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Rabbit Liver Factor D, a Poly(thymidine) Template Stimulatory Protein of DNA Polymerases: Purification and Characterization†

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ABSTRACT: Factor D, a DNA binding protein that enhances the activities of diverse DNA polymerases with a common restricted set of templates, was initially characterized in mouse liver but has resisted extensive purification. In this paper, we report that a similar stimulatory activity can be obtained in highly purified form from nuclei of rabbit hepatocytes. The rabbit liver protein increases the rates at which several DNA polymerases copy sparsely primed natural DNA templates and primed synthetic poly(dT), but it has no effect on the rates of copying of activated DNA or of poly(dG), poly(dA), and poly(dC). Direct binding of the purified stimulatory protein to an oligomer that contains a $(dT)_{16}$ base stretch is visualized by retardation of the nucleoprotein complex on nondenaturing electrophoretograms. In the presence of the enhancing factor, Michaelis constants, K_m, of responsive polymerase for singly primed bacteriophage M13 DNA and for poly(dT), but not for poly(dA), are decreased. Product analysis of M13 DNA primer extension indicates that the rabbit factor augments the apparent processivity of DNA polymerase by decreasing the extent of enzyme pausing at a tract of four consecutive thymidine residues in the template. Gel filtration of the native stimulatory protein yields an apparent relative molecular size of 58 ± 2 kilodaltons. Stimulatory activity is readily inactivated by heat or by trypsin digestion, but it is resistant to micrococcal nuclease, *N*-ethylmaleimide, or calcium ions.

Studies on *Escherichia coli* indicate that DNA binding proteins are required for the replication of DNA (Kornberg, 1980). Several DNA binding proteins from animal cells have been shown to enhance the activities of DNA polymerases [reviewed by Chase and Williams (1986), Richter et al. (1986), and Fry and Loeb (1986)]. However, in contrast to such proteins in prokaryotes, the role of single-strand DNA binding proteins in the replication of eukaryotic cell DNA is debatable (Chase & Williams, 1986; Richter et al., 1986). Yet, analysis of the modes of enhancement of DNA polymerase activity by eukaryotic DNA binding proteins affords insight into the molecular details of DNA synthesis. A unique template-selective DNA polymerase stimulating protein, that has been designated factor D, was isolated recently from regenerating liver of the mouse (Fry et al., 1985). By selectively increasing the affinities of DNA polymerases from diverse sources for sparsely primed DNA and for the synthetic template poly(dT), factor D enhances the rates of their copying (Fry et al., 1987a). The murine stimulatory protein has no significant effect, however, on the efficacies of copying of activated DNA or of poly(dA), poly(dG), poly(dC), poly[d(A-T)], or poly[d(G-C)] (Fry et al., 1985, 1987a). It was demonstrated recently that factor D enhances the copying of singly primed bacteriophage M13 DNA by increasing the efficiency at which either *E. coli* pol I or bovine polymerase α traverses a cluster of at least four contiguous thymidine residues in this template (Fry et al., 1987b). Hence, by binding to DNA, factor D decreases the frequency at which polymerases pause at stretches of consecutive dT residues in synthetic or natural templates, thus increasing the apparent processivity of the enzymes (Fry et al., 1987b).

Definitive studies on factor D and on analogous activities require their purification to a very high degree. Purification of factor D has been hindered, however, by the limited amounts of extractable mouse tissue as well as by the instability of the partially purified murine protein. In this paper, we report the isolation and extensive purification of a DNA binding protein from rabbit liver that stimulates DNA polymerases in a template-specific manner analogous to that of mouse factor D.

Materials and Methods

Materials. Tritium- or α-^{32}P-labeled deoxynucleoside 5′-triphosphates ([α-^{32}P]dNTPs), and adenosine 5′-[γ-^{32}P]tri-

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phosphate ([γ-^{32}P]ATP) were products of New England Nuclear, Boston, MA. Unlabeled dNTPs, *N*-ethylmaleimide (MalNet), dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), leupeptin, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), phenyl-Sepharose CL-4B, denatured DNA–cellulose, salmon sperm DNA, poly(deoxyguanosine) [poly(dG)], poly(deoxyadenosine) [poly(dA)], poly(thymidine) [poly(dT)], poly(deoxyadenosine-thymidine) [poly[d(AT)]], bovine serum albumin (BSA), trypsin, soybean trypsin inhibitor, micrococcal nuclease, and S1 nuclease were supplied by Sigma Chemical Co., St. Louis, MO. Pharmacia supplied Sephadex G-75, poly(deoxycytidine) [poly(dC)], oligo(deoxyguanosine) [$(dG)_{10}$], avian myeloblastosis reverse transcriptase (AMV polymerase), and calf thymus DNA polymerase α. *E. coli* DNA polymerase I (pol I) was produced by BRL, Bethesda, MD. Whatman Inc., Kent, U.K., provided (diethylaminoethyl)cellulose (DEAE-cellulose, DE-52) and phosphocellulose (P-11). Bacteriophage M13mp2 circular single-stranded DNA was a gift from Dr. B. Preston, University of Washington, Seattle. Primers to M13 DNA, 5′-d(TCCCAGTCACGACGT)-3′ and 5′-d(GTTTTCCCAGTCACG)-3′, were synthesized by the phosphoramidite method with an Applied Biosystems 380A DNA synthesizer and purified by thin-layer chromatography (Alvarado-Urbina et al., 1981). The oligonucleotide 5′-d[AATTC$(T)_{16}$G]-3′ was similarly synthesized and purified by thin-layer chromatography followed by electrophoresis on a denaturing gel. End labeling with [γ-^{32}P]ATP of 5′-d[AATTC$(T)_{16}$G]-3′ and of the M13 primers and annealing of the latter to the template were described elsewhere (Fry et al., 1987b). Salmon sperm DNA was maximally activated by limited DNase digestion (Loeb, 1969) or was heat denatured as described (Pritchard & DePamphilis, 1983). Phosphocellulose and DNA–cellulose-purified fractions of DNA polymerases α and β, respectively, were prepared from regenerating mouse liver as described by Silber et al. (1985). Homogeneous Novikoff hepatoma DNA polymerase β was kindly contributed by Dr. R. Meyer, University of Cincinnati.

Preparation of Nuclear Extract. Polymerase-stimulating protein was isolated from salt extract of rabbit liver cells. Female rabbits (New Zealand White) were reared at the Technion Faculty of Medicine vivarium and sacrificed to obtain liver tissue when they reached 2–2.5-kg body weight. All the procedures to prepare the extract and to subsequently purify the factor were performed at 4 °C. Livers were washed immediately after their excision in 0.3 M sucrose/4 mM $CaCl_2$, dried lightly, cut with fine scissors, and resuspended in 9 volumes of the same solution. Cells were disrupted by 40 strokes with a "B" type pestle of a Dounce homogenizer, and the extract was filtered through four layers of cheesecloth. Nuclei were collected by centrifugation at 10000*g* for 10 min, and the nuclear pellet was resuspended in an equal volume of 25 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 9.0, 0.8 M NaCl, 0.5 mM DTT, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 μg/mL leupeptin, 1 mM PMSF, and 40% glycerol. The suspension was kept at 4 °C for 15 min with occasional stirring and then Dounce homogenized. The supernatant fraction of a 20 min 12000*g* centrifugation was collected, and the insoluble pellet was extracted twice more by homogenization in 25 mM Tris-HCl buffer, pH 9.0, 0.4 M NaCl, 0.5 mM DTT, 1 mM PMSF, and 40% glycerol. Pooled extract supernatants were chromatographed on a DE-52 column equilibrated in the above extraction buffer to remove residual DNA (Fry et al., 1985), and salt was subsequently removed by an overnight dialysis of the extract against 100–200 volumes of 25 mM Tris-HCl buffer, pH 8.2, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol.

DEAE-cellulose Column Chromatography. Protein that selectively stimulates the copying of poly(dT) by DNA polymerases was resolved from liver DNA polymerase activity by chromatography of the dialyzed nuclear extract on a DE-52 column (Fry et al., 1985). Stimulatory activity free of DNA polymerases was eluted from DE-52 at 0–50 mM KCl (see Results). Fractions that contained the stimulatory protein were pooled and dialyzed overnight against 100 volumes of 25 mM potassium phosphate buffer, pH 8.0, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol.

Phosphocellulose Column Chromatography. After dialysis, the DE-52-purified stimulatory activity was loaded onto a P-11 column and eluted by a gradient of potassium phosphate buffer, pH 8.0, as described for murine factor D (Fry et al., 1985). Enhancing activity which was eluted from the column at 100–200 mM potassium phosphate (see Results) was dialyzed overnight against 200 volumes of 25 mM Tris-HCl buffer, pH 7.6, 4.0 M NaCl, 0.5 mM DTT, and 1 mM EDTA.

Phenyl-Sepharose Column Chromatography. Dialyzed phosphocellulose-purified stimulatory activity was loaded onto a column of phenyl-Sepharose at a ratio of 1 mg of protein/mL packed column volume. The loaded column was washed with 2 column volumes of the dialysis buffer, and elution was conducted by consecutively passing through the column two single-column volume batches each of 2.5, 1.0, 0.5, and 0.0 M NaCl in 25 mM Tris-HCl buffer, pH 7.6, 0.5 mM DTT, and 1 mM EDTA. Each single-column volume fraction was dialyzed overnight against 300 volumes of 25 mM Tris-HCl buffer, pH 8.2, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol. Stimulatory activity which was eluted in the first column volume wash of 1.0 M NaCl exhibited two major polypeptide bands on denaturing polyacrylamide electrophoretograms (see Results), whereas activity eluting in the second 1.0 M NaCl wash was in a mixture of about six polypeptides (results not shown). Thus, only the first fraction was used for experiments described in this report. This preparation of the enhancing protein was 25–40% pure as judged by densitometry of electrophoretograms, assuming the 63-kilodalton (kDa) band to represent factor D. This factor was stable for at least 4 months when stored at −70 °C with or without 0.3 mg of BSA/mL.

DNA–Cellulose Chromatography. Phenyl-Sepharose-purified enhancing factor, which was dialyzed overnight against 200 volumes of 20 mM Tris-HCl buffer, pH 7.6, 25 mM NaCl, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol, was loaded onto a denatured DNA–cellulose column at a ratio of 5–10 μg of protein/mL packed column volume. The loaded column was washed by 2 volumes of dialysis buffer, and subsequent stepwise elution was conducted by 2 column volumes each of 0.15, 0.25, 0.4, and 2.0 M NaCl in 20 mM Tris-HCl buffer, pH 7.6, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol. The stimulatory activity was eluted from the column at 2.0 M NaCl with a flow rate of 0.6 mL/h. The polymerase stimulatory activity in the DNA–cellulose fraction is unstable, and most of the activity was lost after storage for 6 days at −70 °C even in the presence of 0.3 mg of BSA/mL.

Sephadex G-75 Gel Filtration. Phenyl-Sepharose-purified stimulatory protein in 300–500 μL of 25 mM Tris-HCl buffer, pH 8.2, 1 mM EDTA, 0.5 mM DTT, and 20% glycerol was loaded onto a column of 1.5 × 82 cm of G-75 Sephadex that was equilibrated in the same buffer solution. Fractions were collected in the cold and analyzed for the stimulation of *E. coli* pol I catalyzed incorporation of [^{3}H]dAMP into poly(dA)·poly(dT). The column void volume, V_0, was measured

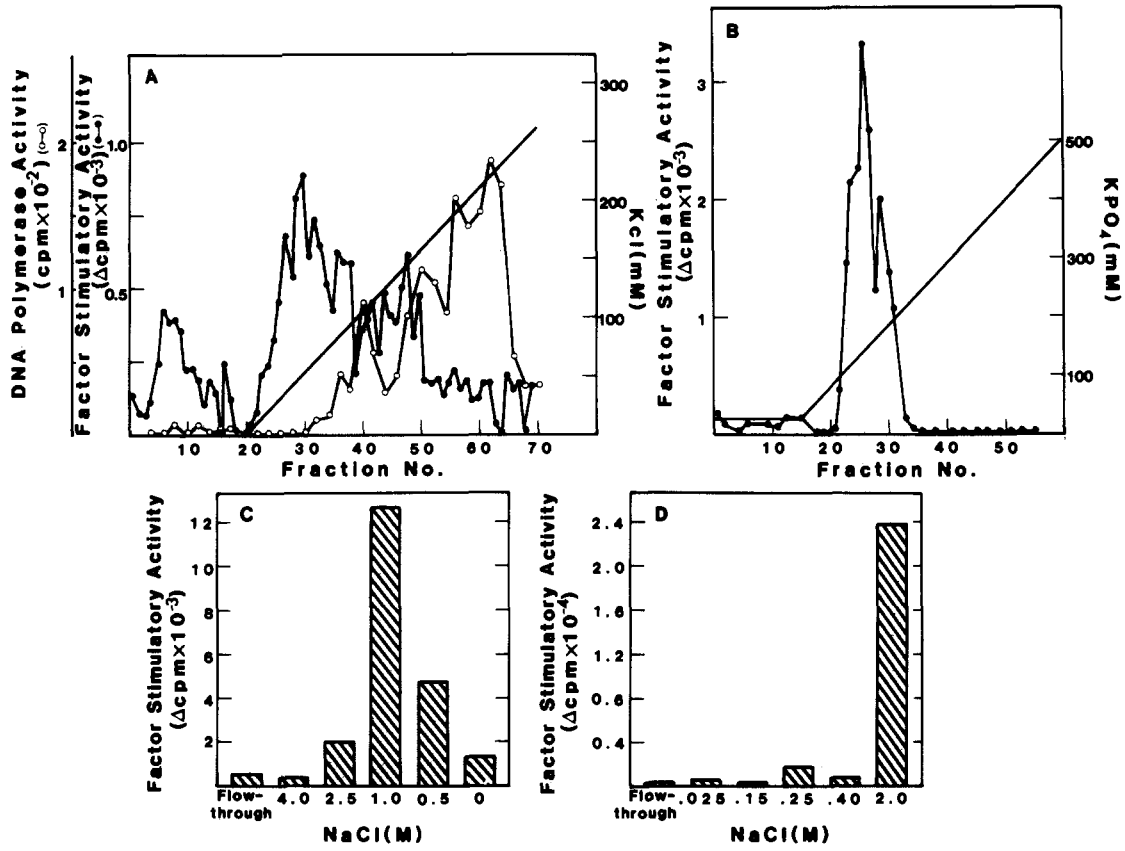

FIGURE 1: Column chromatographic purification of DNA polymerase stimulating protein from nuclei of rabbit hepatocytes. Salt extracts of non-histone nuclear proteins (see Materials and Methods) were loaded onto a DEAE-cellulose column to resolve from rabbit DNA polymerases an activity that enhances *E. coli* pol I catalyzed incorporation of [^{3}H]dAMP into poly(dA)·poly(dT). The isolated enhancing activity which was devoid of polymerase activity was further purified by three subsequent column chromatographic steps. (A) DE-52 chromatogram. Collected fractions were assayed as described under Materials and Methods for net stimulatory activity (●) after subtraction of DNA polymerase activity (○). (B) Phosphocellulose (P-11) chromatogram; (C) phenyl-Sepharose chromatogram; (D) DNA–cellulose chromatogram.

as the exclusion volume of dextran blue, and the molecular weight marker proteins were cytochrome *c* (13.3K), RNase A (12.7K), myoglobin (16.8K), soybean trypsin inhibitor (21.6K), carbonic anhydrase (30.0K), ovalbumin (45K), hemoglobin (65K), and BSA (66K).

Assay Conditions for the Activity of the Stimulatory Protein. Throughout this work, the level of stimulatory protein was measured by the extent of enhancement of the copying of the poly(dT) strand of the duplex poly(dA)·poly(dT) by *E. coli* pol I. Assay conditions were as described for mouse factor D, and units of stimulatory activity were determined from the linear portion of stimulation titration curves using 30–150 ng of protein of phenyl-Sepharose-purified factor per assay. One unit of stimulatory activity is the activity that increases by 1 nmol/h the incorporation of [^{3}H]dAMP into poly(dA)·poly(dT) in a polymerization reaction that is catalyzed by 1 unit of *E. coli* pol I, defined as the activity that incorporates 10 nmol of dNMPs into DNA in 30 min at 37 °C using poly[d(AT)] as primer-template. Stimulation of the extension of 5′-^{32}P-labeled M13 primers was assessed by electrophoresis of DNA synthesis products as described recently (Fry et al., 1978b).

Electrophoresis of Protein–Oligothymidine Complex. Binding of the stimulatory protein to oligo(dT) was monitored by slowed electrophoretic mobility of this oligomer upon formation of a complex with the protein (Fry et al., 1988). Increasing amounts of phenyl-Sepharose-purified stimulatory protein were incubated for 30 min at 4 °C with 30 ng of 5′-[^{32}P]d[AATTC(T)$_{16}$G]-3′ in a final volume of 15 μL of 25 mM Tris-HCl buffer, pH 8.0, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol. The mixtures were then electrophoresed for 135 min at 4 °C on gels of 4% polyacrylamide–0.16% bis(acrylamide) in 10 mM Tris-HCl buffer, pH 8.0, and 1 mM EDTA. Mobilities of free and protein-bound labeled oligomer were visualized by autoradiography of the dried gel.

Analysis of Proteins. Purified enhancing factor was electrophoresed on sodium dodecyl sulfate–10% polyacrylamide slab gels as described by Laemmli (1970). To determine the size of factor D, five electrophoretograms using different preparations of the stimulatory protein were produced. Marker polypeptides were soybean trypsin inhibitor (21.6 kDa), carbonic anhydrase (30 kDa), cross-linked globin dimer (32.5 kDa), ovalbumin (45 kDa), globin trimer (48.8 kDa), globin tetramer (65 kDa), BSA (66 kDa), and globin heptamer (81.2 kDa). Resolved polypeptide bands were stained with ammonical silver (Switzer et al., 1979). The amount of protein was measured with the Bio-Rad protein assay kit.

Assay Conditions for Associated Activities. Levels of DNA polymerases, DNA primase, deoxynucleotidyltransferase, exonuclease, and endonuclease were measured in preparations of the phenyl-Sepharose-purified enhancing factor under conditions for maximum stimulation of DNA polymerase activity as detailed previously (Fry et al., 1985). DNA helix unwinding activity was determined by the method of Koerner and Meyer (1983) as adapted recently (Fry et al., 1987a).

RESULTS

Purification of a DNA Polymerase Stimulating Factor. For the extensive purification of factor D, we used rabbit liver. Extracts of non-histone nuclear proteins were chromatographed consecutively on columns of DE-52, P-11, phenyl-Sepharose, and DNA–cellulose (Figure 1). Stimulatory activity was

Table I: Purification of Factor D from Rabbit Liver[a]

purification step	protein (mg)	act. (units)	sp act. (units/mg)	purification (x-fold)	yield (%)
nuclear extract	1140.0	333.0	0.3	1.0	100.0
DEAE-cellulose	89.1	250.2	2.8	9.3	75.0
phosphocellulose	5.9	120.0	20.3	67.7	36.0
phenyl-Sepharose	0.031	32.2	1040	3460	9.7
DNA–cellulose	0.020	13.3	665	2215	4.0

[a] In this representative purification scheme, 70 g of liver tissue was pooled from two rabbits. Stimulatory activity in different fractions was calculated from the linear portion of the enhancement curve of the copying of poly(dA)·poly(dT) by *E. coli* pol I in the presence of increasing amounts of added protein. Due to high proteolytic and nucleolytic activities in crude whole cell extracts, stimulatory activity was undetectable; the presented purification is, therefore, a minimal estimate relative to activity in the nucleus.

measured by the ability of protein fractions to increase DNA polymerase activity using poly(dA)·poly(dT) as a primer-template. Activity that enhances copying of poly(dT) by pol I is resolved by DEAE–cellulose chromatography of the nuclear extract into two peaks that are eluted from the column in the breakthrough fraction with no salt and by 20–75 mM KCl. In contrast, the rabbit DNA polymerase is eluted at salt concentrations exceeding 70 mM (Figure 1A). Following further purification, the two DE-52-resolved stimulatory peaks were found to possess indistinguishable properties (data not shown). Thus, enhancing activity that elutes at 0–50 mM KCl was routinely pooled for subsequent chromatography on a P-11 column (Figure 1B) followed by phenyl-Sepharose hydrophobic chromatography (Figure 1C) and by DNA–cellulose affinity column purification (Figure 1D). Note that the stimulatory activity binds strongly to DNA–cellulose and requires salt in excess of 0.4 M to be eluted from this column (Figure 1D). Purification data of a typical experiment are compiled in Table I. It was found that whereas the DE-52- and P-11-purified fractions of the stimulatory activity display multiple polypeptide bands on denaturing gel electrophoretograms, phenyl-Sepharose- and DNA–cellulose-purified fractions contain only two polypeptides and a single major polypeptide, respectively (see below). Since the DNA–cellulose fraction of the factor was unstable, as measured by loss of stimulatory activity, during storage at −70 °C, we routinely used for this study the highly purified but nonhomogeneous phenyl-Sepharose preparations. Attempts to stabilize the more pure DNA–cellulose fraction were unsuccessful.

Molecular Size of the Purified Enhancing Factor. To determine the relative molecular size of the native stimulatory factor, phenyl-Sepharose-purified activity was chromatographed on a Sephadex G-75 column. The majority of stimulatory activity exhibits an apparent molecular size of 58 ± 2 kDa as deduced from a gel partition coefficient, K_{av}, of 0.14 (average of three determinations). A minor peak of enhancing activity of 24 ± 3 kDa (K_{av} = 0.29) is also discerned. Denaturing gel electrophoresis of the phenyl-Sepharose-purified activity revealed two predominant polypeptides of 63 and 26 kDa as well as minor bands that intensified after subsequent DNA–cellulose chromatography (results not shown). However, the further purified DNA–cellulose fraction of the enhancing factor displayed a single major polypeptide of 63 kDa which is accompanied by three additional minor polypeptides of 58, 74, and 76 kDa. Gel electrophoresis indicated a tight correlation between the increased specific activity of the factor in the course of its purification (Table I) and the intensification of the 63-kDa polypeptide on gel electrophoretograms (not shown). Furthermore, the distribution of activity eluted from the phenyl-Sepharose column (Figure 1C) is also correlated with the relative intensity of the electrophoresed 63-kDa band (not shown). It is likely, therefore, that the 63-kDa major polypeptide constitutes the enhancing protein. However, although the minor bands seen at 58, 74, and 76 kDa became clearly discernible only after the ultimate DNA–cellulose purification step, we cannot exclude the possibility that the 58-kDa minor polypeptide represents the polymerase stimulator (see Discussion). Since gel filtration and denaturing gel electrophoresis are affected to some extent by properties additional to the protein molecular size, our results only permit us to characterize the rabbit enhancing factor as a monomeric protein of 56–63 kDa.

Table II: Chemical–Physical Properties of Polymerase-Stimulating Factor[a]

treatment	% initial act.	treatment	% initial act.
none	100.0	54 °C, 10 min[d]	3.8
trypsin digestion[b]	4.2	8 mM MalNet[e]	77.7
micrococcal nuclease[c]	98.1	3 mM $CaCl_2$[f]	89.0
48 °C, 10 min[d]	62.0		

[a] Differently treated aliquots of phenyl-Sepharose-purified enhancing factor were assayed for stimulation of copying of poly(dA)·poly(dT) by *E. coli* pol I as described under Materials and Methods. One hundred percent stimulatory activity was 0.1 unit. [b] Factor was incubated for 60 min at 37 °C with 50 μg/mL trypsin. Proteolysis was terminated by addition of 4-fold molar excess of soybean trypsin inhibitor. Undegraded 100% activity was determined for aliquots that were exposed to a mixture of trypsin which was preincubated with excess inhibitor. [c] Aliquots of factor were incubated for 20 min at 37 °C with 33 μg/mL micrococcal nuclease in the presence of 1 mM $CaCl_2$. Hydrolysis was terminated by the addition of 5 mM EGTA. Undigested 100% activity was determined in aliquots that were exposed to 1 mM $CaCl_2$ and then to 5 mM EGTA. [d] Factor was incubated at 4, 48, or 54 °C for 10 min. Activity of 100% is that of the aliquot held at 4 °C. [e] Factor was incubated for 15 min at 4 °C with 8 mM MalNet, and 20 mM DTT was subsequently added for an additional 15 min before assaying stimulatory activity. [f] Factor was incubated with 3 mM $CaCl_2$ for 15 min at 4 °C, and 6 mM EGTA was added for an additional 15 min before assaying the stimulatory activity.

Table III: Measurement of Associated Activities in the Highly Purified Fraction of the Enhancing Factor

measured activity	relative level[a]	measured activity	relative level[a]
DNA polymerase	$\leq 5.0 \times 10^{-3}$	terminal	$\leq 0.3 \times 10^{-3}$
DNA primase	$\leq 0.4 \times 10^{-3}$	deoxy-	
endodeoxyribonuclease[b]	not detected	nucleotidyl	
exodeoxyribonuclease[c]	$\leq 0.5 \times 10^{-3}$	transferase	
		DNA helicase[d]	not detected

[a] Relative level of activities associated with the phenyl-Sepharose fraction of the stimulating factor was calculated as units of associated activity per unit of polymerase enhancing activity. The various activities were assayed as described under Materials and Methods. [b] Endonucleolytic activity was monitored as described (Fry et al., 1985). Less than a single phosphodiester bond in 10^6 molecules of bacteriophage *φX174* DNA was cleaved by 5×10^{-3} unit of the factor. [c] Factor was incubated for 30 min at 37 °C with native or denatured *E. coli* [^{3}H]DNA (3500 cpm/μg). Under these conditions, 2.5 μg of DNase I rendered >95% of the labeled acid soluble, and the factor (1 unit) did not detectably hydrolyze the DNA. [d] Helicase activity was assayed as described under Materials and Methods. No conversion of [^{3}H]DNA duplex into single-stranded DNA was detected after its incubation with 1 unit of factor for 90 min at 37 °C.

Properties of the Highly Purified Enhancing Factor. As seen in Table II, the enhancing activity is resistant to nuclease treatment, but it is inactivated by trypsin digestion and by heating at 48 or 54 °C. Results summarized in Table II also indicate that the rabbit stimulatory protein is insensitive to either MalNet or Ca^{2+} ions. Activities of DNA polymerases, deoxynucleotidyl terminal transferase, DNA primase, endo-

Table IV: Enzyme and Template Specificities of Rabbit Liver Factor D[a]

	DNA template and dXTP substrate											
	activated DNA, [^{3}H]dATP + dNTP		denatured DNA, [^{3}H]dATP + dNTP		primed M13DNA, [^{3}H]dATP + dNTP		poly(dA)·poly-(dT), [^{3}H]dATP + dTTP		poly(dA)·poly-(dT), [^{3}H]dTTP + dATP		oligo(dG)·poly-(dC), [^{3}H]dGTP + dCTP	
DNA polymerase	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)
E. coli pol I	217.4	228.8 (1.05)	37.5	193.9 (5.2)	11.5	69.0 (6.0)	0.2	20.4 (102.0)	6.8	12.0 (1.8)	159.0	168.4 (1.2)
AMV pol	28.5	45.2 (1.6)	3.4	14.2 (4.2)	0.14	0.94 (6.7)	0.2	1.9 (9.5)	3.9	5.1 (1.3)	172.7	113.0 (0.7)
calf thymus pol α	2.8	3.1 (1.1)	1.0	5.5 (5.5)	0.35	2.9 (8.3)	ND	ND	ND	ND	13.5	9.0 (0.7)
mouse pol α	1.9	2.7 (1.4)	0.13	0.62 (4.8)	0.13	0.25 (1.9)	0.02	0.11 (5.5)	0.4	0.6 (1.5)	3.5	8.0 (2.3)
Novikoff pol β	10.4	5.9 (0.6)	0.45	0.17 (0.4)	1.2	0.25 (0.2)	0.22	0.22 (1.0)	0.5	0.16 (0.3)	33.2	38.4 (1.2)

[a] Rates of copying of native and synthetic DNA templates by the different DNA polymerases were determined in the presence (+) or absence (−) of saturating levels of phenyl-Sepharose-purified factor D. DNA polymerase activity is in units × 10^{-3}. Values in parentheses are x-fold stimulation of DNA synthesis by the factor. Presented values are averages of duplicate assays. ND = not determined. Final concentrations of native and synthetic DNA templates in the reaction mixture were as follows: activated DNA, 0.5 mg/mL; denatured DNA, 0.2 mg/mL; poly(dA)·poly(dT), 40 μg/mL; oligo(dG)·poly(dC), 40 μg/mL; at a primer:template molar ratio of 2:1. Hybridization of M13 DNA with 15-mer primer (75 μg of DNA/mL) was performed at a primer:template molar ratio of 3:1.

nuclease, and DNA helix unwinding protein were not detected in the phenyl-Sepharose-purified preparation of the enhancing protein (Table III). Maximum possible levels of these associated activities, as presented in Table III, merely indicate the sensitivity of each assay. Considering the insignificant levels of associated activities, it appears improbable that stimulation of polymerases by the rabbit hepatic protein is due to its association with any one of these known enzymes.

Hepatic Factor Enhances Diverse DNA Polymerases To Copy a Common Limited Set of Templates. Enzyme and template specificities of the rabbit stimulatory activity have been examined. As shown in Table IV, the factor stimulates several DNA polymerases from diverse sources that have varied roles in vivo. Rates of copying of sparsely primed denatured DNA and of singly primed bacteriophage M13 circular single-stranded DNA by AMV polymerase, *E. coli* pol I, and murine and bovine polymerases α are increased 2–7-fold by the rabbit protein, whereas the efficacy of copying of activated DNA remains unchanged (Table IV). Further, the factor stimulates the copying of the poly(dT) strand in the duplex poly(dA)·poly(dT) 5–100-fold, but there is no significant increase in the rate of copying of the poly(dA) strand or on the efficiency of utilization of oligo(dG)·poly(dC) (Table IV). It is also found that the rabbit protein does not change significantly the rates of copying of poly[d(AT)] or of poly-(dG)·poly(dC) by any of the tested enzymes (results not presented). Note, however, that not every polymerase is affected by the enhancing factor and that the activities of Novikoff hepatoma DNA polymerase β (Table IV) and of mouse polymerase β (not shown) remain unchanged in its presence.

Stimulatory Factor Is a DNA Binding Protein That Forms a Complex with Oligothymidine. As demonstrated previously (Figure 1D), the enhancing factor behaves as a typical DNA binding protein that requires salt in excess of 0.4 M to be eluted from DNA–cellulose. To further establish its binding to DNA, the enhancing factor was incubated at 4 °C with denatured DNA and then passed through a DEAE-cellulose column under conditions that were described previously (Fry et al., 1987a). Whereas the enhancing activity is normally weakly retained by DEAE-cellulose (Figure 1A), DNA is strongly absorbed to this anion exchanger and requires salt in excess of 1 M to be eluted from it (Fry et al., 1987a). After being preincubated with DNA, the stimulatory protein becomes tightly bound to DE-52 and can be eluted from it only by 250 mM KCl (results not shown). When the DNA is digested by micrococcal nuclease prior to DE-52 chromatography, factor D activity elutes, as normally, at 0–70 mM KCl (data not shown). It is assumed, therefore, that binding of factor D to DNA is responsible for the tighter adsorption of

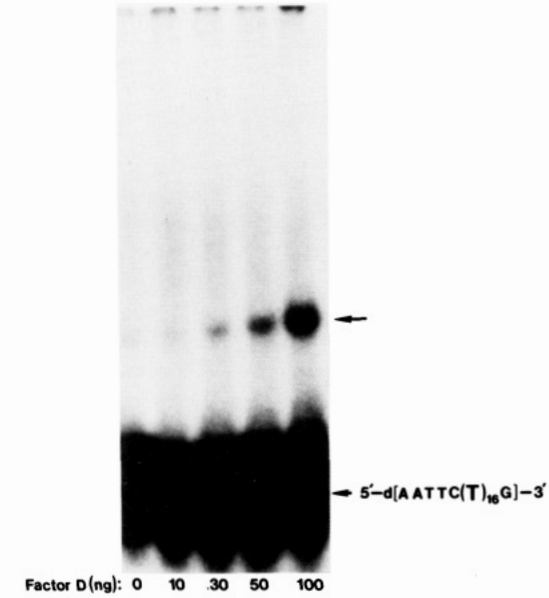

FIGURE 2: Complex formation between the stimulatory protein and the oligomer 5′-d[AATTC(T)$_{16}$G]-3′. Aliquots of phenyl-Sepharose enhancing protein at the indicated amounts were incubated for 30 min at 4 °C with 30 ng of 5′-[^{32}P]d[AATTC(T)$_{16}$G]-3′ in a total volume of 15 μL of 25 mM Tris-HCl buffer, pH 8.0, 0.5 mM DTT, 1 mM EDTA, and 20% glycerol. The samples were subsequently electrophoresed at 4 °C on a nondenaturing polyacrylamide gel as described under Materials and Methods. Shown are autoradiograms of the migration of unbound oligomer and of the retarded protein-bound oligomer (marked by an arrow).

the protein to DE-52. A direct demonstration of the formation of a complex between the stimulatory protein and oligothymidine is provided by retarded electrophoretic mobility of the oligomer on nondenaturing gels in the presence of the enhancing protein. Increasing amounts of the phenyl-Sepharose fraction of the stimulating protein were incubated at 4 °C with 5′-[^{32}P]d[AATTC(T)$_{16}$G]-3′, and the mixtures were electrophoresed on nondenaturing polyacrylamide gels. As seen in Figure 2, an increasing proportion of the labeled oligomer exhibits retarded electrophoretic mobility when mixed with progressively increasing amounts of the enhancing factor.

Enhancing Protein Selectively Decreases the K_m of E. coli Pol I for Poly(dT). Selective stimulation by the rabbit hepatic factor of the copying of only the poly(dT) strand of poly-(dA)·poly(dT) was confirmed by demonstrating that the incorporation of [^{3}H]dAMP, but not of [^{32}P]dTMP, is increased when the double label is added to reaction mixture in which both strands of the duplex are copied either by *E. coli* pol I or by AMV polymerase (data not shown). To analyze ki-

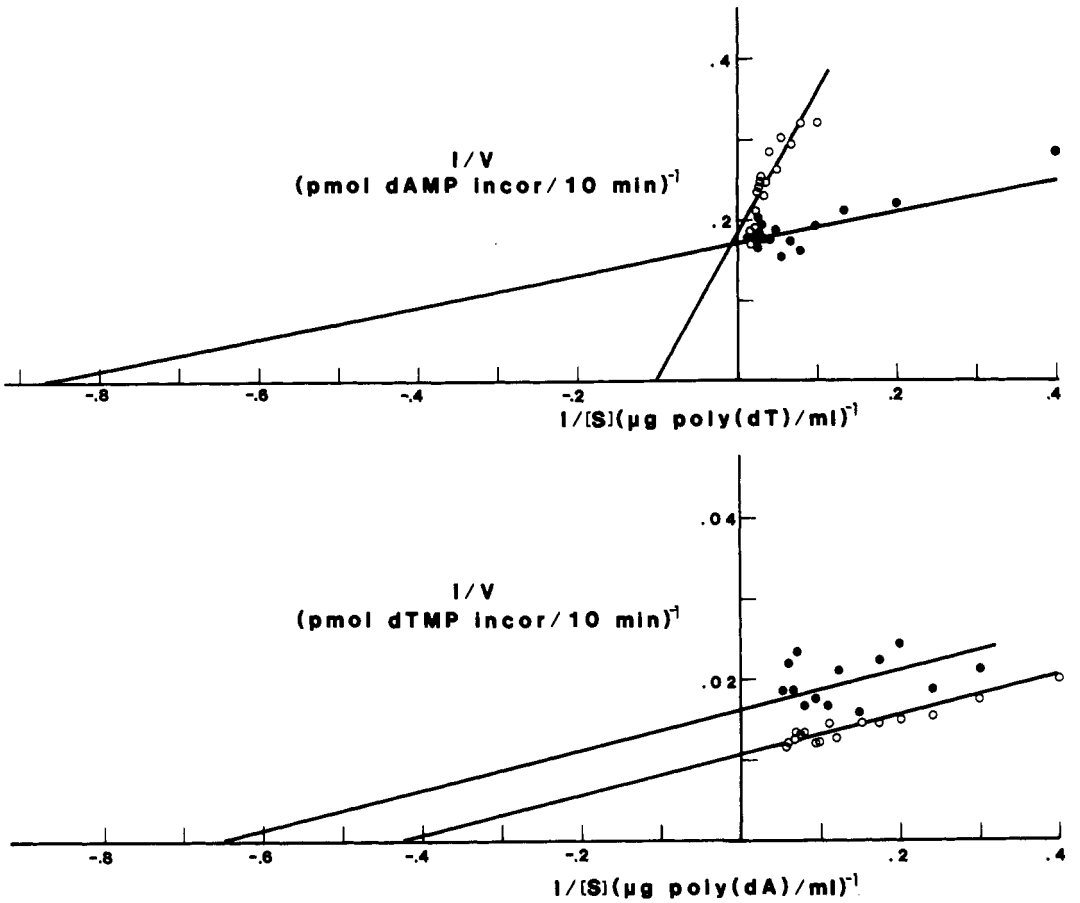

FIGURE 3: Effect of the enhancing protein on the kinetics of copying of poly(dT) and of poly(dA) template strands. *E. coli* pol I (2×10^{-2} unit/50-μL reaction mixture) copied each of the two complementary strands of poly(dA)·poly(dT) in parallel sets of assays that contained either [^{3}H]dATP (620 cpm/pmol) or [^{3}H]dTTP (473 cpm/pmol). Series of assay mixtures with decreasing amounts of poly(dA)·poly(dT) were incubated without or with saturating amounts of phenyl-Sepharose-purified enhancing protein. The final concentration of each copied strand was half the concentration of the 1:1 (w/w) annealed poly(dA)·poly(dT) duplex. (Top) Double-reciprocal plot of copying of the poly(dT) strand with (●) and without (○) enhancing factor. (Bottom) Double-reciprocal plot of copying of the poly(dA) strand with (●) and without (○) enhancing factor.

netically this selective enhancement, values of K_m and of V_{max} of *E. coli* pol I for the poly(dA) and poly(dT) strands were determined in the absence and in the presence of the stimulatory protein. Figure 3 summarizes the results of one of four duplicate experiments which led to essentially the same outcome. As seen in Figure 3, top panel, the K_m of pol I for poly(dT) is decreased by the factor 8.7-fold, declining from 10.0 to 1.15 μg mL^{-1} with no significant change in the maximum velocity of the reaction. By contrast, although the K_m of pol I for poly(dA) is slightly reduced by the factor from 2.4 to 1.55 μg mL^{-1}, the V_{max} also decreases from 95.2 to 60.0 pmol of dTMP incorporated in 10 min with a net effect of a slightly decreased rate of poly(dA) copying (Figure 3, bottom panel). Stimulation of the copying of singly primed M13 DNA by the factor is also found to reflect a reduction in the K_m of pol I for this template from 8.3 to 2.5 μg mL^{-1} with no measurable change in the maximum velocity of the reaction (results not shown).

Rabbit Hepatic Protein Enhances Copying of M13 DNA by Decreasing Polymerase Pausing at Oligo(dT) Template Tracts. To examine whether enhancement by the liver factor of the copying of heteropolymeric DNA and of poly(dT) shares a common mechanism, product analysis of M13 DNA copying was employed to study the sequence specificity of the stimulation of heteropolymeric DNA synthesis. Circular single-strand bacteriophage M13mp2 DNA was primed by 5′-[^{32}P]d(TCCCAGTCACGACGT)-3′ or by 5′-[^{32}P]d-(GTTTTCCCAGTCACG)-3′ which are positioned four and eight bases, respectively, upstream to a tract of four contiguous thymidine template residues. The primed templates were copied for 10 or 20 min by *E. coli* pol I in either the absence or the presence of phosphocellulose-purified stimulatory protein. Products of the DNA primer extension reactions were resolved by DNA sequencing electrophoresis. As seen in Figure 4, progress of pol I along the single-strand template is strongly retarded at the $(dT)_4$ base cluster when it is located four or eight bases downstream to the 3′-hydroxyl terminus of the primer. However, in the presence of the enhancing protein, the polymerase stops less frequently at the $(dT)_4$ base stretch, and there is synthesis of longer DNA products. Note also that although the phosphocellulose fraction of the stimulating protein, which was used in this experiment, was probably slightly contaminated with exonucleolytic activity which trimmed the unextended primer (Figure 4), the factor was still clearly capable of inducing accumulation of longer DNA products past the $(dT)_4$ tract. Comparison of the effect of the factor on elongation of the two differently positioned primers also reveals that in addition to its permissive action on the traversion of the $(dT)_4$ base cluster by pol I, it also decreases polymerase pausing at bases which are immediately adjacent to the primer terminus regardless of their sequence (Figure 4).

Discussion

In the past, we have described the partial purification and characterization of factor D, a DNA binding protein from

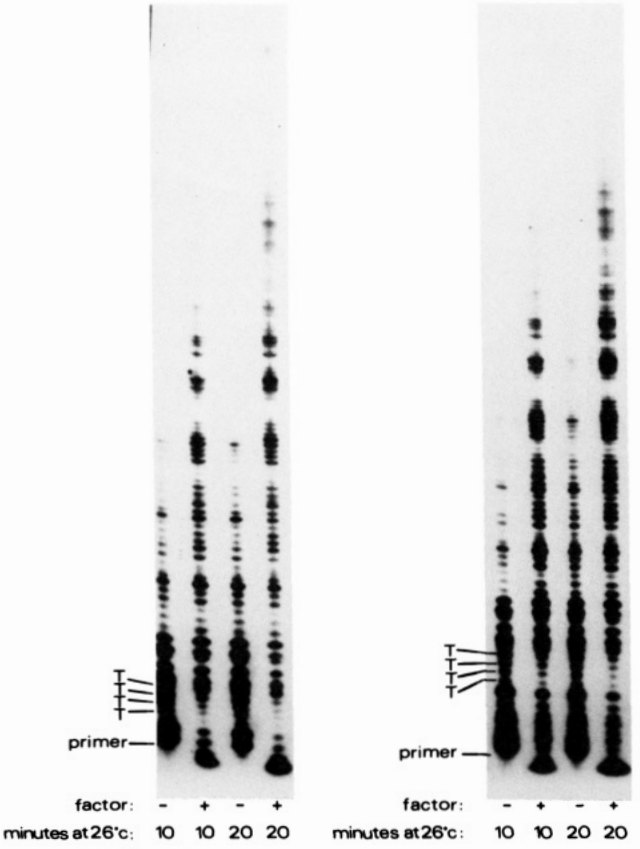

FIGURE 4: Effect of the enhancing protein on the copying by *E. coli* pol I of M13mp2 DNA primed by two differently positioned complementary oligomers. DNA from bacteriophage M13mp2 was annealed to 5-^{32}P-labeled 15-mer primers which complement this template four and eight bases upstream to a $(dT)_4$ base tract (see Materials and Methods). The two differently primed templates were copied at 26 °C for 10 or 20 min by 2.7 units/mL *E. coli* pol I and with 0.0 or 0.3 μg of phosphocellulose-purified stimulatory factor. Primer extension products were resolved by gel electrophoresis and detected by autoradiography, and their base sequence was determined as described (Fry et al., 1987b). The position of the $(dT)_4$ template base cluster relative to the first (left) and second (right) primer termini is indicated.

mouse liver that stimulates diverse DNA polymerases to copy a common restricted set of DNA templates (Fry et al., 1985, 1987a,b). The present paper reports the extensive purification of an analogous enhancing protein from hepatic tissue of a different mammal, the rabbit. The large amount of liver tissue afforded in the rabbit allows purification of the DNA polymerase stimulatory protein to a high degree (Figure 1, Table I). The apparent relative molecular weight of the highly purified factor as deduced from gel filtration is 58K ± 2K (see Results). Upon denatguring gel electrophoresis of the phenyl-Sepharose-purified fraction of the enhancing factor, two predominant polypeptides of 63 and 26 kDa are discerned (R. Sharf and P. Weisman-Shomer, unpublished results). To determine whether the native protein is a monomer of a single 56–63-kDa polypeptide chain or rather a dimer of two 26-kDa polypeptides, it was further purified by DNA–cellulose column chromatography which resulted in complete removal of the 26-kDa polypeptide and maintenance of only the 63-kDa species in the active fraction. However, the DNA–cellulose-purified preparation of the active protein also contains additional minor polypeptides of 58, 74, and 76 kDa. These three minor bands became definitively detectable only after the ultimate DNA–cellulose chromatographic step whereas the 63-kDa polypeptide consistently intensified during progressive purification of the enhancing activity. Further, the amount of the 63-kDa species was positively correlated with the level of stimulatory activity in the various fractions which were eluted by decreasing salt concentration from phenyl-Sepharose (R. Sharf, P. Weisman-Shomer, and M. Fry, unpublished results). It is most likely, therefore, that the major 63-kDa polypeptide represents the active stimulatory protein although we cannot unambiguously exclude the possibility that the 58-kDa minor species consists this activity. Whichever the active polypeptide is, however, it is clear that the enhancing factor from rabbit hepatocytes is a monomeric protein of 58–63 kDa. Gel filtration of mouse factor D on a BioSil column resolved activity peaks of 10 and 20 kDa (Fry et al., 1985). To reevaluate the discrepant molecular sizes of the otherwise similar murine and rabbit factors, the two proteins were chromatographed in sequence on the same Sephadex G-75 column. It was observed that in contrast to the rabbit protein that migrated as a (58 ± 2)-kDa molecule, mouse factor D exhibited on the same column an apparent size of 37 kDa (R. Sharf and M. Fry, unpublished results). The different molecular sizes exhibited by mouse factor D on BioSil and on Sephadex columns may reflect an unusual shape of the molecule or can result from limited proteolysis of the stimulatory protein into fragments that maintain enhancing activity. It is obvious, however, that filtration of the rabbit and mouse proteins on even the same molecular sieve yields discrepant molecule sizes. In contrast to their different molecular sizes, all the other tested properties of the rabbit and mouse proteins appear indistinguishable.

In semblance with mouse factor D, the rabbit enhancing protein is also a DNA binding protein (Figure 1D) that forms a complex with oligothymidine (Figure 2). Elsewhere, we show that the enhancing protein from rabbit liver requires stretches of oligo(dT) for binding to single-stranded DNA oligomers and that it does not bind significantly to double-stranded DNA or to single-stranded oligomers that do not contain contiguous thymidine residues (Fry et al., 1988). Both murine and rabbit proteins similarly increase the efficiency at which AMV polymerase, *E. coli* pol I, and mammalian polymerases α copy sparsely primed DNA and poly(dT). However, neither affects the rates of copying of activated DNA or of any other synthetic polydeoxynucleotide [compare Fry et al. (1987a) and Table IV of this work]. Interestingly, both mouse factor D (Fry et al., 1985, 1987a) and rabbit factor (Table IV) do not significantly stimulate the copying of any tested template by DNA polymerases of the β class. Further, in resemblance to mouse factor D (Fry et al., 1987a), the enhancing protein from rabbit liver decreases K_m values of *E. coli* pol I for sparsely primed DNA and for poly(dT) but not for poly(dA) (Figure 3). Most importantly, mouse and rabbit factors both appear to enhance the copying of heteropolymeric DNA by specifically increasing the efficiency at which polymerases traverse clusters of oligo(dT) in the template. As demonstrated in Figure 4, the rabbit factor alleviates a $(dT)_4$ replication barrier in bacteriophage M13 DNA whether this base cluster is located four or eight bases downstream to the primer's 3′-hydroxyl terminus. A similar sequence-specific enhancing effect has been reported recently for mouse factor D (Fry et al., 1987b). Hence, by decresing polymerase pausing at a specific site on the template, the length of product DNA is increased. We do not believe, however, that the augmented apparent processivity is due to an increase in the enzyme's inherent processivity. Rather, by binding to the template, factor D allows the polymerase to traverse $(dT)_n$ clusters at a higher efficiency, and longer DNA product molecules are accumulated. It is also noted that in addition to their sequence-selective per-

missive action, both the rabbit factor (Figure 4) and mouse factor D (Fry et al., 1987b) enhance the polymerization of the first few nucleotides immediately adjacent to the primer terminus without apparent sequence dependency.

Evidence has been gathered, therefore, to show that both mouse and rabbit hepatic tissues contain similar DNA binding proteins that enhance the activities of several DNA polymerases by increasing their ability to processively traverse sequences of a number of contiguous thymidine template residues. Despite their considerble similarity, the murine and rabbit proteins differ in their molecular sizes, and the precise relationship between these two analogous proteins remains to be determined. Efforts are presently made to raise monospecific antibodies against the highly purified rabbit factor in order to study its possible cross-antigenicity with mouse factor D.

Factor D from either mouse or rabbit cells clearly differs from other polymerase-enhancing DNA binding proteins that were characterized in mammalian cells. First, stimulation of DNA polymerases by factor D is not species specific, and activities of several heterologous polymerases are affected by this protein (Fry et al., 1987a; Table IV in this paper). By contrast, enhancement of DNA synthesis by a murine single-strand DNA binding protein (Otto et al., 1977), calf thymus UP-1 (Herrick & Alberts, 1976), and mouse HDI (Detera et al., 1984) is restricted to polymerase α. Second, whereas several DNA binding proteins enhance polymerase activity through destabilization of duplex DNA (Herrick & Alberts, 1976; Bonne et al., 1979; Planck & Wilson, 1980), factor D is devoid of helicase activity (Fry et al., 1987a; Table III in this paper). Most interestingly, factor D is the only mammalian DNA binding protein, of which we are aware, that selectively binds to oligo(dT) stretches and exclusively enhances their copying by polymerases in both natural and synthetic DNA templates (Fry et al., 1985, 1987a,b, 1988; this paper). Last, factor D clearly differs from the recently described polymerase δ stimulatory protein cyclin/PCNA (Tan et al., 1986; Bravo et al., 1987; Prelich et al., 1987). Cyclin is a dimer of two 37-kDa polypeptides that enhances the copying of poly(dA) template strand by only polymerase δ without having an effect on *E. coli* pol I or polymerase α (Tan et al., 1986). Further, whereas the expression of cyclin is regulated during the cell cycle (Bravo et al., 1987; Prelich et al., 1987), factor D activity remains essentially the same in chromatin from quiescent and dividing mouse hepatocytes (Z. Renart and M. Fry, unpublished results).

The presence in two mammalian species of proteins with the same sequence specificity of DNA binding and of polymerase stimulation may allude to the importance of their role in vivo. Even short stretches of oligo(dT)·oligo(dA) within heteropolymeric DNA have an unusual conformation which induces bending of the double helix (Koo et al., 1986; Hagerman, 1986; Griffith et al., 1986). Such homogenic base clusters are widespread in the mammalian genome and have been noted, for instance, in mouse satellite DNA (Horz & Altenburger, 1981) and in the nontranscribed regions of ribosomal DNA genes (Kuehn & Arnheim, 1983). Factors of the type observed in the mouse and rabbit might interact specifically with such tracts in order to change their unusual conformation in preparation for their efficient replication. However, such specific interaction may also serve to organize these sequences for compact packaging in chromatin or, alternatively, to change their accessibility to other affector molecules.

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Registry No. Pol I, 9012-90-2; poly(dA), 25086-81-1; poly(dT), 25191-20-2.

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