoxyribonucleotidyl terminal transferase, and endo- or exodeoxyribonuclease.

The replication of genomic DNA in eucaryotes is mediated by a number of enzymes and proteins that are thought to form a multiprotein replicative complex (Noguchi et al., 1983; Reddy & Pardee, 1983; Ottiger & Hübscher, 1984). Within that putative complex, DNA polymerase α is deemed to play a central role in the nucleotide selection and polymerization processes (Reddy & Pardee, 1983; Hübscher, 1983a,b). Although the precise molecular structure of polymerase α is still under debate, there is a general agreement that this high molecular weight enzyme consists of a number of subunits and that it copurifies and interacts with primase (Hübscher et al., 1982; Tseng & Ahlem, 1983; Hübscher, 1983b; Yagura et al., 1983; König et al., 1983; Wang et al., 1984), P^1,P^4-bis(5′-adenosyl) tetraphosphate (Ap_4A) binding protein (Rapaport et al., 1981; Baril et al., 1983; Rapaport & Feldman, 1984), and primer recognition proteins (Lamothe et al., 1981; Pritchard & DePamphilis, 1983; Pritchard et al., 1983). The identification and characterization of polypeptides, which together with the polymerase α catalytic core form an active replicative complex, is essential for the elucidation of the details of DNA replication in animal cells. In addition to the extensively investigated catalytic subunit of polymerase α, DNA primase was recently shown to be either a dissociable component of polymerase α or an inseparable part of the polymerase molecule (Tseng & Ahlem, 1983; Hübscher, 1983b; Yagura et al., 1983; König et al., 1983; Wang et al., 1984). Several investigators have identified additional protein factors that associate with polymerase α to increase its affinity to various primer templates. Protein cofactors that enhance the activity of polymerase α with sparsely primed DNA templates such as denatured DNA have been described (Otto et al., 1977; Richter et al., 1978; Novak & Baril, 1978). Two such stimulatory cofactors, proteins C_1C_2 from human and monkey cells, were found to act as primer recognition proteins (Lamothe et

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al., 1981; Pritchard & DePamphilis, 1983; Pritchard et al., 1983). Recently described DNA-binding proteins from calf thymus were found to block the nonproductive binding of polymerase α to the template and thus to increase its affinity to the 3′-hydroxyl ends of the primer (Sapp et al., 1985).

In this work we describe the partial purification and characterization of a protein from mouse liver that stimulates the utilization of selected templates by homologous and heterologous polymerase α. This protein enhances the activity of polymerase α with denatured DNA, and is thus designated factor D, and with singly primed single-stranded circular phage DNA. In contrast to all the previously described stimulatory proteins, factor D is sequence-selective with synthetic DNA: Whereas it strongly increases the rate of copying of poly(dT) by polymerase α, it has no effect on the utilization of poly(dA), poly(dG), and poly(dC) templates at any primer to template ratio. Possible significance of the sequence specificity of factor D is discussed.

Materials and Methods

Materials. Salmon sperm DNA was a product of Sigma Chemical Co., St. Louis, MO. Maximally activated DNA was prepared as described by Loeb (1969). Heat-denatured DNA was prepared, stored at 4 °C, diluted, and denatured a second time immediately prior to use, as described recently by Pritchard & DePamphilis (1983). Deoxy[8-^{3}H]adenosine 5′-triphosphate ([^{3}H]dATP) and deoxy[8-^{3}H]guanosine 5′-triphosphate ([^{3}H]dGTP), as well as deoxycytidine 5′-[α-^{32}P]triphosphate and deoxythymidine 5′-[α-^{32}P]triphosphate, were products of New England Nuclear, Boston, MA. P-L Biochemicals Milwaukee, WI, supplied calf thymus DNA polymerase α, 2′,3′-dideoxythymidine (d_2TTP), poly(deoxycytidine) [poly(dC)], poly(deoxythymidine) [poly(dT)], poly(deoxyadenosine) [poly(dA)], the hybrid poly(dA)·poly(dT), alternating heteropolymer poly[d(AT)], oligo(deoxyguanosine) [$(dG)_{12-18}$], poly(deoxyguanosine) [poly(dG)], and the hybrid poly(deoxycytosine)–poly(deoxyguanosine) [poly(dG)·poly(dC)], oligodeoxyadenosine [$(dA)_{12-18}$], and oligo(deoxythymidine) [$(dT)_{12-18}$]. Sigma Chemical Co., St. Louis, MO, was the supplier of ribonucleoside 5′-triphosphates (rNTPs), native and denatured DNA–cellulose, trypsin and soybean and pancreatic inhibitors of trypsin, aphidicolin, dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), leupeptin, *N*-ethylmaleimide (MalNet), bovine serum albumin (BSA), phosphocreatinine and creatinine phosphokinase, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), adenosine 5′-triphosphate (ATP), and the unlabeled deoxynucleoside 5′-triphosphates (dNTPs) 2′-deoxyguanosine 5′-triphosphate (dGTP), thymidine 5′-triphosphate (dTTP), 2′-deoxyadenosine 5′-triphosphate (dATP), and 2′-deoxycytidine 5′-triphosphate (dCTP). (Diethylaminoethyl)cellulose (DEAE-cellulose, DE-52) and phosphocellulose (P-11) were purchased from Whatman, Ltd., Kent, United Kingdom. Hydroxylapatite was obtained from Bio-Rad, Richmond, CA. Phage ϕX174 DNA and the complementary synthetic oligonucleotide primer 5′-d(GGAAAAGCGAGGGTAT)-3′ were the generous gift of Dr. L. A. Loeb, University of Washington. Phage M-13 circular single-stranded DNA was generously given by Dr. B. Preston, University of Washington.

Animals and Partial Hepatectomy. Female mice at age 6–10 weeks (ICR strain) were used. The animals were born and maintained at the Technion Faculty of Medicine vivarium and fed ad libitum. Liver regeneration was induced in the mice by partial hepatectomy as recently described (Fry et al., 1984), except that during surgery the animals were anesthetized by ether instead of by tribromoethyl alcohol.

Isolation of Chromatin. Mice were sacrificed 72 h after partial hepatectomy, and liver cell nuclei and chromatin were isolated by modifications of the methods of Lynch et al. (1975) and Knopf & Weissbach (1977), as recently described (Fry et al., 1984).

Solubilization of Chromatin-Bound DNA Polymerases and Factor D. DNA polymerases α and β and factor D were quantitatively solubilized by repeated salt extractions of chromatin. Salt (0.8 M NaCl) in 25 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 8.5, 0.5 mM DTT, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 μg/mL leupeptin, and 40% glycerol was added to an equal volume of chromatin suspension. Extraction with salt was conducted at 4 °C for 30 min with occasional moderate stirring. Solubilized chromatin proteins were separated from the insoluble residue by centrifugation at 12000*g* for 15 min. The supernatant was collected, and the insoluble pellet was resuspended in 0.4 M NaCl in 25 mM Tris-HCl buffer, pH 8.5, 0.5 mM DTT, 1 mM EDTA, 10 μg/mL leupeptin, and 40% glycerol and extracted as above. This salt-extraction step was repeated twice. About 40–50% of the activities of chromatin-bound polymerase α and β and factor D were solubilized in the first step, 15–25% in the second step, and 10–15% in the last step. Nearly quantitative solubilization of chromatin-associated polymerases and factor D was therefore attained. To remove residual DNA from the combined salt-extraction supernatants, they were passed through a column of DE-52 equilibrated with 25 mM Tris-HCl buffer, pH 8.2, 0.4 M NaCl, 0.5 M DTT, 1 mM EDTA, and 20% glycerol. Loading of the column was at a proportion of 5–10 mg of extract protein/mL of packed column volume. Salt was subsequently removed from the protein solution by dialysis overnight against 500 volumes of 25 mM Tris-HCl, pH 8.2, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol.

DEAE-cellulose Chromatography of Chromatin Extract. To separate the activities of DNA polymerases α and β and of factor D, the combined dialyzed salt extracts were chromatographed on DE-52 columns. Extract was loaded onto a DE-52 column equilibrated with 25 mM Tris-HCl buffer, pH 8.2, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol at 2.5–3.0 mg of protein/mL of packed column volume. The flow-through volume was collected in fractions, each constituting 20% of the packed column volume. The loaded column was further washed with 1 column volume of the equilibration buffer, and fractions were collected as described above. Proteins that remained adsorbed to the ion exchanger were subsequently eluted by applying to the column a linear gradient of 6 column volumes each of 0.0–500 mM KCl in 25 mM Tris-HCl buffer, pH 8.2, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol. Eighty fractions, each constituting 10% of the packed column volume, were collected at a rate of one fraction in 2–3 min. Fractions were assayed for polymerases α and β and factor D activities. Polymerase α was eluted from the column as a wide heterogeneous peak at 150–250 mM KCl. This activity was 88–92% inhibited by 10 μg/mL aphidicolin, a specific inhibitor of DNA polymerase α, and 90–97% by 4 mM MalNet to which DNA polymerase α, but not polymerase β, is sensitive (Fry, 1983; Hübscher, 1983) (results not shown). The pooled activity of polymerase α was stored at −75 °C until used. Polymerase β, which was completely resistant to 10 μg/mL aphidicolin, 75–89% resistant to 4 mM MalNet, and 90% sensitive to 50 μM d_2TTP, was eluted at 40–90 mM KCl and pooled separately. Factor D was not absorbed to DE-52 under the described experimental conditions and was collected in the flow-through and column-wash fractions. The factor

was free of DNA polymerase activity.

Phosphocellulose Column Chromatography of Factor D. Factor D, collected in the flow through of DE-52, was loaded at 2 mg of protein/mL of packed column volume of P-11, equilibrated with 100 mM potassium phosphate (K_3PO_4) buffer, pH 8.0, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol. The loaded column was washed with 1 volume of equilibration buffer, and the adsorbed factor activity was eluted with a linear gradient of 5 column volumes each of 100–500 mM K_3PO_4 buffer, pH 8.0, containing 0.5 mM DTT, 1 mM EDTA, and 20% glycerol. Fifty fractions, each constituting 10% of the packed column volume, were collected at a rate of one fraction every 2 min. Factor D eluted from P-11 at 150–250 mM K_3PO_4.

Hydroxylapatite Column Chromatography of Factor D. Phosphocellulose-purified factor D was dialyzed overnight against 500 volumes of hydroxylapatite equilibration buffer, which consisted of 5 mM K_3PO_4, pH 7.6, 400 mM KCl, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol. Dialyzed factor D was loaded onto hydroxylapatite columns at a ratio of 1 mg of protein/mL of packed column volume. The loaded column was subsequently washed with one packed column volume of the equilibration buffer. No factor D activity was detected in the column-wash fractions; it was eluted from the hydroxylapatite columns with a linear gradient of 6 column volumes each of the equilibration buffer and of a high-salt buffer consisting of 200 mM K_3PO_4, pH 7.6, 500 mM KCl, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol. Fifty fractions were collected, a fraction volume being 20% of the packed column volume. Prior to assay of activities therein, the collected fractions were dialyzed overnight against 400 volumes of 20 mM Tris-HCl buffer, pH 7.5, which contained 50 mM NaCl, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol. Factor D was eluted at 40–70 mM K_3PO_4.

DNA–Cellulose Chromatography of Factor D. A column of native DNA–cellulose (2–3-mL packed column volume) was equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl, 0.5 mM DTT, 1 mM EDTA, 100 μg/mL BSA, and 20% glycerol. Dialyzed hydroxylapatite-purified factor D was loaded onto the DNA–cellulose column at a ratio of 1 mg of protein/mL of packed column volume. The loaded column was washed 3 times with 1 column volume of the equilibration buffer. The adsorbed factor was eluted by three similar stepwise washes with equilibration buffer that contained 0.2, 0.4, 0.6 and 2.0 M NaCl, in succession. The collected active fractions were dialyzed overnight against 300 volumes of 20 mM Tris-HCl buffer, pH 7.5, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol and assayed for activity. Factor D was eluted from native DNA–cellulose at 2.0–0.40 mM NaCl. A similar elution pattern of the factor was observed with denatured DNA–cellulose. DNA–cellulose-purified factor D was stored at −75 °C in the presence of 300 μg/mL BSA, for up to 3 months without detectable loss of stimulatory activity.

Gel Filtration. Molecular size of partially purified factor D was determined by filtration on Bio-Sil HPLC gel column TSK-125 (Bio-Rad). An aliquot of 150 μL of DE-52-purified factor D was filtered at a rate of 1 mL/min through the column. Molecular weight markers were BSA (64 000), ovalbumin (45 000), myoglobin (17 500), and cyanocobalamine (1355). Recovery of factor D activity was about 50%.

Purification of DNA Polymerase α. Mouse liver DNA polymerase α that was eluted from DE-52 columns was further purified by P-11 chromatography as described recently (Silber et al., 1985). Polymerase α was eluted from P-11 as a wide peak that often displayed two maxima at about 240 mM and 270 mM K_3PO_4 buffer. The two peaks of polymerase activity, designated α_1 and α_2, were pooled separately, dialyzed against 25 mM Tris-HCl, pH 7.6, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol, and stored at −75 °C with 300 μg/mL BSA until used. Either P-11-purified mouse liver polymerase α_1 or α_2 or calf thymus polymerase α (P-L Biochemicals) was used to assess the stimulatory effect of factor D with similar results.

Purification of DNA Polymerase β. Mouse liver polymerase β that was eluted from DE-52 columns was sequentially purified by P-11 and DNA–cellulose column chromatographies as described by Silber et al. (1985).

Assay Conditions for Activity of DNA Polymerases. The activities of purified DNA polymerases α and β were assayed for 10–30 min at 37 °C in a 50-μL mixture that contained 25 mM Tris-HCl buffer, pH 7.6, 1 mM DTT, 5 mM $MgCl_2$, 25 μM each of dATP, dGTP, dCTP, and dTTP, and glycerol at a final concentration of 26%. Tritium- or ^{32}P-labeled substrates were added at specific activities, as detailed under Results. Activated DNA or denatured DNA templates were present at final concentrations of 400 and 200 μg/mL, respectively. Primed φX174 DNA was added at 4–8 μg/mL. Synthetic DNA primer templates were each added to a final concentration of 40 μg/mL. Activities of chromatin-bound polymerases α and β were assayed as described previously (Fry et al., 1984). DNA polymerization was terminated essentially as described by Battula & Loeb (1974), except that the final acid-insoluble precipitate was solubilized by Soluene (Packard Co.) and incorporated radioactivity was monitored in a toluene-based scintillation liquid. One unit of polymerase activity was defined as that activity which catalyzed the polymerization of 1 nmol of deoxynucleoside monophosphates in 1 h at 37 °C.

Assay Conditions for Activity of Factor D. Factor D was detected in chromatin or in partially purified fractions by its ability to stimulate the in vitro copying of heat-denatured DNA by mouse liver or calf thymus DNA polymerase α. DNA polymerization was conducted in parallel for 30 min at 37 °C, with and without added factor D. Activity of factor D was deduced from the difference between extent of DNA synthesis by polymerase α in its presence and the sum of activities of polymerase and factor assayed separately. Stimulation of copying of denatured DNA was directly related to the amount of factor D added to a constant amount of polymerase α until saturation with factor was attained. Activity of factor D was assessed at the linear portion of the stimulation curve (excess polymerase α). One unit of stimulatory activity was defined as that amount of factor that increased 2-fold the activity of 9×10^{-2} unit of polymerase α with denatured DNA template at 37 °C for 30 min. With denatured DNA and synthetic polydeoxythymidine templates, maximum activity of factor D and linear stimulation curves were attained in the presence of 50 mM KCl in the reaction mixture, whereas with primed φX174 single-stranded DNA 50 mM salt was inhibitory and maximum stimulation was observed at 10 mM KCl.

Assay Conditions for Activities of Exo- and Endodeoxyribonucleases. The possible association of purified factor D with exo- or endonucleases was examined as follows: For the detection of exonucleolytic activity, factor D was incubated at 37 °C for 30 min with tritium-labeled native or denatured DNA under assay conditions for factor activity. The lower limit of sensitivity of this nuclease assay was 0.5×10^{-3} nmol of [^3H]dNMP released per 1.0 nmol of stimulation of dNMP incorporation. Endonuclease activity was assessed by incubating factor D under its assay conditions with single-stranded circular φX174 DNA, transfecting *Escherichia coli* spheroplasts with the incubated DNA, and assessing loss of biological

activity of the phage DNA. Since a single nick formed in the closed DNA circle suffices to eliminate plaque formation, this assay is exceedingly sensitive. In a typical experiment, 1.5×10^{11} ϕX174 DNA molecules were either exposed to 0.2 units of factor D or incubated in its absence. *E. coli* spheroplasts were transfected with both factor-treated and untreated phage DNA molecules, and plaque numbers were determined. Efficiency of transfection allows for the detection of one nick formed in 10^6–10^7 DNA molecules.

Assay Conditions for DNA Primase Activity. Assay conditions for maximum stimulatory activity of factor D were used to detect the possible presence of DNA primase with the following modifications: Poly(dC), poly(dT), or phage M-13 single-stranded DNA served as unprimed templates, and either a complementary single ribonucleotide triphosphate or all four ribonucleotide triphosphates were added at 100–300 μM. Incorporation into acid-insoluble product of complementary tritium-labeled dNMP was followed as described for assay of DNA polymerase activity.

Other Methods. Amount of protein was determined with the Bio-Rad protein assay kit. Determination of DNA in chromatin was performed according to the method of Burton (1956).

Results

A Chromatin-Bound Factor Stimulates Copying of Denatured DNA by Polymerase α. In the past, we have found that DNA polymerase α, which is associated with chromatin, copies denatured DNA at a high efficiency (Kaftory & Fry, 1978; Weisman-Shomer et al., 1979). To inquire whether the efficient copying of denatured DNA by chromatin-bound polymerase α is due to its possible interaction with a specific, chromatin-associated stimulating factor, the effect of added chromatin on the utilization of activated and denatured DNA by partially purified DNA polymerase α was measured. Partially purified mouse liver DNA polymerase α, which copied denatured DNA at a relatively low rate, was admixed with chromatin, and the copying of denatured DNA was found to exceed by about 30% the summed activities of chromatin and isolated polymerase, which were assayed separately. No such synergistic effect was observed for the copying of activated DNA. Further, chromatin-bound polymerase α was inactivated by either 5.0 mM MalNet or 1.5 mM $CaCl_2$, and excess inactivators were complexed by 20 mM DTT and 5.0 mM EGTA, respectively. Although the inactivated chromatin was devoid of detectable polymerase α activity and did not support copying of denatured DNA, upon its addition to mouse liver polymerase α, it enhanced utilization of heat-denatured DNA by the purified enzyme. These results suggested the possibility that chromatin contains a MalNet- and Ca^{2+}-insensitive factor that stimulates either chromatin-bound or exogenously added polymerase α to preferentially copy denatured DNA. In consideration of its specific stimulation of copying of denatured DNA, the putative stimulatory factor was designated factor D.

Partial Purification of Factor D. To separate factor D from polymerases α and β, salt extracts of mouse liver chromatin were chromatographed on DE-52 columns as described under Materials and Methods. Eluted fractions were assayed for activities of DNA polymerases and factor D (see Materials and Methods). Complete resolution of factor D from both polymerases α and β was attained when both the DE-52 column equilibration buffer and the loaded extract were devoid of salt (Figure 1A). Presence of even 25 mM K_3PO_4 in the equilibration buffer led to exclusion of factor D from the column together with polymerase β (results not shown). As

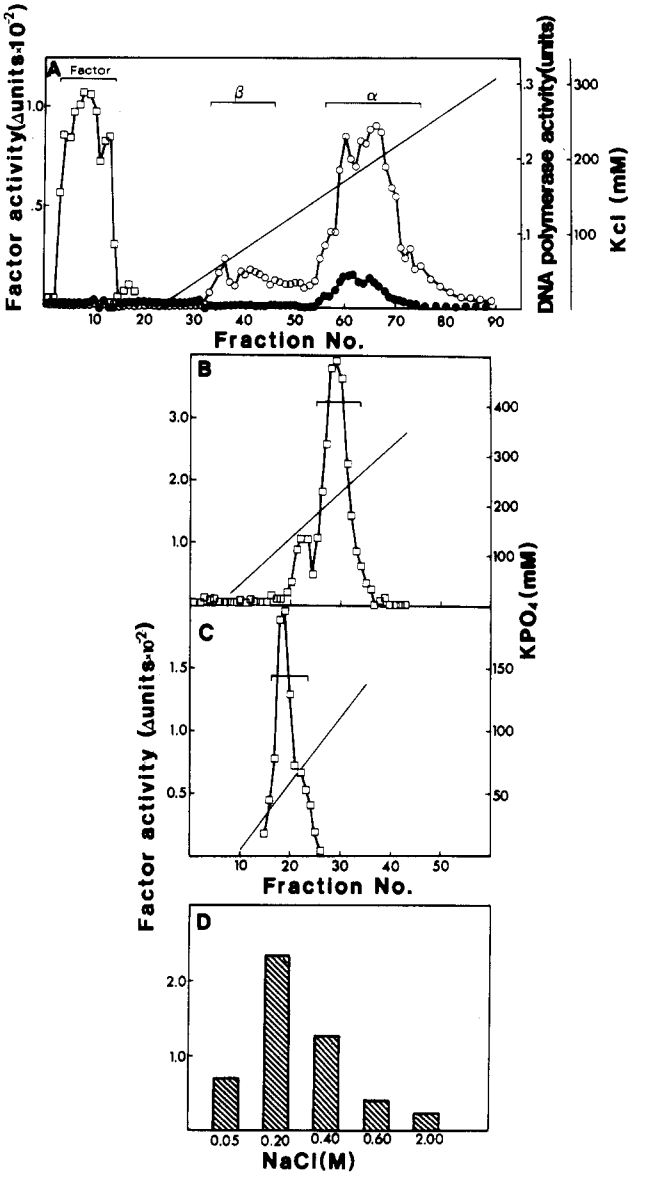

FIGURE 1: Column chromatographic purification of factor D. Chromatin was isolated from 25 regenerating mouse livers, and DNA polymerases α and β and factor D were extracted with 0.4 M NaCl. Activities of polymerases α and β were resolved by DE-52 chromatography, and the isolated stimulatory factor was sequentially purified by three additional chromatographic steps. (A) DEAE-cellulose (DE-52) chromatogram. Collected fractions were assayed as described under Materials and Methods for factor D stimulatory activity (□) and for DNA polymerases activity in parallel assays that contained either activated DNA (○) or denatured DNA (●). Polymerases α and β were distinguished by their characteristics response to aphidicolin, MalNet, and d_2TTP (see Results). Factor D (fractions 3–11 of DE-52) was collected and purified by consecutive chromatographic steps on columns of (B) phosphocellulose (P-11), (C) hydroxylapatite, and (D) DNA–cellulose.

seen in Figure 1A, at low ionic strength factor D does not absorb to DEAE-cellulose and is completely separated from polymerase α and β, which are eluted at 50–100 mM KCl and 150–200 mM KCl, respectively. Polymerase β was 97 and 90% resistant to 20 μg/mL aphidicolin and 4 mM MalNet, respectively, whereas 90, 95 and 20% of the activity of polymerase α was inhibited by 20 μg/mL aphidicolin, 4 mM MalNet, and 25 μM d_2TTP. Polymerase activity that eluted between 100 and 140 mM KCl contained a mixture of polymerases α and β, as assessed by intermediate response to inhibitors. Note that the partially purified polymerase α, but not polymerase β, copies denatured DNA as also was found

Table I: Purification of Factor D[a]

purification stage	protein (mg)	factor D activity (units)	factor D sp act. ($\times 10^{-2}$ units/mg)	purification (x-fold)	yield (%)
total cell extract	1375.0	ND			
chromatin	63.0	2.52	4.0	1.0	100.0
chromatin extract	44.4	1.91	4.6	1.1	75.8
DEAE-cellulose	1.4	0.56	40.0	10.0	22.2
phosphocellulose	0.1	0.30	300.0	75.0	11.9
hydroxylapatite	0.006	0.17	2833.3	708.2	6.8

[a] In this representative purification scheme about 18 g of regenerating liver tissue that was collected from 18 partially hepatectomized mice was used. Activity of factor D was not determined in total cell extracts, and purification is relative to the activity of factor in crude chromatin. Protein concentration in the hydroxylapatite fraction was measured in an aliquot that was concentrated 45-fold. Activity of factor D was determined in all fractions by titration with denatured DNA. Purification at the DNA–cellulose step was not determined since the amount of recovered protein was below detection.

Table II: Effect of Factor D on Copying of Natural and Synthetic DNA by Calf Thymus Polymerase α[a]

template[b]	polymerase	substrate(s)[c]	polymerase act. ($\times 10^{-3}$ units)	stimulation of polymerase α by factor D (x-fold)
activated DNA (salmon sperm)	α	dNTPs + [^{3}H]dATP	282.8	
	α–D	dNTPs + [^{3}H]dATP	281.2	0.99
denatured DNA (salmon sperm)	α	dNTPs + [^{3}H]dATP	21.0	
	α–D	dNTPs + [^{3}H]dATP	57.3	2.73
primed φX174	α	dNTPs + [^{32}P]dTTP	3.2	
	α–D	dNTPs + [^{32}P]dTTP	12.1	3.78
poly(dG)·poly(dC)	α	dCTP + [^{3}H]dGTP	2.3	
	α–D	dCTP + [^{3}H]dGTP	2.5	1.09
$(dT)_{12-18}$·poly(dA)	α	[^{32}P]dTTP	0.15	
	α–D	[^{32}P]dTTP	0.03	0.13
poly[(dAT)]	α	dTTP + [^{3}H]dATP	2.4	
	α–D	dTTP + [^{3}H]dATP	2.6	1.08
poly(dA)·poly(dT)	α	dTTP + [^{3}H]dATP	1.6	
	α–D	dTTP + [^{3}H]dATP	21.6	13.5
$(dA)_{12-18}$·poly(dT)	α	[^{3}H]dATP	6.9	
	α–D	[^{3}H]dATP	21.2	3.07

[a] DNA polymerization with and without factor D was conducted as described under Materials and Methods, except for the copying of φX174 DNA, which was performed at 30 °C to stabilize the primer-template hybrid. With all primer templates, maximum stimulatory activity of factor D was obtained in the presence of 50 mM KCl in the reaction mixture, except for copying of φX174 DNA, which was maximally stimulated in the presence of 10 mM KCl. Factor D (P-11) was added to saturation to 0.3 unit of calf thymus polymerase α (mixture is designated α–D in this table). All the results are averages of triplicate or quadruplicate determinations, which deviated by less than 5%. [b] Final concentrations of natural and synthetic templates were as specified under Materials and Methods. Phage φX174 DNA was primed with a synthetic 15-mer primer at a primer to template molar ratio of 2:1. Oligo(dT) and oligo(dA) were annealed to their respective templates at a ratio of 2:1 (w/w, primer to template). [c] Specific activities (cpm/pmol of dNMP) of the various substrates were as follows: dNTPs labeled with [^{3}H]dATP, 221; dNTPs + [^{32}P]dTTP, 250; [^{3}H]dGTP, 1286; [^{32}P]dTTP, 359; dTTP + [^{3}H]dATP, 442; [^{3}H]dATP, 885.

for chromatin-associated enzymes. Sequential purification steps of factor D by chromatography on columns of P-11, hydroxylapatite, and DNA–cellulose is depicted in panels B, C, and D, respectively, of Figure 1. Note that factor D behaves as a moderate double-strand DNA binding protein with most of its activity eluting at 0.20–0.40 M NaCl (Figure 1D). Similar moderate binding of factor D was observed with columns of single-strand DNA–cellulose (results not shown). Purification of factor D from crude chromatin and yield of purified factor are summarized in Table I.

Factor D Preferentially Stimulates Utilization of Poly(dT) by Polymerase α. Previously described eucaryotic proteins were found to stimulate the utilization by polymerase α of self-primed, heat-denatured DNA and of other sparsely primed natural and synthetic DNA templates (Otto et al., 1977; Richter et al., 1978; Novak & Baril, 1978; Lamothe et al., 1981; Pritchard & DePamphilis, 1983; Pritchard et al., 1983). Factor D superficially resembles these proteins in that it stimulates utilization of denatured DNA.

To learn whether or not the mouse liver protein is analogous to some of these stimulatory and primer-recognition protein factors, we investigated its enzyme and template preferences. Utilization of several natural and synthetic DNA templates by polymerases α from mouse liver and calf thymus and by mouse liver polymerase β was studied. Shown in Table II are some of the results obtained for calf thymus polymerase α. Very similar results were observed with mouse liver polymerase α. Factor D was found to be devoid of detectable stimulatory effect on polymerase β from mouse liver with any of the templates that were tested (results not shown). Whereas copying of activated DNA by polymerase α is not affected by factor D, the rates of utilization of both heat-denatured DNA and singly primed φX174 DNA are enhanced 2.7-fold and 3.8-fold, respectively, by the factor (Table II). The most dramatic stimulatory effect of factor D on polymerase α is observed, however, with a synthetic poly(dT) template: The rate of copying of the poly(dC) strand in the duplex poly(dC)·poly(dG), as well as utilization of the alternating double-stranded polymer poly[d(AT)] and copying of the poly(dA) strand in oligo(dT)·poly(dA), is not affected by factor D. By clear contrast, copying of poly(dT) primed with $oligo(dA)_{12-18}$ and with poly(dA) is stimulated 3-fold and 13.5-fold, respectively, by factor D. Similar preference for poly(dT) template was observed with mouse liver polymerase α, whereas activity of murine polymerase β with poly(dT) or any other template was unaffected by the factor (data not shown). In addition to their strong stimulatory effect on the utilization of sparsely primed natural DNA, the extensively described primer-recognition proteins C_1C_2 enhance the activity of polymerase α on synthetic templates that are primed with highly

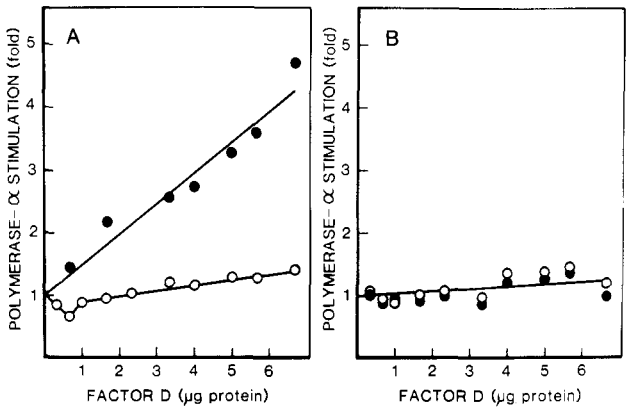

FIGURE 2: Template sequence dependent stimulation of polymerase α activity by factor D. DNA synthesis directed by calf thymus polymerase α (0.8 units per assay) was monitored in the presence of increasing amounts of the P-11 fraction of factor D. Poly(dA)·poly(dT) and poly(dG)·poly(dC) at 40 μg/mL served as templates, and DNA polymerization was conducted for 30 min at 37 °C in the presence of 50 mM KCl, as described under Materials and Methods. Factor D without polymerase α did not exhibit polymerase activity with any of the tested homopolymers. Factor D was stabilized with BSA, which comprised 86% of the added protein. (A) Copying of poly(dA)·poly(dT). Specific activities of dNTP substrates were 311 and 933 cpm/mol of [α-^{32}P]dTTP and [^{3}H]dATP, respectively. Incorporation in the complete absence of factor D (no stimulation) was 0.92 and 2.13 pmol of [^{32}P]dTMP and [^{3}H]dAMP, respectively. Incorporation of [^{3}H]dAMP (●); incorporation of [^{32}P]dTMP (○). (B) Copying of poly(dG)·poly(dC). Specific activities of dNTP substrates were 467 and 1774 cpm/pmol of [α-^{32}P]dCTP and [^{3}H]dGTP, respectively. Incorporation in the complete absence of factor D (no stimulation) was 2.16 and 2.19 pmol of [^{32}P]dCMP and [^{3}H]dGMP, respectively. Incorporation of [^{3}H]dGMP (●); incorporation of [^{32}P]dCMP (○).

dilute complementary oligonucleotides (Pritchard & DePamphilis, 1983; Pritchard et al., 1983). We find that, in contrast to C_1C_2, factor D exerts maximum stimulatory effect on utilization of oligo(dA)·poly(dT) at primer to template ratios of 1:2 (w/w) (Table II). Use of this primer at lower primer to template ratio leads to strongly diminishing enhancing effect of the factor (not shown). By analogy, factor D has no effect on the rate of copying of oligo(dG)·poly(dC) at primer to template ratios ranging between 1:2 and 1:200 (w/w) (data not shown). These results distinguish factor D from the previously described C_1C_2 primer-recognition factors. The most characteristic and interesting aspect of factor D activity is its preference for poly(dT) template. To substantiate this property, the following experiment was conducted: Rates of copying by polymerase α of both respective strands of the duplex templates poly(dG)·poly(dC) and poly(dA)·poly(dT) were measured in the presence of increasing amounts of factor D. Simultaneous monitoring of utilization of both strands in each duplex was attained by use of ^{3}H- and ^{32}P-labeled complementary dNTP substrates. Results of this experiment are presented in Figure 2. It is clear that whereas the incorporation of [^{3}H]dAMP is linearly enhanced by increasing amounts of factor D, the utilization of the poly(dA) template strand is not affected by the factor and incorporation of [^{32}P]dTMP remains unaltered in the presence of increasing levels of the stimulatory protein (Figure 2A). Both copying of the poly(dC) and poly(dG) strands in poly(dG)·poly(dC) are essentially unaffected by factor D as reflected by the unchanged rates of incorporation of [^{3}H]dGMP and [^{32}P]-dCMP, respectively (Figure 2B). We conclude that factor D possesses specific stimulatory activity only with stretches of poly(dT) template among all the synthetic templates that were examined.

Additional Characterization of Factor D. (*A*) *Factor D Is a Protein.* Phosphocellulose-purified factor D was incubated at 37 °C with 0.08–0.33 mg/mL trypsin for 60 min. Proteolysis was terminated with the addition of 5-fold excess (w/w) of pancreatic or soybean trypsin inhibitors, and stimulation of utilization of denatured DNA by calf thymus polymerase α was determined. At the range of trypsin concentrations used, this protease inactivated factor D by 83–92%, whereas trypsin inhibitors alone had no effect on the factor's enhancing ability. That factor D behaves as a protein was further supported with the finding that it is heat-labile: In the presence of 0.3 mg/mL BSA, factor D is inactivated, with a half-life time of 7 min upon heating at 46 °C; it is also 100% inactivated within 10 min by heating by 51 °C (data not shown).

(*B*) *Molecular Size.* Gel filtration of DE-52 or phosphocellulose fractions of factor D on a Bio-Sil TSK-125 column yielded a major peak of stimulatory activity at an apparent size of 10 kDa and a secondary peak at 20 kDa.

(*C*) *Sensitivity to Inhibitors.* Factor D was found to be completely resistant to the sulfhydryl group interactive agent MalNet. The DNA–cellulose fraction of factor D was incubated at 4 °C for 15 min in the presence of 0.06–10.0 mM MelNet, and 20 mM DTT was subsequently added for an additional 15 min to complex the free MalNet. Polymerase α was added to monitor the stimulatory activity of factor D with denatured DNA. No detectable decrease in the stimulatory activity of the factor was observed at any concentration of MalNet, whereas partially purified polymerase α was completely inactivated by 4–10.0 mM MalNet under the same incubation conditions. The activity of factor D was also unaffected by calcium ions. Isolated factor was incubated at 4 °C for 15 min with 0.1–3.1 mM $CaCl_2$, and a 4-fold excess of EGTA was added for an additional 15 min to complex free ions. No loss of factor D stimulatory activity was detected at all $CaCl_2$ concentrations, whereas purified polymerase α was 60% inactivated by 1.5 mM $CaCl_2$, in line with a previous observation of Eichler et al. (1977).

(*D*) *Factor D Is Devoid of Detectable Deoxyribonuclease.* It has been shown that limited endonucleolytic digestion that creates 3′-OH primer termini renders self-primed (loop-back) denatured DNA a more efficient template for polymerase α (Wang et al., 1976). To examine whether factor D contains endonuclease activity, 0.2 units of P-11 fraction of the factor was incubated under assay conditions for stimulatory activity with circular ϕX174 DNA (see Materials and Methods). Upon transfection of the treated phage DNA into *E. coli* spheroplasts, no discernible decline in phage plaque formation was observed. The sensitivity of the assay allowed us to determine that in the presence of factor D less than one phosphodiester bond in 5×10^6 molecules of ϕX174 DNA was hydrolyzed. With both tritium-labeled duplex and denatured DNA substrates, it was found that less than 0.5×10^{-3} nmol of [^{3}H]dNTP was exonucleolytically rendered acid-soluble per each nanomole of dNMP incorporated by polymerase α in the presence of 1 unit of factor D. Lastly, to critically test whether factor D activates denatured DNA by nucleolytic digestion or by any other irreversible modification, the template was preincubated with factor D at 37 °C for 30 min, after which the factor was inactivated by either trypsin digestion or by heating at 51 or 100 °C for 10 min. It was found that denatured DNA that was exposed to factor D was equally efficient as a template for polymerase α as untreated DNA or as DNA preincubated in the absence of factor D. Hence, factor D does not exert its stimulatory activity by nucleolytic

digestion or by any other irreversible change of the template DNA.

(*E*) *Factor D Is Devoid of Detectable DNA Polymerase, Primase, and Terminal Transferase.* To test whether the stimulatory activity of factor D is due to associated DNA polymerase, DNA primase, or deoxyribonucleotidyl terminal transferase activities, these enzymes were assayed in phosphocellulose and DNA–cellulose fractions of factor D. All three activities were undetectable to a level of less than $(0.5–1.0) \times 10^{-3}$ unit of enzyme activity per unit of factor D stimulatory activity.

Discussion

Described in the present work are the partial purification from mouse liver and the characterization of a polymerase α stimulatory protein, designated factor D. The enhancing effect of factor D is similar for polymerases α from calf thymus and from mouse liver. Its major distinguishing property is selectivity for sequence of the copied synthetic DNA template. Of the various homopolymeric and alternating synthetic templates that were tested, only the copying of poly(dT) is stimulated up to 13.5-fold by factor D. In addition, factor D increases the rate of utilization by polymerases α of heat-denatured DNA and of primed, single-stranded φX174 DNA. In contrast, factor D has no discernible effect on the activity of mouse hepatic DNA polymerase β with any tested natural or synthetic template.

Chromatographic behavior (Figure 1), molecular size, thermolability, and inactivation by trypsin (see Results) identify factor D as a protein. Factor D is distinguished from polymerase α both in crude chromatin and after their partial purification by its resistance to MalNet and to inactivation by calcium ions (see Results). Further, it appears that the stimulatory activity of factor D is not due to its association with DNA synthesizing or hydrolyzing enzymes: Partially purified factor D is found to be devoid of detectable levels of DNA polymerase, DNA primase, terminal transferase, and endo- or exodeoxyribonuclease (see Results). In the presence of factor D, the rate of utilization by polymerase α of heat-denatured DNA and of primed circular φX174 DNA is increased 2–4-fold, whereas the copying of activated DNA is unaffected (Table II). Several authors have reported on protein cofactors of polymerase α that increase its activity with denatured DNA (Otto et al., 1977; Richter et al., 1978; Novak & Baril, 1978). Analogous proteins from human cells (Lamothe et al., 1981) and monkey cells (Pritchard & DePamphilis, 1983; Pritchard et al., 1983) have been identified as primer-recognition proteins that increase the affinity of polymerase α to 3′-hydroxyl termini of highly dilute primers on denatured DNA and synthetic templates. Although factor D superficially resembles these proteins in that it stimulates the activity of polymerase α with sparsely primed heteropolymeric DNA, it is clearly distinguishable from any of the previously described cofactors of polymerase α. First, factor D enhancing activity is diminished with the progressive decrease in the ratio of oligo(dA) primer to the poly(dT) template, and it has no detectable effect on the utilization of oligo(dT)·poly(dA) and oligo(dG)·poly(dC) at any primer to template ratio (Table II and accompanying text). It appears, therefore, that factor D does not decrease the K_m of the polymerase to the 3′-OH end of the primer, as do primer-recognition cofactors of polymerase α. More importantly, unlike all previously described enhancer proteins of polymerase α, factor D exhibits clear template-sequence preference in stimulating the utilization by polymerase α of synthetic deoxyribonucleotide polymers. The activity of polymerase α with poly(dT) template is enhanced in the presence of factor D by 3- and 13-fold with oligo(dA) and poly(dA) primers, respectively (Table II). In contrast, factor D has no discernible effect on the rate of copying of poly(dG), poly(dA), and poly(dC) annealed to either oligo- or poly(deoxynucleotide) complementary primers (Table II, Figure 2). In fact, in the presence of factor D or some other protein that copurifies with it, the rate of copying of poly(dA) template is reduced (Table II). It appears, therefore, that factor D enhances only the utilization by polymerase α of stretches of poly(dT) template. This unique characteristic of factor D is further delineated by the observation that factor D has no detectable effect on the rate of copying of the alternating heteropolymer poly[d(AT)] (Table II). Hence, the presence of dispersed dTMP residues in the copied template does not suffice to elicit stimulatory activity of factor D. It could be argued that factor D is an Ap_4A binding protein and that this dinucleotide primes poly(dT) in the primer templates oligo(dA)·poly(dT) and poly(dA)·poly(dT) (Rapaport et al., 1981). However, we find that factor D does not support copying of unprimed poly(dT) and only stimulates copying of primed φX174 but not of unprimed M13 DNA. It appears, therefore, that factor D cannot act as an alternate source of primer and probably does not contain Ap_4A.

It is not clear whether factor D exerts its stimulatory activity by interacting with the polymerase α molecule, with the copied template, or with both. Some interaction between factor and enzyme is indirectly suggested by a change in the thermolabilities of both proteins upon their mixing together (M. Fry, J. Lapidot, and P. Weisman-Shomer, unpublished results). However, as suggested by its moderate DNA binding properties (Figure 1D), it may well be that factor D also interacts with the template DNA. Yet preincubation of factor D with the template does not appear to induce an irreversible modification in the copied template (see Results).

At present, one can only speculate on the biological significance of factor D. It is unclear whether or not the primary in vivo activity of factor D is its enhancing effect on DNA polymerization by polymerase α. One possibility is that factor D interacts with poly(dT)-rich stretches of DNA and melts secondary structures that impede the activity of polymerase α in vitro. Hence, factor D may well be a structural protein that is bound in chromatin to dT-rich tracts in DNA. Such stretches comprise the mouse satellite DNA that constitutes a considerable portion of the genome and whose sequence is CTTTTTAGT (John & Miklos, 1979). A more tempting speculation is that factor D acts in vivo as a replicative cofactor of polymerase α. A wide range of results on the ability of polymerase α to utilize poly(dT) template have been reported [reviewed by Fry (1983)]. It may be that in some cases purification of the polymerase involved removal of factor D and thus selective reduction in the efficacy of poly(dT) copying. Note, for instance, the recent report on the low rate of poly(dT) replication by the isolated catalytic subunit of polymerase α (Karawaya et al., 1984). The intriguing specificity of factor D for poly(dT) synthetic template may indicate that it serves as a template sequence recognition accessory protein for polymerase α. It is not clear, however, whether the stimulation by factor D of copying of heteropolymeric denatured and φX174 DNA is due to specific enhancement of the replication of dTMP-rich tracts in these templates. This question is presently under investigation.

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Oligomers with Alternating Thymidine and 2′-Deoxytubercidin: Duplex Stabilization by a 7-Deazapurine Base†

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ABSTRACT: Self-complementary oligonucleotides with an alternating sequence of 2′-deoxytubercidin and thymidine and a chain length of 6 and 12 monomeric units have been synthesized by employing the phosphoramidite technique on solid support. The modified nucleoside **1a** used in these experiments has been prepared from 4-chloro-2-(methylthio)pyrrolo[2,3-*d*]pyrimidine and the halogenose **6** by applying phase-transfer conditions. The oligomers containing 7-deaza-2′-deoxyadenosine (**1a**) are compared to those having a parent d(A-T) sequence. Replacement of the adenine by the 7-deazaadenine base results in duplex stabilization of the oligomers $d(A\text{-}T)_3$ and $d(A\text{-}T)_6$. This is demonstrated by an increased T_m and a decreased susceptibility of $d(c^7A\text{-}T)_3$ and $d(c^7A\text{-}T)_6$ toward nuclease S1.

In a previous publication (Seela & Kehne, 1985) we have reported the synthesis of an appropriately protected phosphoramidite of 2′-deoxytubercidin (**1a**) that was employed in oligonucleotide synthesis in solution. By this means 2′-deoxytubercidylyl-(3′,5′)-2′-deoxytubercidin has been prepared; it exhibits unusual properties toward nucleoside processing enzymes like nucleases. To extend our studies from di-

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