Purification and Characterization of Primer Recognition Proteins from HeLa Cells†

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ABSTRACT: We have purified to homogeneity the primer recognition proteins (PRP) from human HeLa cells. PRP is associated with DNA polymerase α complex in HeLa cells. Purified PRP is free of DNA polymerases α, β, and δ, deoxyribonuclease, DNA primase, ATPase, topoisomerase, and DNA ligase activities. The protein structure of the PRP was defined by sodium dodecyl sulfate gel electrophoresis, which revealed two polypeptides of 36 000 Da (PRP 1) and 41 000 Da (PRP 2). The two polypeptides are associated in a complex in the native state. The Stokes radius of the PRP complex by gel filtration is 40.5 Å and the sedimentation coefficient in glycerol gradients is 5.7 S. Purified PRP, which exhibits no DNA polymerase activity, completely restores the activity of DNA polymerase α on templates with low primer to template ratios such as heat-denaturated DNA, poly(dA)–oligo(dT), and singly primed M13 single-stranded DNA. Experiments using various amounts of PRP, DNA polymerase α, and DNA indicate that a concentration dependence exists between these components in the DNA replication process. Amino acid composition analysis indicates that the PRP is rich in hydrophobic amino acids.

DNA replication in eukaryotes is a tightly regulated process that occurs only in the S phase of the cell cycle. It involves multiple stages during which many protein–protein and protein–DNA interactions are involved (Kornberg, 1980, 1988a; Campbell, 1986). A prerequisite in elucidating the mechanisms and regulation of eukaryotic DNA replication is the identification and characterization of the important protein participants in this process. Considerable evidence suggests DNA polymerase α to be a key enzyme in DNA replication (Wong et al., 1989). Recently, another eukaryotic DNA polymerase, DNA polymerase δ, has also been implicated to be involved in the DNA replication process (Wong et al., 1989). In the past few years, investigations into the mechanism of DNA replication in both prokaryotes and eukaryotes have led to the proposal of a dimeric DNA polymerase complex catalyzing the replication of both the leading and lagging strands of a replication fork (McHenry, 1988). Utilizing the simian virus 40 in vitro DNA replication system, models of the eukaryotic replication fork have been developed with DNA polymerase δ as the leading strand replicase and DNA polymerase α as the lagging strand replicase (Fairman et al., 1988).

DNA replication is semidiscontinuous, with continuous DNA synthesis on the leading strand and discontinuous synthesis on the lagging strand. Discontinuous synthesis involves the repeated initiation of Okazaki fragments an average of once every 135–150 bases (Hay & DePamphilis, 1982) and requires DNA polymerase α to initiate DNA synthesis on the RNA primer made by the DNA primase, utilizing the exposed single-stranded DNA as template. Thus, a lower primer to template ratio exists on the lagging strand of the replication fork. While DNA polymerase α is efficient in copying substrates with high primer to template ratios, such as DNase I activated DNA, it is very inefficient in copying substrates with low primer to template ratios, such as denatured DNA (Lamothe et al., 1981; Pritchard and DePamphilis, 1983; Novak and Baril, 1978). A multiprotein complex of DNA polymerase α, namely, DNA polymerase α_2, was isolated (Vishwanatha et al., 1986a) that can utilize templates with low primer to template ratios as effectively as DNase I activated DNA. This is due to the presence of two cofactor proteins, which have been called primer recognition proteins (Pritchard et al., 1983).

Primer recognition proteins have been identified in HeLa cells (Novak & Baril, 1978; Lamothe et al., 1981; Vishwanatha et al., 1986a) and monkey CV1 cells (Pritchard & DePamphilis, 1983; Pritchard et al., 1983). In the presence of primer recognition proteins, selection of template initiation sites and the lengths of RNA primers synthesized by DNA primase–polymerase α were shown to be strongly affected (Vishwanatha et al., 1986b).

In this report, we describe the purification of the primer recognition proteins to homogeneity and the physical and enzymatic characterization of the polypeptides, in an attempt to understand the physiological role of these proteins in DNA replication.

Experimental Procedures

Materials. Unlabeled ribo- and deoxyribonucleotides were from P-L Biochemicals and Boehringer-Mannheim and [^{3}H]dTTP was from ICN Radiochemicals. Calf thymus DNA was from Calbiochem-Behring. Pancreatic DNase I and *E. coli* DNA polymerase I were from Boehringer. DEAE-cellulose (grade 52)[1] and phosphocellulose (P-11) were from Whatman. The Superose-12 FPLC column was from Pharmacia. DNA-cellulose was prepared as described (Alberts and Herrick, 1971) with heat-denatured or native calf thymus DNA. Pancreatic DNase I activated calf thymus DNA was prepared according to a published procedure (Baril et al., 1977). Heat-denatured DNA was prepared from purified calf

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[1] Abbreviations: DEAE-cellulose, [(diethylamino)ethyl]cellulose; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PRP, primer recognition proteins; CAPS, 3-(cyclohexylamino)propanesulfonic acid; PEG, poly(ethylene glycol); PCNA, proliferating cell nuclear antigen.

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thymus DNA under conditions critical for observing the maximum stimulation of DNA polymerase α by the stimulatory factors as described (Pritchard & DePamphilis, 1983). Bacteriophage M13 virion DNA was prepared according to published procedures (Sambrook et al., 1989) and singly primed M13 single-stranded DNA was prepared by annealing the sequencing primer (17-mer) at a 3-fold excess to the virion DNA. Homopolymer template primers were from P-L Biochemicals.

Cell Culture. HeLa S3 cells were maintained in suspension cultures in Joklik's modified Eagle's medium supplemented with 5% defined iron-supplemented calf serum (Hyclone) at 37 °C. Cells were harvested at a density of (0.5–1.0) × 10^6 cells/mL, while they were in the log phase of growth. Cells were monitored for mycoplasma contamination by using a Mycotrim TC detection system (Hana Biologics, Inc.). Cell pellets were rinsed with Hank's balanced salt solution twice and stored at −80 °C.

Preparation of Cell Extract. Frozen cell pellets were rapidly thawed at 30 °C and cell extract was prepared by a modification of the procedure of Weaver and DePamphilis (1982). All operations were performed at 4 °C. Briefly, the cell paste was resuspended in 4 volumes of 10 mM Tris-HCl, pH 7.8, 50 mM potassium acetate, 0.5 mM magnesium acetate, 1 mM EDTA, 1 mM PMSF, 0.5 mM DTT, and 10% (v/v) glycerol. Cells were homogenized by 30 strokes with pestle B of a Dounce homogenizer. Nuclei were separated from the cytoplasmic fraction by centrifugation at 2000*g* for 10 min. The nuclear pellet was resuspended in 3 volumes of 50 mM potassium phosphate buffer, pH 7.2, containing 20 mM KCl and 1 mM DTT, and extracted by the addition of an equal volume of 0.8 M potassium phosphate buffer, pH 7.2, with 1 mM DTT. The extraction was allowed to proceed for 1 h with slow rotation. The soluble nuclear extract was collected by centrifugation in a Beckman SW 28 rotor at 27 000 rpm for 1 h. The postmicrosomal supernatant was prepared from the cytoplasmic fraction by first centrifuging the cytoplasmic fraction at 10000*g* in a Sorvall SS34 rotor for 20 min to separate the mitochondrial pellet and then centrifuging the supernatant in a Beckman 60Ti rotor at 100000*g* for 1 h to separate the microsomal pellet. The postmicrosomal supernatant was combined with the soluble nuclear extract and the KCl concentration was adjusted to 0.5 M by addition of 4 M KCl. This combined extract was passed through a 50-mL column of DEAE-cellulose (DE-52), equilibrated in the same buffer to remove nucleic acids. The DE-52 column was washed with one column volume of buffer. The unbound fraction contained most of the protein and all of the DNA polymerase α and primer recognition protein activities. This served as the starting fraction for further purification.

Immunoaffinity Purification of Pol α. Immunoaffinity purified pol α was prepared by using the protocol of Wold et al. (1989) with some modifications. In initial experiments, the immunoaffinity matrix was prepared as described by Wold et al. (1989). Subsequently, immunoaffinity columns made by coupling pol α specific monoclonal antibody SJK 287-38 to cyanogen bromide activated Sepharose 4B (10 mg of antibody per mL of matrix), following the instructions of the manufacturer, were used. No differences were observed in the performance of these two matrices. HeLa cell pol α_2 (Vishwanatha et al., 1986a) was chromatographed over a 5-mL immunoaffinity column and immunoaffinity-purified pol α was collected as per Wold et al. (1989).

Velocity Gradient Centrifugation. Sedimentation studies were performed in the Beckman SW 50.1 rotor in linear 15–35% (v/v) glycerol gradients containing 50 mM potassium phosphate buffer, pH 7.2, 1 mM EDTA, 1 mM dithiothreitol, and 20 mM KCl (buffer A). The sample load was 200 μL. Centrifugation was carried out for 40 h at 40 000 rpm and 4 °C. Fractions were collected from the bottom of the tube and were assayed for stimulation of DNA polymerase α. In parallel gradients, marker proteins such as catalase (11.0 S), aldolase (7.35 S), bovine serum albumin (4.7 S), and cytochrome *c* (1.8 S) were centrifuged under the conditions described above.

Gel Filtration on Superose 12 FPLC. A prepacked FPLC column (10 mm × 300 mm) of Superose 12 (Pharmacia) was equilibrated and developed in buffer A containing 0.2% Triton X-100. Fractions of 0.5 mL were collected and assayed for activity. The column was calibrated with blue dextran (V_0), aldolase (158 000 Da), bovine serum albumin (67 000 Da), and cytochrome *c* (13 000 Da).

Polyacrylamide Gel Electrophoresis. Electrophoresis under denaturing conditions was performed on 4–15% gradient polyacrylamide separating gel with a 3% stacking gel containing SDS under conditions described by Laemmli (1970). Electrophoresis under nondenaturing conditions was performed essentially by the same procedure as above but without SDS in the electrophoresis and sample buffers. The gels were fixed and stained with silver reagent by the procedure of Morrissey (1981).

Enzyme Assays. DNA polymerases α and β were routinely assayed on pancreatic DNase I activated calf thymus DNA (Baril et al., 1977) at 35 °C for 30 min. DNA polymerase α activity with heat-denatured calf thymus DNA was assayed according to Lamothe et al. (1981). One unit of DNA polymerase α or β activity is defined as 1 nmol of dNTP incorporated per hour at 35 °C.

DNA primase activity was routinely measured according to a previously published procedure (Vishwanatha & Baril, 1986). Exonuclease activity was assayed by using native or heat-denatured *Escherichia coli* [^{3}H]DNA as previously described (Vishwanatha et al., 1986a).

The DNA-dependent ATPase activity was measured by the conversion of [γ-^{32}P]ATP to a charcoal-nonadsorbable form. In a reaction volume of 25 μL, the enzyme fraction was incubated with 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM DTT, 1 mM [γ-^{32}P]ATP (2000–3000 cpm/nmol), and 5 μg of heat-inactivated bovine serum albumin with or without 0.5 μg of DNA as a cofactor. After a 30-min incubation at 30 °C, 75 μL of a 10% (v/v) suspension of activated charcoal in 0.25 N HCl, 0.025 M KH_2PO_4, and 0.025 M $Na_4P_2O_7$ was added and the mixture was allowed to stand on ice for 5 min. After centrifugation at 10000*g* for 5 min, the supernatant was withdrawn and radioactivity was determined by liquid scintillation spectrometry.

Assay for Primer Recognition Proteins. The DNA polymerase α cofactor primer recognition proteins were detected by their ability to stimulate DNA polymerase α on heat-denatured calf thymus DNA template. In a 100-μL reaction, DNA polymerase α was combined with primer recognition proteins and a reaction mix consisting of 50 μg/mL heat-denatured calf thymus DNA, 1 mg/mL heat-inactivated bovine serum albumin, 20 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 1.8% PEG 8000, 100 μM dATP, dCTP, and dGTP, and 50 μM [^{3}H]dTTP (100–200 cpm/pmol). Incubation was at 35 °C for 30 min and the reaction was terminated and processed as for DNA polymerase α. For the purpose of purification, one unit of primer recognition protein activity is defined as a 100% stimulation of DNA polymerase

Table I: Summary of Primer-Recognition Protein Purification[a]

fraction no.	fraction	vol (mL)	protein (mg/mL)	activity		purification (fold)
				total (units)	specific (units/mg)	
1	DEAE-cellulose	92.0	6.3	18276	32	1
2	phosphocellulose	105.0	0.96	39060	379	12
3	DNA-cellulose	120.0	0.36	50400	1167	36
4	Sephacryl S-300	48.0	0.04	18144	9450	295

[a] The starting material was 73 g wet weight of HeLa cells grown in suspension culture. One unit of activity represents 100% stimulation of DNA polymerase α activity on denatured DNA template in 1 h at 35 °C.

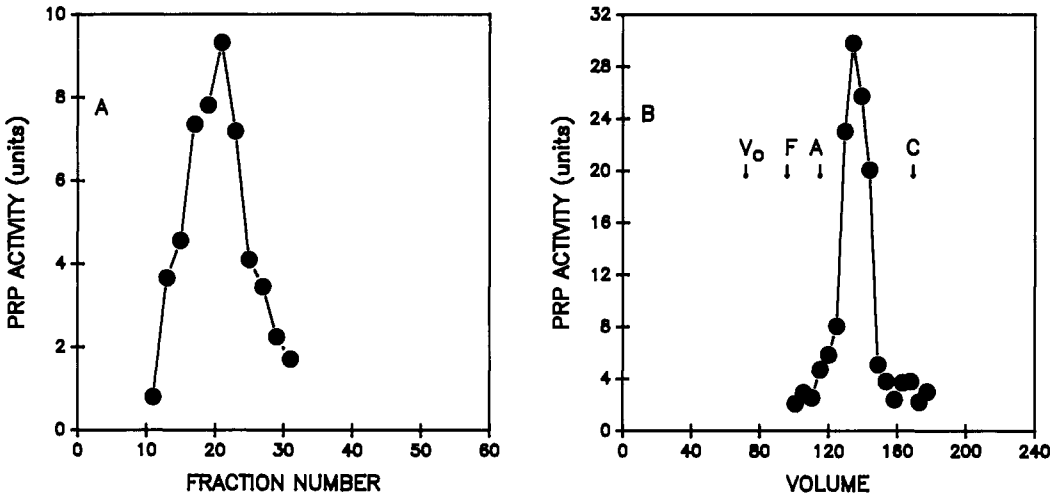

FIGURE 1: Purification of PRP. (A) Phosphocellulose chromatography. The unbound protein fraction from the DE-52 chromatography (fraction 1, Table I) was precipitated with 50–70% saturation of ammonium sulfate, dialyzed against buffer A, and chromatographed on a 50-mL column of phosphocellulose. The bound proteins were eluted with a 400-mL linear gradient of 20–700 mM KCl in buffer A. An aliquot of each fraction was used to measure stimulation of DNA polymerase α activity on denatured DNA template as described under Experimental Procedures. (B) Sephacryl S-300 gel filtration of PRP. Fraction 3 was concentrated with Omegacell membrane concentrator and loaded on a 3 cm × 40 cm column of Sephacryl S-300 column equilibrated and developed in buffer A. Fractions of 4.8 mL were collected and assayed for PRP activity. The column was calibrated with blue dextran (V_0), ferritin (F), aldolase (A), and cytochrome c (C), and their elution positions are indicated by arrows.

α activity in this assay. For the majority of experiments, the pol α used was immunoaffinity-purified as described above.

Amino Acid Composition Analysis. For amino acid composition analysis, proteins were subjected to SDS–PAGE and then electroblotted onto an Immobilon-P transfer membrane (Millipore) in 10 mM CAPS and 10% methanol, pH 11, at 300 mA for 90 min at room temperature (Yuen et al., 1989). Quantitative transfer of proteins onto membranes was seen with this protocol. After staining and destaining of the membrane, the primer-recognition protein bands were excised and subjected to acid hydrolysis. Amino acid composition was determined by using the Pico-Tag amino acid analysis system (Millipore).

Other Methods. Protein concentrations were determined by the method of Bradford (1976) using the dye reagent and protein standard (BSA) purchased from BioRad. Conductivity measurements were made with an Orion Model 101 conductivity meter. Diluted protein samples were concentrated by using Centricon-10 microconcentrators (Amicon) for smaller volumes and an Omegacell stirred cell concentrator (Pharmacia) for larger volumes, using membranes with a molecular weight cutoff of 10000.

RESULTS

Purification of Primer Recognition Proteins. Primer recognition proteins were purified from the combined soluble nuclear extract and postmicrosomal supernatant of HeLa cells by the procedure summarized in Table I. The cell extract prepared as described under Experimental Procedures was chromatographed over a 50-mL DEAE-cellulose (DE-52) column that had been previously equilibrated with buffer A. The column was then washed with 100 mL of buffer A followed by a 400-mL linear gradient of 20 to 350 mM KCl in buffer A. The unbound proteins in the column wash contained a majority of the activity that stimulates the ability of DNA polymerase α to recognize and then extend the 3′-OH end of a polynucleotide primer such as heat-denatured calf thymus DNA. All of the DNA polymerase α activity and a small fraction of the stimulatory activity were bound to the column and eluted as a single peak of activity at 90 mM KCl (data not shown). The DE-52 flowthrough fraction was then precipitated between 50% and 70% saturation of ammonium sulfate, dissolved, and dialyzed with buffer A (fraction 1). Fraction 1 was contaminated with activities of DNA polymerases β and δ and ATPase.

The stimulatory activity in fraction 1 was further purified by chromatography on a 50-mL column of phosphocellulose (P-11) equilibrated in buffer A. After the column was washed with two column volumes of buffer A, it was developed with a 400-mL linear gradient of 20 to 700 mM KCl in buffer A. Fractions of 6.5 mL were collected and an aliquot of each fraction was assayed for pol α stimulation on a heat-denatured DNA template (Figure 1). The stimulatory activity eluted in a single peak at around 130 mM KCl. The fractions containing this activity were pooled and dialyzed against buffer A (fraction 2). We were able to separate DNA polymerase δ activity from the stimulatory activity. However, DNA polymerase β and ATPase activities still persisted in fraction 2.

Fraction 2 was chromatographed on a column of double-stranded DNA-cellulose (15 mL) connected in series to a column of single-stranded DNA-cellulose (15 mL), both of which were equilibrated with buffer A. DNA polymerase β

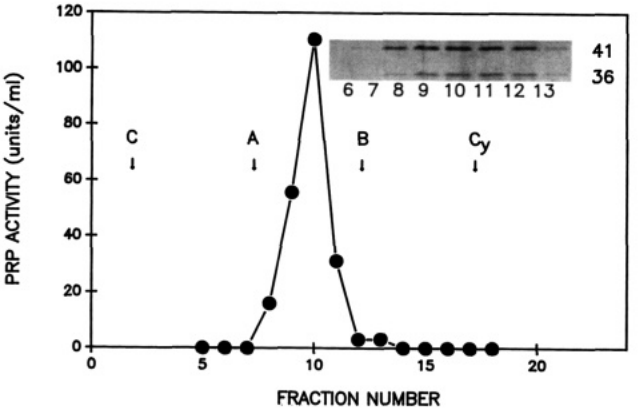

FIGURE 2: Glycerol gradient centrifugation of PRP. Purified PRP (fraction 4, Table I) was concentrated to small volumes by using a Centricon-10 centrifugal concentrator and layered on a 15–35% (v/v) glycerol gradient in buffer A prepared in polyalomer tubes for the Beckman SW 50.1 rotor. Centrifugation was at 40 000 rpm for 40 h at 4 °C. Fractions were collected from the bottom of the tube and assayed for PRP activity. The positions of marker proteins run on parallel gradients are indicated (C, catalase; A, aldolase; B, bovine serum albumin; Cy, cytochrome *c*). The inset shows the SDS–PAGE of peak fractions. The relative molecular weights are indicated on the side.

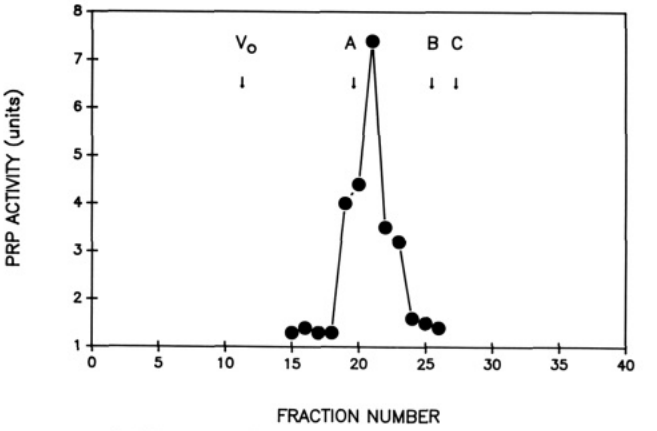

FIGURE 3: Gel filtration of PRP on Superose 12 FPLC. PRP from DNA-cellulose chromatography (fraction 3, Table I) was concentrated by using membrane concentrators to small volumes and chromatographed over a prepacked Superose 12 FPLC column (10 mm × 300 mm) equilibrated in buffer A containing 0.2% Triton X-100. Fractions of 0.5 mL were collected and assayed for PRP activity. The positions of marker proteins used to calibrate the column are indicated (V_0, void volume; A, aldolase; B, bovine serum albumin; C, cytochrome *c*). The PRP activity elutes with the peak at a position corresponding to a Stokes radius of 40.5 Å.

bound to the double-stranded DNA-cellulose column, while the PRP activity did not bind to either column. The DNA-cellulose flow through (fraction 3) was concentrated by using membrane concentrators to smaller volumes.

Fraction 3 was subjected to gel filtration over a 3 cm × 40 cm column of Sephacryl S-300 equilibrated in buffer A. The column was developed in buffer A. The ATPase activity eluted in the void volume and the DNA polymerase α stimulatory activity eluted in a single peak of activity. The fractions containing stimulatory activity were pooled, dialyzed against buffer A containing 50% glycerol, and stored at −80 °C (fraction 4). The stimulatory activity was stable under these conditions for over 3 months.

Data for a typical purification are shown in Table I, starting from 73 g wet weight of HeLa cells. The procedure described here has been used in over 10 such preparations with HeLa cells and human placenta and was found to be reproducible. Specific activities of the primer recognition proteins in fraction 4 ranged from 3000 to 12 000 units/mg in the various preparations.

Velocity Sedimentation and Gel Filtration Analysis. Sedimentation analysis of primer recognition proteins in linear 15–35% (v/v) glycerol gradients reveals that the proteins sediment as a complex at 5.7 S (Figure 2). When the active fractions were analyzed by denaturing gel electrophoresis, two polypeptides corresponding to 41 000 Da and 36 000 Da were seen (Figure 2, inset). Upon gel filtration on Superose 12 FPLC, PRP sediment as a single peak corresponding to a Stokes radius of 40.5 Å (Figure 3). In our pilot experiments, we found that inclusion of 0.2% Triton X-100 was helpful in better resolution of PRP and hence the Superose 12 gel filtration was performed in buffer A with 0.2% Triton X-100. The presence of Triton X-100 did not alter the elution profile of PRP. From the method of Siegel and Monty (1966), and assuming a partial specific volume of 0.725, the native molecular weight of PRP may be calculated as 96 000. The apparent native molecular weight, according to a plot of log molecular weight against V_e/V_0, was 120 000, indicating that the PRP is asymmetric.

Subunit Composition of PRP. Analysis of the proteins in various stages of purification by denaturing polyacrylamide gel electrophoresis (Figure 4a) shows that the PRP are composed of two polypeptides of 41 000 Da and 36 000 Da. This was also observed in active fractions from velocity gradient centrifugation (Figure 2, inset). In the highly purified fraction (lane 1 of Figure 4a), the two polypeptides comprise greater than 95% of the protein that was loaded on that lane. A minor polypeptide of 33 000 Da is seen at various levels in different fractionations and appears to be a degradative product of the 36 000-Da polypeptide. We designate the 36 000-Da polypeptide as PRP 1 and the 41 000-Da polypeptide as PRP 2. Upon electrophoresis under nondenaturing conditions, purified PRP migrate as a single complex of 340 000 Da (Figure 4b). We have been unable to elute the PRP activity from the native PAGE gels under a variety of elution conditions.

The Absence of Contaminating or Associated Enzymatic Activities. We have analyzed the PRP (fraction 4) for various enzymatic activities that might affect DNA synthesis. An ATPase activity that is DNA-independent cofractionates with PRP through DNA-cellulose chromatography. However, this activity elutes in the void volume of a Sephacryl S-300 gel filtration column. The purified PRP lack detectable activities of DNA polymerases α, β, and δ, DNA primase, deoxyribonuclease, DNA-dependent ATPase, RNase H, topoisomerase, or DNA ligase. In particular, PRP lack any polymerase activity on either activated DNA or templates such as heat-denatured DNA, poly(dA-dT), or poly(dA)–oligo(dT) (20:1).

Enzymological Characterization. The primer recognition proteins were discovered on the basis of their ability to stimulate homologous DNA polymerase α on templates with low primer to template ratios (Pritchard et al., 1981). DNA polymerase α utilizes such DNA templates very inefficiently, while in the presence of PRP, pol α is as efficient on these templates as on activated DNA, its preferred template. By combining PRP (fraction 4) with various amounts of immunoaffinity-purified DNA polymerase α, up to 10-fold stimulation of pol α activity on heat-denatured DNA (Figure 5a) can be seen, while DNA polymerase α by itself is ineffective on this template. Stimulation of up to 10-fold has been observed with other templates such as singly primed M13 virion DNA and poly(dA)–oligo(dT)$_{10}$. By use of a fixed amount of immunoaffinity-purified DNA polymerase α and various amounts of PRP (Figure 5B), it can be seen that a linear relationship exists between stimulation of DNA polymerase

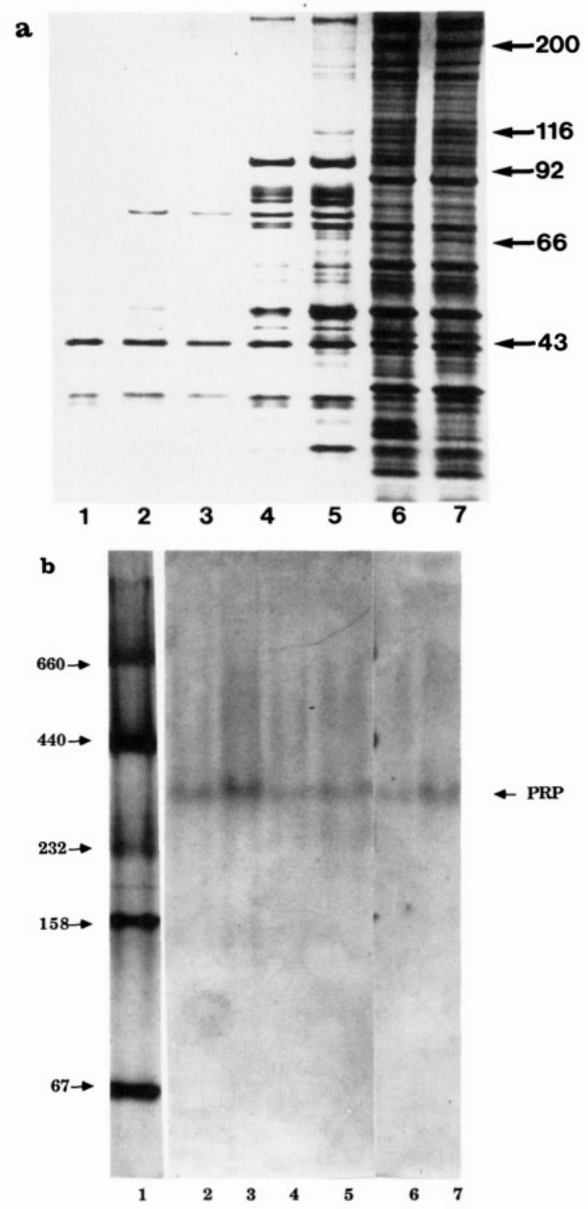

FIGURE 4: (a) Polypeptide composition of PRP by SDS–polyacrylamide gel electrophoresis. Fractions at various stages of purification of the PRP were subjected to electrophoresis on a 4–15% gradient polyacrylamide gel containing SDS. The gel was fixed and stained with silver reagent. Positions of the marker proteins run on the same gel are as indicated. The different fractions shown are Superose 12 (lane 1), fraction 3 (lanes 2 and 3), fraction 2 (lane 4), fraction 1 (50–70% ammonium sulfate) (lane 5), DE-52 flow-through (lane 6), and crude extract (lane 7). From a plot of R_f versus log moleular weight, the two polypeptides in lane 1 have relative molecular weights of 41 000 and 36 000, respectively. (b) Nondenaturing PAGE of PRP. Purified PRP (lane 1, part a) was subjected to electrophoresis under nondenaturing conditions on a 4–15% gradient polyacrylamide gel. The gel was fixed and stained with silver reagent. Positions of the marker proteins are indicated. Lanes 2–7 contain purified PRP from three different fractionations. Lanes 3, 5, and 7 contain twice the amount of protein in lanes 2, 4, and 6.

α and amount of PRP at low concentrations of PRP and a maximal stimulation occurs at 3 μg of PRP. Higher concentrations of PRP actually inhibit the stimulation. This indicates that a concentration dependence exists in the stimulatory process. This was further confirmed by varying the amounts of denatured DNA template while keeping the PRP and DNA polymerase α constant (Figure 5C). The optimal concentration of denatured DNA in the reaction is 40 μg/mL. Stimulation of pol α is not due to an increase in processivity but is due to the improved ability of the pol α-PRP complex

Table II: Effect of PRP on the First dNTP Incorporation at a DNA Primer

template/primer	DNA synthesis[a] pol α	DNA synthesis[a] pol α + PRP	fold stimulation
heat-denatured CTDNA (dCTP)	4.8	34.8	7.3
M13 DNA: sequencing primer (dGTP)	0.7	6.5	9.3

[a] DNA synthesis reactions were set up in standard pol α reaction mixture containing 40 μg/mL of DNA with the nucleotide indicated in parentheses being the only dNTP present. The [α-^{32}P]dNTP was at 100 μM and 10 000 cpm/pmol. Results are presented as picomoles per hour at 35 °C and have been corrected for background radioactivity in tubes where either enzyme or DNA was omitted.

Table III: Stabilization of Pol α Complex by PRP[a]

[KCl] (mM)	pol α (units)	pol α + PRP (units)
25		
100		89 (86)
300	138	62

[a] DNA polymerase α with or without an excess of PRP (fraction 4, Table I) was chromatographed separately over DEAE-Biogel A columns and the columns were eluted with five column volumes of buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol) containing indicated amounts of KCl. Aliquots of each fraction were assayed for pol α activity with DNase I activated or heat-denatured (in parentheses) DNA templates. During chromatography of pol α + PRP, free PRP was found in 25 mM KCl fraction and could be detected by its stimulation of pol α. Results are indicated as nanomoles of dNMP incorporated per hour at 35 °C.

in recognizing the primer in substrate with low primer to template ratios. This was demonstrated by measuring the incorporation of the first dNTP in a truncated DNA synthesis assay by using singly primed M13 virion DNA or heat-denatured calf thymus DNA (Table II). In the presence of PRP, pol α was seven to nine times more effective at incorporating the first nucleotide at the primer as compared to pol α alone. This is a distinguishing feature of PRP from other pol α stimulatory proteins that need addition of several dNTPs to observe stimulation of DNA synthesis. Purified PRP does not stimulate the activities of DNA primase, DNA polymerases β, γ, or δ, and *E. coli* DNA polymerase I.

In the presence of PRP, pol α exists as a complex whose chromatographic behavior is different from that of pol α alone (Vishwanatha et al., 1986a). While pol α_2 complex elutes from DEAE-Biogel A (BioRad) at 100 mM KCl, pol α alone elutes at 300 mM KCl. As shown in Table III, PRP can stabilize this complex formation. PRP by itself elutes in the flow-through fraction of the column and pol α by itself binds tightly and elutes at 300 mM KCl. However, preincubation of pol α and PRP results in stabilization of the pol α-PRP complex, which elutes at 100 mM KCl.

The PRP activity is stable to heat denaturation (42 °C for up to 15 min), it has a pH optimum of 7.0–8.5 and an ATP requirement of 1 mM, and the stimulatory activity is enhanced in the presence of 1.8% (v/v) poly(ethylene glycol) 8000 in the reaction.

Amino Acid Composition of PRP. Purified PRP (fraction 4) was subjected to amino acid composition analysis according to procedures detailed under Experimental Procedures and the results are presented in Table IV. Both of the polypeptides are rich in nonpolar (hydrophobic) amino acids (68% of amino acids in PRP 1 and 74% of amino acids in PRP 2 are hydrophobic) and contain more basic amino acids than acidic amino acids. Consequently, their pI is basic, and by IEF, we have found both PRP polypeptides to have pI around 7.2 (data not shown). Hydrophobic interactions play a major role in

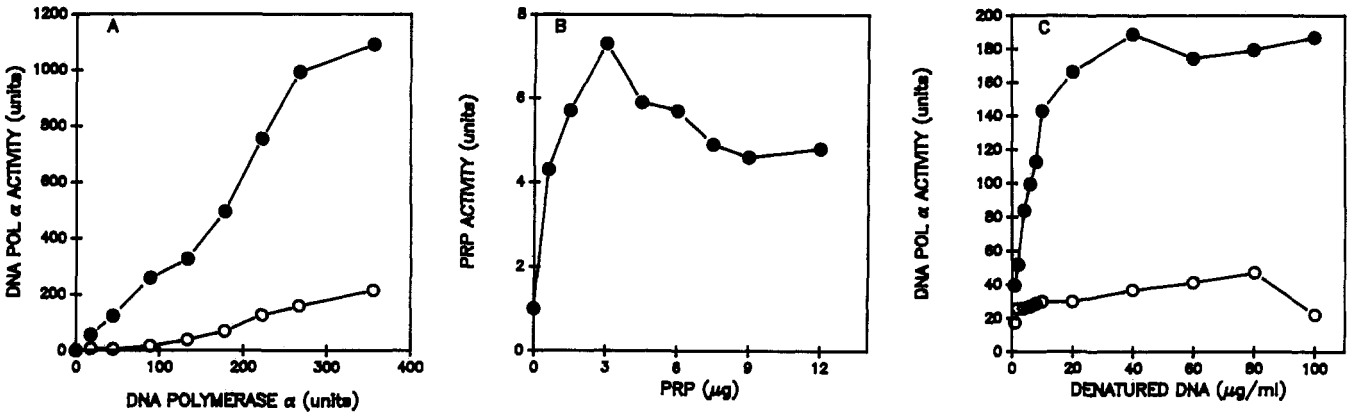

FIGURE 5: Effects of the concentrations of PRP, denatured DNA, and DNA polymerase α on DNA polymerase α activity with denatured DNA. (A) Different concentrations of immunoaffinity-purified DNA polymerase α (as units of activity on DNase I activated DNA) were incubated without (○) or with (●) fraction 4 in the standard PRP reaction mixture for 30 min at 35 °C. One unit of DNA polymerase α activity is the incorporation of 1 nmol of dNMP/h at 35 °C. (B) Various concentrations of PRP (fraction 4) were incubated with 1 unit of immunoaffinity-purified DNA polymerase α in the standard PRP reaction mixture at 35 °C for 30 min. The results are indicated as units of PRP activity. (C) DNA polymerase α was incubated without (○) and with (●) PRP (fraction 4) in the standard PRP reaction mix with various concentrations of heat-denatured DNA template for 30 min at 35 °C. The results are indicated as DNA polymerase α activity.

Table IV: Amino Acid Composition of PRP

	mol of amino acid/mol of protein	
	PRP1[a]	PRP2[b]
aspartic acid	6.6	4.6
glutamic acid	6.8	4.7
serine	22.3	28.4
glycine	22.3	39.8
histidine	8.3	10.9
arginine	6.3	6.2
threonine	14.1	15.1
alanine	53.7	62.0
proline	12.9	21.5
tyrosine	19.6	9.8
valine	26.2	37.7
methionine	24.3	34.2
cystine	ND[c]	ND[c]
isoleucine	40.9	38.4
leucine	36.4	34.6
phenylalanine	12.3	22.7
lysine	23.2	24.9
tryptophan	ND[c]	ND[c]

[a] Values are normalized to a molecular weight of 36 000. [b] Values are normalized to a molecular weight of 41 000. [c] Not determined.

interaction of PRP with DNA polymerase α (Vishwanatha et al., 1986a).

Discussion

Current models of a DNA replication fork in both prokaryotes (Kornberg, 1988; McHenry, 1988) and eukaryotes (Fairman et al., 1988) envision the DNA synthesis on both the lagging strand and leading strand to be catalyzed by an asymmetric DNA polymerase complex. From their studies on SV40 DNA replication in vitro, Prelich and Stillman (1988) and Fairman et al (1988) have proposed that DNA polymerase δ with its auxilliary protein, PCNA, catalyzes the leading strand replication, while DNA polymerase α, with its tightly associated DNA primase activity, catalyzes lagging strand replication. The discontinuous DNA synthesis on the lagging strand requires DNA primase–polymerase α to initiate RNA primers and extend these primers with DNA synthesis utilizing from 40 to 300 bases of single-stranded DNA template downstream (Pritchard et al., 1983). Thus, the template available to DNA polymerase α on the lagging strand is a long single-stranded template with a low primer to template ratio. Purified DNA primase–polymerase α is inefficient on such templates, indicating the need for accessory proteins in DNA synthesis on the lagging strand. A majority of DNA polymerase α in HeLa cells (Vishwanatha et al., 1986a) and CV1 cells (Pritchard and DePamphilis, 1983) has been shown to exist in a multiprotein form that includes primer recognition proteins. The primer recognition proteins restore the activity of DNA polymerase α on templates with low primer to template ratios to that observed on DNase I activated DNA.

Although primer recognition proteins have been identified and partially purified from human HeLa cells and monkey CV1 cells, their precise polypeptide composition has not been defined. In this report we have described the structure and catalytic properties of the HeLa primer recognition proteins.

HeLa cell PRP has been identified in a complex with pol α_2 (Vishwanatha et al., 1986a). To separate the PRP from DNA polymerase α, hydrophobic chromatography using nonionic detergents had to be employed. This results in extensive loss of activities as well as poor reconstitution when combined (unpublished observations). Therefore, we have used an alternate procedure and we find PRP to exist as a free complex that does not bind to DEAE-cellulose, whereas DNA polymerase α binds to the matrix. We have purified PRP independent of the contaminating enzymatic activities. The PRP exist as a complex in the native state exhibiting a sedimentation coefficient of 5.7 S (Figure 3) and a Stokes radius of 40.5 Å (Figure 2), upon gel filtration. The PRP are composed of two polypeptides of 41 000 Da and 36 000 Da (Figure 4).

Recently, Tsurimoto and Stillman (1989) have isolated a cellular replication factor, called RF-C, that is required for coordinated synthesis of leading and lagging strands in SV40 in vitro DNA replication. RF-C is composed of several protein bands, with polypeptides of 41 000 and 37 000 Da being the major components. It is possible that these two polypeptides represent the PRP function and that PRP is part of the RF-C complex. The molecular mass of PRP 1 is similar to that of PCNA. However, PRP 1 is not PCNA, as it does not stimulate pol δ activity and the amino acid compositions of the two are very different.

PRP imparts the capability of DNA synthesis on denatured DNA to DNA polymerase α (Figure 5A). Our data indicate that optimal stimulation depends on precise concentrations of PRP, DNA polymerase α, and denatured DNA template (Figure 5) in the reaction. Excess of PRP reduces the ability of DNA polymerase α to function on denatured DNA. The amino acid composition (Table IV) confirms the findings that

PRP is highly hydrophobic (Vishwanatha et al., 1986a), as the two polypeptides are dominated by hydrophobic amino acids.

The physiological role of PRP in eukaryotic cells has not been established. They help DNA primase–polymerase α in locating either RNA or RNA–DNA primers at replication forks (Pritchard et al., 1983) and may function as proteins involved in switching from transcription of initiator RNA to replication in DNA synthesis in vivo (Vishwanatha et al., 1986b). With the availability of homogeneous PRP preparations, it should be possible to define the physiological role of these proteins.

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DNA Repair within Nucleosome Cores of UV-Irradiated Human Cells†

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ABSTRACT: We have compared the distributions of repair synthesis and pyrimidine dimers (PD) in nucleosome core DNA during the early (fast) repair phase and the late (slow) repair phase of UV-irradiated human fibroblasts. As shown previously [Lan, S. Y., & Smerdon, M. J. (1985) *Biochemistry 24*, 7771–7783], repair synthesis is nonuniform in nucleosome core particles during the fast repair phase, and the distribution curve can be approximated by a model where repair synthesis occurs preferentially in the 5′ and 3′ end regions. In this report, we show that, during the slow repair phase, [^{3}H]dThd-labeled repair patches are much more uniformly distributed in core DNA, although they appear to be preferentially located in sequences degraded slowly by exonuclease III. This change in distribution cannot be explained by an increase in patch size during slow repair, since the size of these patches actually decreases to about half the size measured during the fast repair phase. Furthermore, PD mapping within core DNA at the single-nucleotide level demonstrated that, at least within the 30–130-base region from the 5′ end, there is little (or no) selective removal of PD during the fast repair phase. However, the nonuniform distribution of repair synthesis obtained during fast repair throughout most of the core DNA region (~40–146 bases) is accounted for by the nonuniform distribution of PD in core DNA. The near-uniform distribution of repair synthesis observed during slow repair may result from more extensive nucleosome rearrangement and/or nucleosome modification during this phase.

Excision repair of ultraviolet (UV)[1] damage to DNA of human cells is associated with at least two distinct phases [reviewed in Smerdon (1989)]. Between 50 and 75% of the pyrimidine dimers (PD) are removed rapidly from the genome, and the remaining PD are removed much more slowly. During each of these phases, the repair process is associated with nucleosome rearrangements and, following repair synthesis and ligation, newly repaired regions rapidly become associated with

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[1] Abbreviations: PD, cis-syn cyclobutane pyrimidine dimers; UV, ultraviolet; ESS, endonuclease-sensitive sites.

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