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A 17S Multiprotein Form of Murine Cell DNA Polymerase Mediates Polyomavirus DNA Replication In Vitro

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Abstract We have identified and purified a multiprotein form of DNA polymerase from the murine mammary carcinoma cell line (FM3A) using a series of centrifugation, polyethylene glycol precipitation, and ion-exchange chromatography steps. Proteins and enzymatic activities associated with this mouse cell multiprotein form of DNA polymerase include the DNA polymerases α and δ, DNA primase, proliferating cell nuclear antigen (PCNA), DNA ligase I, DNA helicase, and DNA topoisomerases I and II. The sedimentation coefficient of the multiprotein form of DNA polymerase is 17S, as determined by sucrose density gradient analysis. The integrity of the murine cell multiprotein form of DNA polymerase is maintained after treatment with detergents, salt, RNase, DNase, and after chromatography on DE52-cellulose, suggesting that the association of the proteins with one another is independent of nonspecific interaction with other cellular macromolecular components. Most importantly, we have demonstrated that this complex of proteins is fully competent to replicate polyomavirus DNA in vitro. This result implies that all of the cellular activities required for large T-antigen dependent in vitro polyomavirus DNA synthesis are present within the isolated 17S multiprotein form of the mouse cell DNA replication activities. A model is proposed to represent the mammalian *M*ultiprotein DNA *R*eplication *C*omplex (MRC) based on the fractionation and chromatographic profiles of the individual proteins found to co-purify with the complex.

Key words: mammalian cells, DNA replication, multiprotein complex, polyomavirus, DNA polymerase

The mechanism and regulation of mammalian cell chromosomal DNA replication is not fully understood. To date several mammalian enzymes and proteins have been shown to be required for DNA replication in vitro [reviewed in Kelly, 1988; Challberg and Kelly, 1989; Stillman, 1989; Hurwitz et al., 1990; Malkas et al., 1990a]. Many of these proteins were identified using a simian virus 40 (SV40) based in vitro DNA replication system [Li and Kelly, 1984], and include: DNA polymerase α-primase, DNA polymerase δ, proliferating cell nuclear antigen (PCNA); RP-A (a.k.a. RF-A or HSSB); topoisomerases I and II; and RF-C or Activator 1 (A-1) protein complex. Over the last several years a growing body of evidence has suggested that DNA synthesis may be mediated by the concerted action of DNA replication proteins, once these proteins are organized into multiprotein structures [reviewed in Mathews and Slabaugh, 1986; Fry and Loeb, 1986; Malkas et al., 1990a]. However, the manner in which these proteins associate with one another, as well as their ability to act in concert to efficiently replicate DNA is not well defined.

Reports regarding the isolation of large complexes of proteins for DNA synthesis from ex-

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tracts of eukaryotic cells suggested that these complexes were relatively unstable when subjected to the usual conditions used for protein purification [reviewed in Mathews and Slabaugh, 1986; Fry and Loeb, 1986; Malkas et al., 1990a]. In addition, the lack of a functional assay to test the DNA replication ability of these multiprotein complexes made the purification and characterization of these putative multiprotein DNA synthesis complexes difficult. The first description of a multiprotein complex from eukaryotic cells that fully supported all phases of the replication of DNA was that of Jazwinski and Edelman [1984]. These authors reported the isolation of a two million dalton complex from yeast cells that had associated DNA polymerase I, DNA primase, DNA ligase, and topoisomerase II activities. They also showed that this multiprotein complex could efficiently replicate, in vitro, yeast extrachromosomal 2 micron DNA. A multiprotein form of DNA polymerase was isolated from human (HeLa) cells [Hickey et al., 1988; Baril et al., 1988; and Malkas et al., 1990b]. The complex was observed to sediment at 18-21S during velocity sedimentation analyses. Some of the proteins identified in the human cell 18-21S multiprotein complex fraction included: DNA polymerase α, DNA primase, topoisomerase I, DNA ligase, RNase H, PCNA, and a DNA-dependent ATPase. This multiprotein complex was further purified by Q-Sepharose chromatography and shown to be fully competent in supporting origin specific and large T-antigen dependent SV40 DNA replication in vitro [Malkas et al., 1990b]. In this paper, we report the identification and characterization of a readily sedimentable form of DNA polymerase-primase from murine cells that is fully capable of supporting DNA replication in vitro using the polyomavirus (PyV) based DNA synthesis system [Murakami et al., 1986; Dermody et al., 1988]. We conclude that there is now sufficient evidence to suggest that DNA synthesis is mediated by a multiprotein complex in mammalian cells.

METHODS

Cell Culture

Suspension cultures of mouse FM3A cells [Nakano, 1966] were adapted from monolayer cultures. The cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated calf and fetal bovine serum. Exponentially growing cells (5×10^5 cells/ml of medium) were harvested and washed three times with phosphate buffered saline (PBS): 8.4 mM Na_2HPO_4, 0.137 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4. The cells were then pelleted by low speed centrifugation (200*g*, 5 min, 4°C), and the cell pellets stored at −80°C till fractionation.

Fractionation Procedures

FM3A cells were fractionated essentially as described by Malkas et al. [1990b], with some variations, and as outlined in Figure 1. In brief, frozen cell pellets (20–30 g wet *weight) were* quickly thawed and resuspended in 2–3 volumes of buffer containing: 50 mM Tris-HCl, pH 7.5; 0.25 M sucrose; 5 mM $MgCl_2$; 0.1 mM each of phenylmethyl sulfonyl fluoride (PMSF), which was dissolved in isopropyl alcohol, and aminoacetonitrile hemisulfate (AAN), pH 7.5; 1 mM dithiothreitol (DTT). The resuspended cells were homogenized using a loose-fitting Dounce homogenizer. The homogenate was then centrifuged for 10 min at 500*g*, and the crude nuclear and cytosolic (LS-1) fractions were each col-

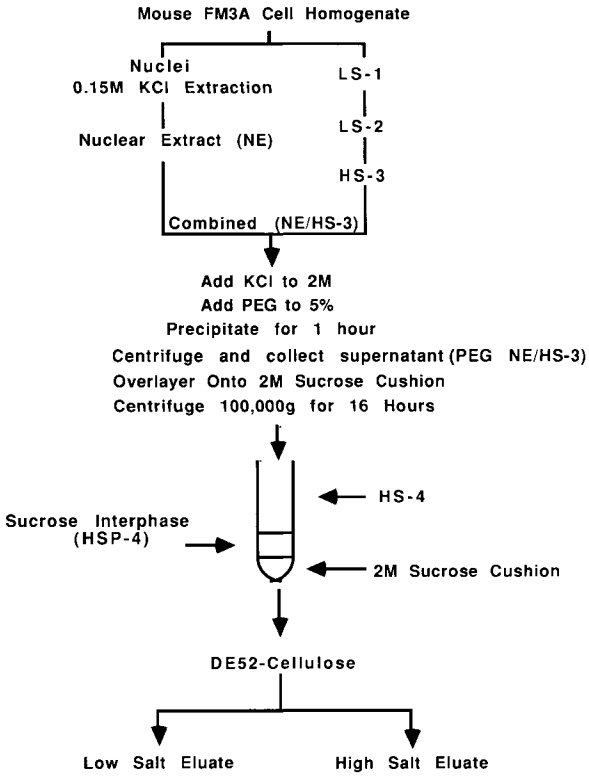

Fig. 1. The flow diagram of the isolation scheme used to purify the mouse cell multiprotein DNA replication complex (MRC). A detailed description of the isolation scheme is presented in Methods.

lected separately. Mitochondria were removed from the LS-1 fraction by centrifugation at 27,000 *g* for 15 min, and the resultant supernatant was designated as an LS-2 fraction. The LS-2 fraction was then subjected to centrifugation at 100,000 *g* for 60 min to remove microsomes. The post-microsomal supernatant (HS-3) was collected.

The crude nuclear pellet was resuspended in 2 volumes of a buffer containing: 50 mM Tris-HCl, pH 7.5; 1 mM DTT; 0.15 M KCl; 5 mM each of EDTA-Na_3 and EGTA-Na_3; 0.1 mM each of PMSF and AAN. The resuspended nuclei were gently stirred for 2 h at 4°C. The extracted nuclei were centrifuged for 60 min at 100,000*g* and the supernatant (NE) was collected. The NE and HS-3 fractions were pooled and made 2 M in KCl. Polyethylene glycol (PEG; Sigma Chemical Company, St. Louis, MO; molecular weight 8,000) was added to a final concentration of 5% and the mixture stirred gently for 1 h at 4°C. PEG precipitated material was pelleted by centrifugation for 30 min at 16,000*g*, and the supernatant (PEG NE/HS-3) was collected. The PEG NE/HS-3 was dialyzed (to remove the PEG) for 3 h against 2 changes of a buffer containing: 50 mM Tris-HCl, pH 7.5; 1 mM DTT; 1 mM each of EDTA-Na_3 and EGTA-Na_3; 0.1 mM each of PMSF and AAN; 50 mM KCl. The dialyzate was clarified by centrifugation for 10 min at 13,000*g*. The clarified PEG NE/HS-3 fraction was layered over a 2 M sucrose cushion and subjected to centrifugation at 100,000*g* for 16–18 h at 4°C. The material above the sucrose interphase was collected and designated the HS-4 fraction. The sucrose interphase fraction was collected and designated HSP-4.

DE52-Cellulose Column Chromatography

The FM3A HSP-4 fraction was dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA-Na_3, 10% glycerol, 5 mM KCl. The dialyzate was loaded onto a column of DE52-cellulose (25 mg protein/1 ml matrix) pre-equilibrated in 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA-Na_3, 10% glycerol, 5 mM KCl (see Fig. 1). The column was washed with 8 column volumes of the column pre-equilibration buffer. The column flow-through and wash fractions were pooled and designated the low salt eluate. Matrix bound protein was then eluted by 8 column volumes of 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA-Na_3, 10% glycerol, 1 M KCl, and the collected fraction designated the high salt eluate. Both the low and high salt eluates were dialyzed into 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA-Na_3, 10% glycerol, and stored in aliquots at −80°C.

Enzyme Assays

DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures [Lamothe et al., 1981; Vishwanatha et al., 1986]. One unit of DNA polymerase activity equals 1 nmol of total ^{3}H-TTP incorporated into DNA per h at 35°C.

DNA primase activity with polydT template was assayed according to the procedure described in Lamothe et al. [1981] and Vishwanatha et al. [1986]. One unit of DNA primase activity equals 1 nmol of total ^{3}H-ATP incorporated into polydT template.

DNA helicase. The helicase substrate was a radiolabelled 17 mer oligodeoxynucleotide annealed with single-stranded circular M13 mp19 DNA (positive strand). DNA helicase activity was assayed by measuring the ability to unwind the ^{32}P-17 mer-M13 substrate. The 10 μl reaction mixture contained 20 mM Tris-HCl, pH 7.4, 5 mM DTT, 2 mM ATP, 50 μg/ml BSA, 3,000 cpm ^{32}P-17 mer-M13 substrate, and protein fraction. The reaction was carried out at 37°C for 30 minutes. The reaction products were then resolved on a 12% nondenaturing polyacrylamide gel [Sambrook et al., 1989]. Quantitation was performed using a Betascope 603 (Betagen, Waltham, MA).

Topoisomerase I activity was assayed as described in Hickey et al. [1988] for the relaxation of pJLPYO plasmid DNA [Dermody et al., 1988].

Immunodetection of PCNA, DNA Ligase I, Topoisomerase II, and DNA polymerase δ

Denaturing polyacrylamide gel electrophoretic analysis, using 20 μg/lane of each of the various protein fractions was performed as previously described [Laemmli, 1970]. Western blot analysis of these proteins was performed as described in Malkas et al. [1990b]. To detect DNA polymerase δ polypeptide a polyclonal antibody prepared against the C-terminal peptides of the DNA polymerase δ [Yang et al., 1992] was used. The anti-PCNA antibody was purchased from Boehringer-Mannheim (Indianapolis, IN). The anti-DNA ligase I antibody was a gift from Dr. Alan Tomkinson. The anti-topoisomerase II antibody was a gift from Dr. Leroy Liu.

Purification of PyV Large T-Antigen

PyV large T-antigen is purified from baculovirus (vEV55PyT) infected Sf21 insect cells [Rice et al., 1987] using immunoaffinity chromatography on a column containing a monoclonal antibody from hybridoma F4 reactive for PyV large T-antigen [Murakami et al., 1986; Dermody et al., 1988].

In Vitro PyV DNA Replication Assay

Assay reaction mixtures (25 μl) contained 80 mM Tris-HCl, pH 7.5; 7 mM $MgCl_2$; 1 mM DTT; 0.1–0.2 mg of mouse fraction protein; 0.5–1.0 μg of purified PyV large T-antigen; 30 ng of plasmid pJLPYO containing an insert of PyV replication origin DNA sequences [Dermody et al., 1988]; 100 μM each dTTP, dATP, dGTP; 200 μM each rCTP, rGTP, UTP; 4 mM ATP; 25 μM (^{32}P)dCTP; 40 mM creatine phosphate; 1 μg of creatine kinase. The reaction was incubated for 2 h at 35°C. The replication reaction was stopped by adding 100 μg of carrier RNA and 0.1% sodium dodecyl sulfate (SDS). The replication assay reaction products were processed using either agarose gel electrophoresis, to visualize the replication products [Dermody et al., 1988], or by DE81 filter binding [Sambrook et al., 1989], to quantitate the amount of radiolabel incorporated into the replication products. One unit of in vitro DNA replication activity equals 1 pmol of total ^{32}P-TTP incorporated in DNA per 2 h at 35°C.

Assessment of Integrity of the Murine Cell Multiprotein Form of DNA Polymerase

The FM3A PEG NE/HS-3 fraction (see Fig. 1) (50 mg in total) was incubated under one of the following conditions for 1 h, with gentle rocking, at 4°C: 2 M KCl; 0.5% NP-40; 2.5% butanol/5% triton X-100; DNase (70 U) or RNase A and RNase T_1 (80 U each). Fractions treated with 2 M KCl, 0.5% NP-40 or 2.5% butanol/5% triton X-100 were then dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM each of EDTA-Na_3 and EGTA-Na_3, 0.1 mM each of PMSF and AAN, 50 mM KCl for 3 h. EDTA was added to a final concentration of 10 mM to the fraction treated with either the RNases or DNase to inactivate these nucleases. HSP-4 and HS-4 fractions were then prepared from the treated PEG NE/HS-3 fraction as described previously.

Velocity Sedimentation Analysis

A 9 ml 10–30% sucrose gradient in 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA-Na_3, 0.5 M KCl, was formed over a 1.0 ml cushion of 2 M sucrose in polyallomer tubes. A 0.5 ml aliquot of protein fraction was loaded onto the preformed gradient and centrifuged at 100,000*g* for 16 h at 4°C using a Sorvall TH641 rotor. After centrifugation, the gradient was collected by puncturing the bottom of the centrifuge tube and collecting the gradient dripping from the tube. Marker proteins (porcine thyroglobulin, 19S; horse spleen apoferritin, 17S; and yeast alcohol dehydrogenase, 7S) were sedimented on parallel gradients to verify that the velocity sedimentation gradients were isokinetic.

RESULTS

Mouse Cell DNA Replication Proteins Co-Fractionate as a Readily Sedimentable Form

To determine whether mouse cell DNA polymerase can be isolated in a readily sedimentable form, as had been previously observed for the DNA polymerase isolated from human cells [Malkas et al., 1990b], we subjected mouse FM3A cells to the fractionation scheme outlined in Figure 1. The PEG NE/HS-3, HS-4 and HSP-4 fractions were collected and assayed for DNA polymerase activity, as well as for the presence of other DNA replication proteins and enzymes.

The majority of the DNA polymerase α and DNA primase enzymatic activities isolated from the FM3A cells was observed to partition with the sedimentable HSP-4 fraction following PEG precipitation of the NE/HS-3 fraction from the mouse cell homogenate (Table I). This pattern of partitioning for the DNA polymerase α and DNA primase enzymatic activities had been ob-

TABLE I. DNA Replication Enzyme Activities Fractionate With HSP-4

				PEG		
Fraction[a]	NE/HS-3	HS-4	HSP-4	NE/HS-3	HS-4	HSP-4
DNA polymerase α	186	15	170	209	9	186
DNA primase	70	5	57	48	1	25

[a]Total units of activity for DNA polymerase and DNA primase as defined in the Methods section.

served previously during the isolation of the 18-21S HeLa cell multiprotein DNA polymerase complex [Malkas et al., 1990b]. The ability of the DNA polymerase α and DNA primase activities to preferentially partition to the HSP-4 fraction is independent of any potential aggregation that may have been promoted by PEG precipitation of the NE/HS-3 fraction. This was demonstrated by the fractionation of mouse cells in the absence of the PEG precipitation step. In the *fractionations* performed in the absence of the PEG precipitation step, the DNA polymerase α and DNA primase activities were observed to still preferentially partition with the HSP-4 fraction (Table I).

DNA polymerase δ [Byrnes et al., 1976; Lee et al., 1984] has been shown to be required for the synthesis of SV40 replication origin containing DNA in vitro [Prelich et al., 1987]. To determine whether the DNA polymerase δ polypeptide was associated with the DNA polymerase-primase enriched mouse HSP-4 fraction Western blot analyses of the mouse cell derived PEG NE/HS-3, HS-4 and HSP-4 fractions were performed using a polyclonal antibody prepared against the C-terminal peptide of DNA polymerase δ [Yang et al., 1992]. These analyses clearly demonstrate the presence of this DNA replication essential enzyme in the PEG NE/HS-3 fraction (Fig. 2). In addition, it was observed that the DNA polymerase δ polypeptide is exclusively associated with the mouse HSP-4 fraction (Fig. 2).

Western analyses of the mouse cell protein fractions demonstrated that the DNA ligase I polypeptide (Fig. 3) is also exclusively associated with the mouse HSP-4 fraction, as is the topoisomerase II polypeptide (Fig. 4A). In addition, several topoisomerase II proteolytic fragments were also observed in the mouse HSP-4 fraction (Fig. 4A).

A DNA helicase activity also is primarily associated with the HSP-4 fraction (Fig. 4B). The presence of DNA helicase activity in both the NE/HS-3 and the HSP-4 was detectable at protein levels slightly in excess of 100 ng/reaction; while helicase activity was barely detectable in the HS-4 at 10 times this protein level.

Topoisomerase I activity and PCNA polypeptide were also detected in the HSP-4 fraction (Fig. 5A,B), but unlike the DNA polymerases α and δ, DNA primase, DNA ligase I, DNA helicase, and topoisomerase II, these proteins are found in the HS-4 fraction as well. This result suggests that the topoisomerase I and PCNA

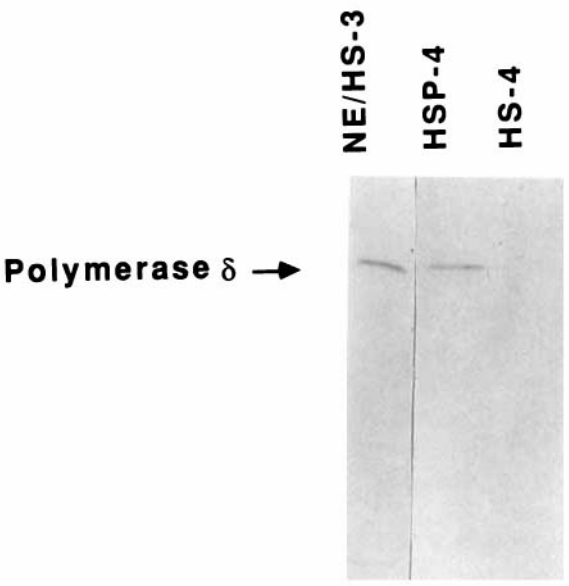

Fig. 2. Western blot analysis for the presence of DNA polymerase δ polypeptide in the NE/HS-3, HS-4, HSP-4 fractions. Twenty micrograms of each protein fraction were size fractionated on an 8% polyacrylamide gel. The proteins were transferred to a nitrocellulose filter membrane and then visualized by sequentially incubating the filter with a 1:50 dilution of a mouse polyclonal anti-polymerase δ anti-serum, followed by a 1:1,000 dilution of an alkaline phosphatase conjugated sheep anti-mouse IgG, and then a solution containing 5-bromo-4-chloro-3-indolyphosphate-p-toluidine salt (BCIP) and nitroblue tetrazolium (NBT).

polypeptides may not be tightly associated with the DNA polymerases, DNA primase, DNA ligase, topoisomerase II and helicase. Alternatively, the abundance of topoisomerase I and PCNA, relative to the other proteins, might influence their distribution during the fractionation.

The co-fractionation of the mouse cell DNA polymerases α and δ, DNA primase, DNA ligase I, DNA helicase, topoisomerases I and II, and PCNA proteins with the HSP-4 fraction suggests that these activities are associated in a readily sedimentable form.

The Sedimentation Coefficient of the Mouse HSP-4 DNA Polymerase Is 17S

The HS-4 and HSP-4 fractions were subjected to velocity sedimentation analysis in 10–30% sucrose gradients containing 0.5 M KCl (see Methods). The gradient fractions were analyzed for DNA polymerase activity, and the S-values were estimated by comparison of the observed migration distances of the polymerase with those of marker proteins on parallel gradients (see

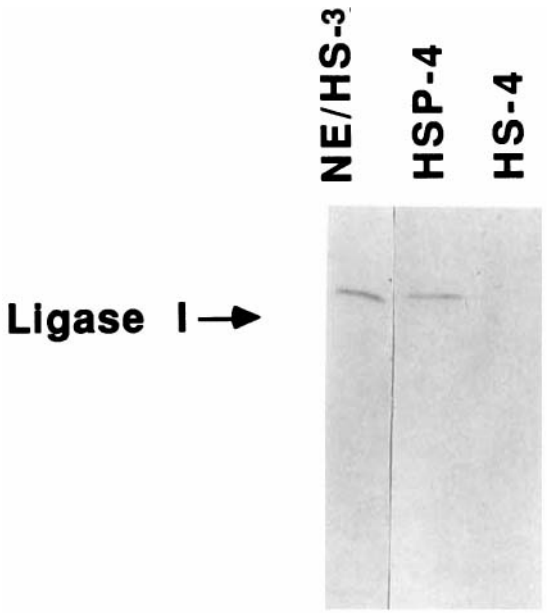

Fig. 3. Western blot analysis for the presence of DNA ligase I polypeptide in the NE/HS-3, HS-4, HSP-4 fractions. Twenty micrograms of each protein fraction was size fractionated through an 8% polyacrylamide gel and then transferred to a nitrocellulose filter membrane. The membrane was treated with sequential incubations of a 1:500 dilution of a rabbit polyclonal antibody to calf thymus DNA ligase I, followed by a 1:1,000 dilution with alkaline phosphatase conjugated goat anti-rabbit IgG, and then a solution containing BCIP and NBT.

Methods). The DNA polymerase activity associated with the HSP-4 and HS-4 fractions had sedimentation coefficients of 17S and 7S, respectively (Fig. 6). The higher S-value of the polymerase activity in the HSP-4 fraction accounts for its sedimentation to the sucrose interphase following centrifugation of the PEG NE/HS-3 fraction (see Methods).

The Mouse Cell 17S Form of DNA Polymerase in the Readily Sedimentable HSP-4 Fraction Supports the In Vitro Replication of DNA

Since the mouse cell DNA polymerases α and δ, DNA primase, DNA helicase, topoisomerases I and II, DNA ligase I, and PCNA proteins partition with the readily sedimentable HSP-4 fraction, an attempt was made to determine whether this fraction could support the in vitro replication of DNA. This was assessed by determining whether the mouse HSP-4 fraction was efficient for the in vitro replication of PyV origin containing DNA (Fig. 7). It was observed that the mouse PEG NE/HS-3 fraction is fully competent in supporting PyV origin containing DNA replication in vitro (Fig. 7). A DE81 filter binding analysis (see Methods) of the ^{32}P-dTTP radiolabeled DNA replication products formed in the reaction showed that the highest level of ^{32}P-dTTP incorporation into DNA occurred when the PEG NE/HS-3 fraction was incubated in the presence of the PyV large T-antigen (Fig. 7A). A negligible amount of ^{32}P-dTTP radiolabel was incorporated in those replication reactions lacking the PyV large T-antigen (Fig. 7A). The PyV large T-antigen dependent replication products were resistant to digestion by DPN I endonuclease (Fig. 7B), indicating that bona fide semiconservative replication of the offered supercoiled PyV replication origin containing DNA had occurred [Murkami et al., 1986]. When the mouse NE/HS-3 fraction was subfractionated into the HS-4 and HSP-4 fractions the ability to support in vitro large T-antigen dependent PyV replication was found to reside exclusively with the sedimentable HSP-4 fraction (Fig. 7A,B). These data indicate that all of the cellular DNA replication factors required to carry out large T-antigen dependent PyV DNA replication are associated with the mouse sedimentable HSP-4 fraction. These results also strongly suggest that the 17S multiprotein form taken by the mouse cell DNA polymerase in the sedimentable HSP-4 fraction permits these cellular factors to function coordinately to carry out all of the reactions required for DNA synthesis.

The Co-Sedimentation of the Mouse Cell DNA Replication Proteins Is Independent of an Association With Other Cellular Components

To determine whether the co-sedimentation of the mouse cell DNA replication proteins was the result of non-specific aggregation with a cellular component, such as a fragment of RNA, DNA, or membrane, we attempted to disrupt any such interaction by treating the mouse PEG NE/HS-3 fraction with the following reagents: high salt (2 M KCl); RNase; DNase; the nonionic detergent NP-40 (0.5%); or 2.5% n-butanol/5% triton X-100 (see Methods). After treatment of the mouse PEG NE/HS-3 fraction with these reagents, HS-4 and HSP-4 fractions were prepared (Fig. 1). The HS-4 and HSP-4 fractions were assayed for DNA polymerase, DNA primase, and in vitro PyV DNA replication activities (Table II). Co-fractionation of the mouse cell DNA polymerase, DNA primase, and in vitro PyV DNA replication activities to the HSP-4

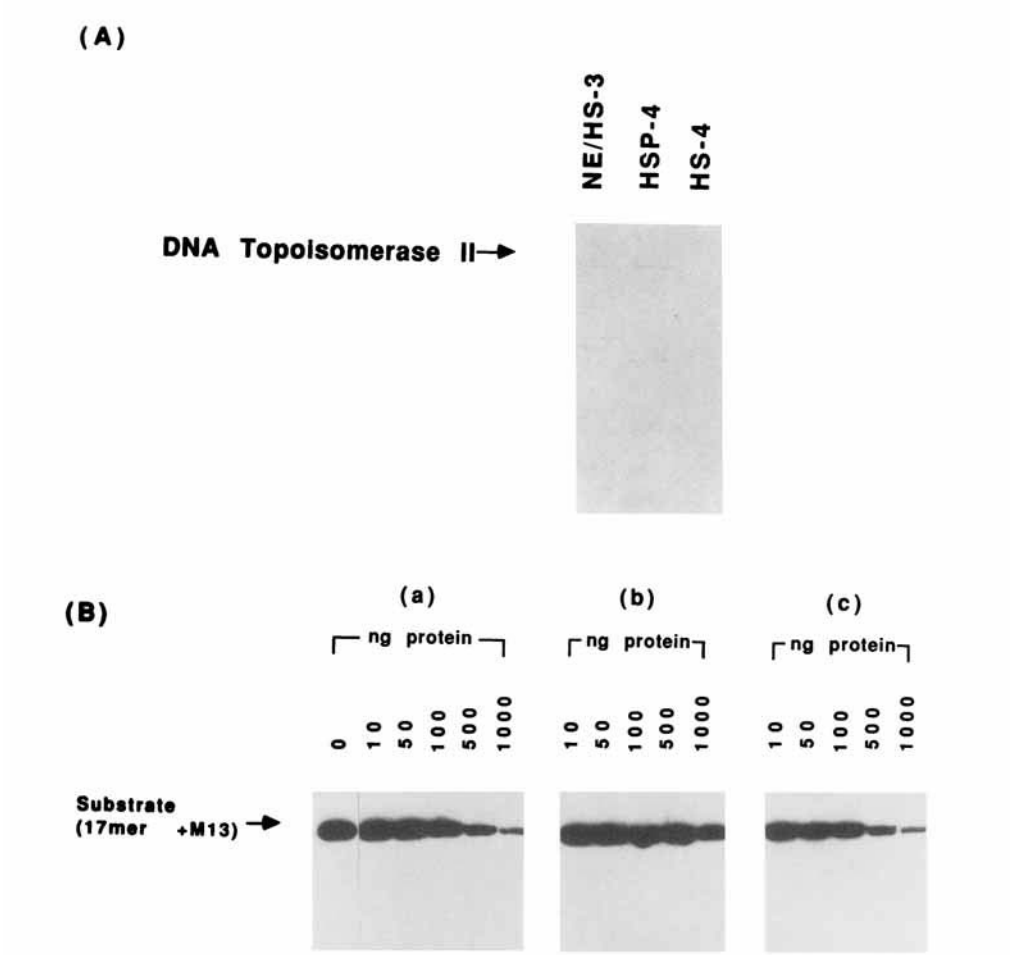

Fig. 4. Assaying for the presence of DNA topoisomerase II and DNA helicase in NE/HS-3, HSP-4, and HS-4 fractions. **A:** the result of a Western blot analysis for the presence of topoisomerase II polypeptide. The protein amount in each fraction was 20 μg. The primary antibody was a mouse monoclonal antibody to human topoisomerase II diluted 1:1,000 in Tris-buffered saline. The secondary antibody was an alkaline phosphatase conjugated sheep anti-mouse IgG diluted 1:1,000. **B:** an analysis of the DNA helicase unwinding activity in the (a) NE/HS-3, (b) HS-4, (c) HSP-4 fractions. The DNA band observed in the gel lanes migrates through a 12% polyacrylamide gel at the position expected for a single-stranded M13 DNA to which a ^{32}P-radiolabeled 17-mer oligonucleotide primer has been hybridized. The protein amounts in each fraction are indicated as nanograms of protein. Details of the procedures are described in Methods. A decrease in the intensity of the DNA band indicates that DNA helicase has unwound the 17-mer primer from the M13 DNA.

fraction was still observed even after treatment of the mouse PEG NE/HS-3 fraction with the various reagents (Table II).

Further Purification of the Mouse Cell 17S Multiprotein Form of DNA Polymerase

The mouse cell 17S multiprotein form of DNA polymerase in the HSP-4 fraction was further purified by DE52-cellulose chromatography (see Methods). The DE52-cellulose elution profile of the mouse HSP-4 fraction total protein, DNA polymerase, DNA primase, and in vitro PyV DNA replication activities are shown in Table III. The total protein of the HSP-4 fraction was observed to equally distribute between the DE52-cellulose low and high salt eluates, while a higher proportion of the DNA polymerase and primase activities was observed in the column high salt eluate. Moreover, the HSP-4 fraction in vitro PyV DNA replication activity was observed to preferentially elute from the DE52-cellulose under the high salt conditions. Denaturing polyacrylamide gel electrophoretic analysis of the DE52-cellulose high salt eluate revealed that the fraction contained approximately 30 polypeptides (data not shown).

The sedimentation coefficient for the DNA polymerase in the DNA replication competent DE52-cellulose high salt eluate was determined on a 10–30% sucrose gradient containing 0.5 M

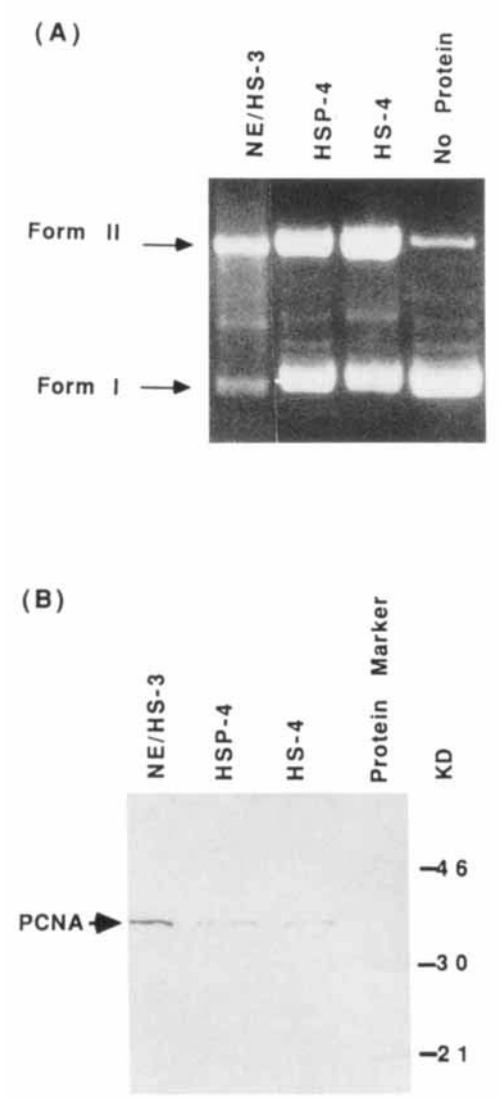

Fig. 5. Assaying for the presence of topoisomerase I and PCNA in the NE/HS-3, HSP-4, and HS-4 fractions. **A:** the degree of conversion of a supercoiled Form I plasmid DNA to a relaxed open circle form II DNA following the incubation of 1.3 μg of supercoiled plasmid DNA (pJLPYO) with 5 μg of each protein fraction. The position of the supercoiled DNA (Form I) and the relaxed open circular DNA (Form II) are indicated with arrows. The degree of relaxation correlates directly with the level of topoisomerase I activity present in each fraction tested. The reaction was incubated at 30°C for 10 min, and then stopped by the addition of 1% SDS. **B:** Western blot analysis of these same fractions for the presence of PCNA polypeptide. Each lane contained 20 μg of protein. The primary antibody was a mouse anti-rabbit PCNA monoclonal antibody diluted 1:1,000 in TBS. The secondary antibody is an alkaline phosphatase conjugated sheep anti-mouse IgG antibody also diluted 1:1,000 in TBS. A solution containing the alkaline phosphatase substrates BCIP and NBT was used to visualize the protein band.

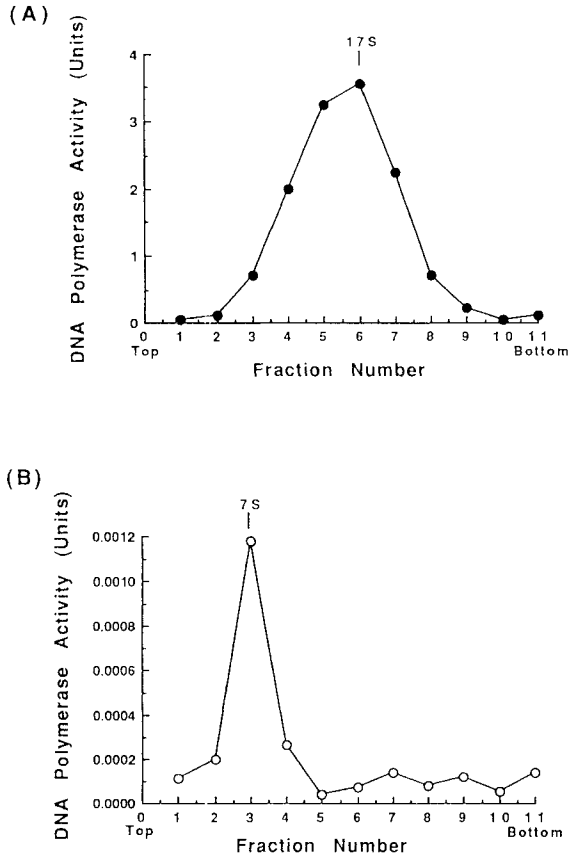

Fig. 6. Sucrose gradient profiles of DNA polymerase activity for **(A)** the HSP-4 and **(B)** the HS-4 fractions. Details of the experimental procedures are described in the Methods section. Units of DNA polymerase activity are as defined in Methods.

KCl (see Methods). It was observed that the replication competent multiprotein form of mouse DNA polymerase in the DE52-cellulose high salt eluate maintained a sedimentation coefficient of 17S (Fig. 8).

The replication competent DE52-cellulose high salt eluate was also examined by Western blotting and activity measurements in order to determine the presence of DNA polymerase δ, topoisomerases I and II, DNA ligase I, PCNA, and DNA helicase. As shown in Figure 9A–F, these proteins are present in the high salt DE-52 eluate of the HSP-4 fraction. Furthermore, these proteins co-chromatograph with the 17S form of DNA polymerase in the high salt eluate of the DE52-cellulose. Analyses of the peak 17S DNA polymerase fraction (Fig. 8, fraction 5), derived from the sucrose gradient analysis of the DE52-cellulose high salt eluate for the presence of the DNA replication essential proteins, indicate that these proteins co-sediment with the DNA repli-

(A)

pmoles 32-P-TTP incorporated/hr at 35 C ^{0}

Fraction	+/- PYV Large T-Antigen +T	+T	-T	-T
NE/HS-3	85.3	84.9	0.7	0.9
HS-4	0.1	0.2	0.5	0.3
HSP-4	57.4	59.2	0.2	0.6

(B) DPNI Digestion of The Replication Products

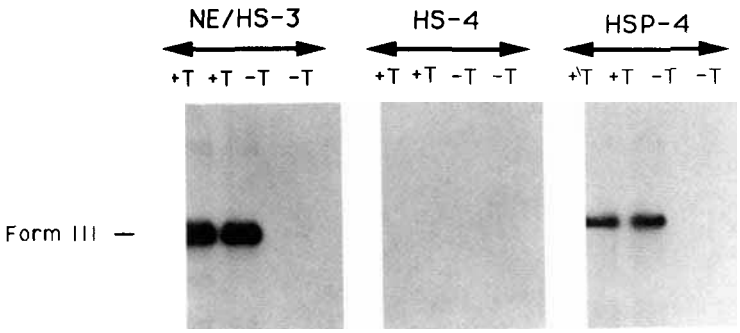

Fig. 7. The in vitro PyV DNA synthesis activities of the NE/HS-3, HS-4, HSP-4 fractions. Fifty micrograms of protein from each fraction was used in these assays in the presence (+T) or the absence (−T) of the PyV large T-antigen, as described in Methods. **A:** the number of pmol of ^{32}P-TTP incorporated per hour into PyV-ori containing plasmid DNA PJLPYO at 35°C. **B:** Neutral 1% agarose gel electrophoretic analysis of the in vitro polyoma virus DNA replication assay products formed in the absence and the presence of T-antigen by the indicated fractions. The DNA products were digested with 10 U of the restriction endonucleases DPNI and Sal I at 37°C for 2 h in order to linearize the plasmid DNA and digest repaired or not fully replicated DNA products.

cation competent 17S DNA polymerase (data not shown).

Through the DE52-cellulose stage of the purification, the DNA polymerase α activity has been purified 112-fold over the activity present in the NE/HS-3 fraction and approximately 340-fold over the activity in the crude cell extract.

DISCUSSION

There have been a number of reports regarding the isolation of large complexes of proteins for DNA synthesis [reviewed in Mathews and Slabaugh, 1986; Fry and Loeb, 1986; Malkas et al., 1990a]. One of the first reports described the co-sedimentation of some of the enzymes for deoxyribonucleotide metabolism with the DNA synthetic enzyme DNA polymerase α from homogenates of regenerating rat liver and hepatomas [Baril et al., 1974]. Similarly, in prokaryotes, T4 bacteriophage ribonucleotide reductase was reported to form a complex with other enzymes for deoxyribonucleotide metabolism [Mathews and Slabaugh, 1986; Reddy et al., 1978]. The association of this bacteriophage multiprotein complex was shown to regulate the activity of the respective enzymes, and in addition the T4 DNA polymerase was shown to interact with the complex [Tomich et al., 1974]. The channeling of uridine or UTP into dTTP and hydroxymethyl dCTP synthesis was demonstrated for the complex [Mathews and Slabaugh, 1986; Reddy et al., 1978]. The actual

TABLE II. Percentage of In Vitro DNA Replication and Enzymatic Activities Associated With HS-4 and HSP-4 Fractions Following Treatment of the PEG NE/HS-3 Fraction With Various Agents*

	% DNA polymerase α		% DNA primase		% PyV DNA replication activities	
Reagent	HS-4	HSP-4	HS-4	HSP-4	HS-4	HSP-4
Untreated	16	84	5	95	0	100
RNase	20	80	5	92	0	100
DNase	18	83	9	91	0	100
NP-40	23	77	3	97	0	100
Butanol/ Triton	20	80	10	90	0	100
2 M KCl	16	84	4	96	0	100

*The total unit activity for DNA polymerase, DNA primase, and PyV DNA replication remaining in the HS-4 and the HSP-4 was determined following the treatment of the PEG treated NE/HS-3 fraction with the agents indicated in the table. The sum of the total number of units of catalytic activity present in the HS-4 and the HSP-4 was then assigned a value of 100%, and the percent of activity contributed by each fraction to the total was calculated.

TABLE III. The Distribution of Enzymatic and In Vitro DNA Replication Activities Following DE52-Cellulose Chromatography

Fraction	Low salt eluate	High salt eluate
DNA polymerase (total units)[a]	14.4	22.0
DNA primase (total units)[a]	1.5	3.3
[32-P]TTP incorporated into PyV-ori containing plasmid DNA (pmols)[a]	12.0	171.0
Total protein (mg)	15.0	14.5

[a]As defined in Methods.

isolation and characterization of the complex, however, was hampered by its fragility.

Recently published experiments using the vaccinia viral system suggest that a 34 kDa viral DNA binding protein specifically interacts with the viral ribonucleotide reductase [Davis and Mathews, 1993]. From this observation the authors suggest that there is an association between deoxynucleotide triphosphate (dNTP) synthesis and DNA replication in the vaccinia viral system. Reddy and Pardee [1980] isolated a complex of proteins for DNA synthesis from hamster fibroblast cells (CHEF) that contained nascent DNA, as well as enzymes for deoxyribonucleotide metabolism and DNA synthesis. The complex was reported to effectively couple ribonucleotide reduction and dNTP synthesis with the subsequent incorporation of the newly made dNTPs into DNA in vitro [Reddy and Pardee, 1982]. The ability of the complex to actually channel ribonucleotide-diphosphate (rNDP) into DNA, however, had been questioned by the work of Spyrou and Reichard [1983] who reported that rNDP was preferentially incorporated into RNA by the complex. The work of Spyrou and Reichard [1983] is not in conflict with the existence of a multiprotein complex for DNA synthesis. Rather, their findings suggest that in eukaryotic systems ribonucleotides are not channeled into DNA by such a complex. Obviously, much work still needs to be done to fully describe the possible physical interaction of the enzymes involved in the de novo synthesis of dNTP with the DNA replication apparatus in eukaryotic systems.

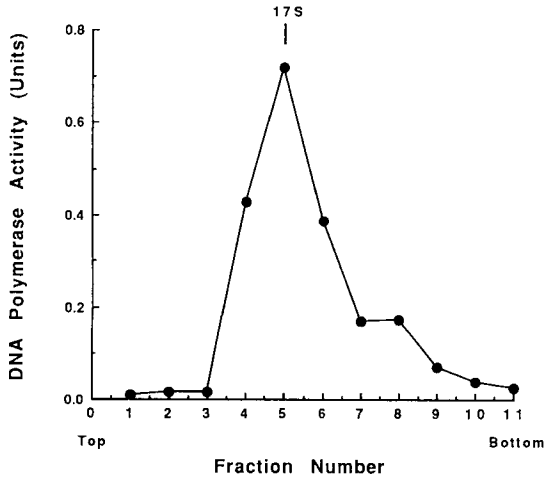

Fig. 8. Sucrose gradient profile for DNA polymerase activity in the DE52 cellulose high salt eluate fraction. Details of the procedures used for running the gradient as well as the unit definition and the assignment of size to the peak fraction are described in Methods.

The actual role of the eukaryotic nuclear architecture in the organization of the DNA synthesizing apparatus is not clear at this time. The work of de Bruyn Kops and Knipe [1988] suggests that the herpesvirus DNA binding protein ICP8 can play a role in the organization of DNA replication proteins at discrete sites within the cell nucleus. Also, a recently published immunolocalization study of RP-A in *Xenopus* egg extracts revealed that this protein is associated with subnuclear foci which the authors pro-

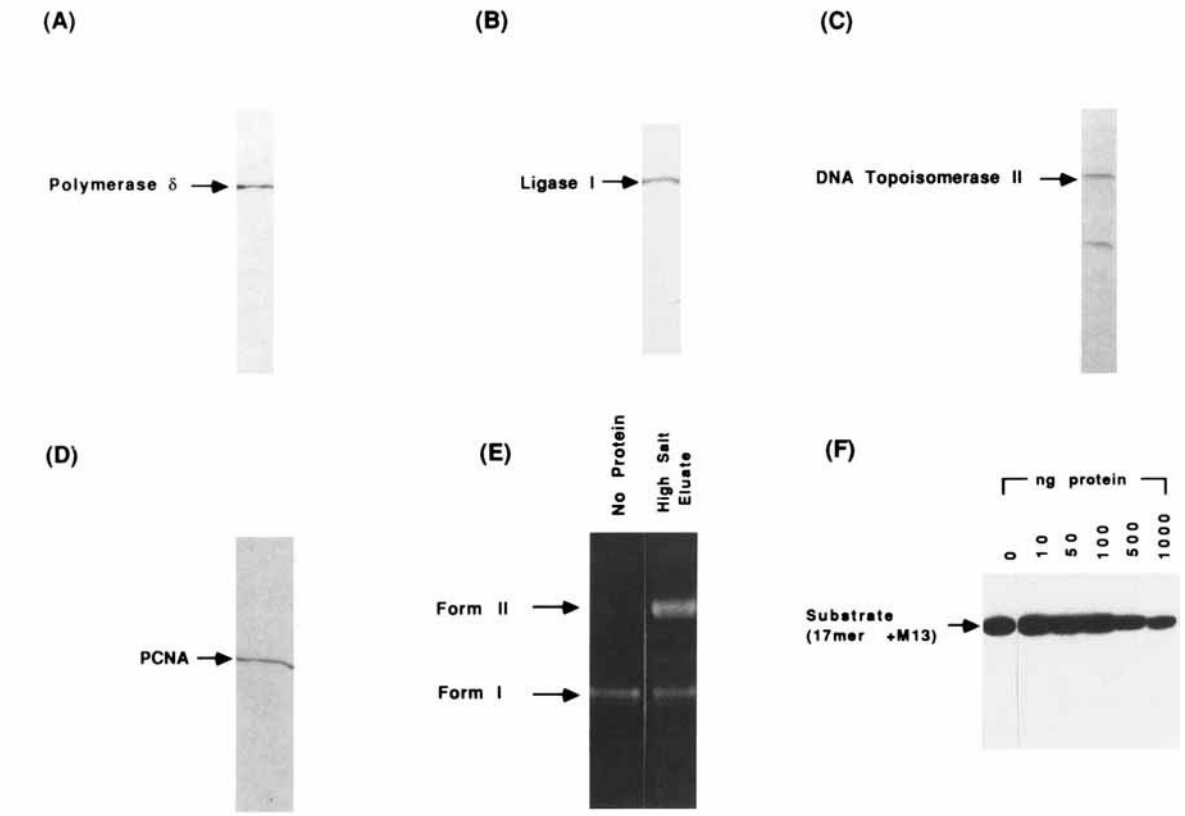

Fig. 9. DNA replication proteins detected in the DE52-cellulose high salt eluate fraction. Panels A–D depict the Western blot analyses for DNA polymerase δ (**A**), DNA ligase I (**B**), DNA topoisomerase II (**C**), and PCNA (**D**), respectively. The primary and secondary antibodies as well as the enzyme substrates used to identify these proteins were the same as those used to identify these proteins in the HSP-4 fraction. Twenty micrograms of protein were used in each lane. **E:** the agarose gel electrophoretic analysis of the relaxed plasmid DNA products formed by the topoisomerase I present in the high salt eluate. The experimental conditions used for the assay were the same as described in Figure 5A. **F:** an analysis of DNA helicase unwinding activity present in the high salt eluate fraction. The assay conditions were as described in Methods, and the experimental design was as described in the legend for Figure 4B.

posed to be an assembly of prereplication centers [Adachi and Laemmli, 1992]. The prereplication centers form post-mitotically during the breakdown of the nuclear membrane, and are absent during the later stages of DNA replication, suggesting that the centers are assembled and poised for the initiation of DNA synthesis in distinct subnuclear regions. The nuclear matrix has also been implicated in chromosomal DNA replication [Tubo and Berezney, 1987a,b; Jackson and Cook, 1986a; Berezney and Coffey, 1975]. Nakamura et al. [1984] performed indirect immunofluorescence microscopy studies on human cells using a monoclonal antibody directed against DNA polymerase α. Microscopy at high magnification revealed a granular pattern of the fluorescence in the nuclei of the cells. The fine granules and meshwork of the fluorescence resembled the pattern of nuclear matrix. Nakayasu and Berezney [1989] used fluorescent microscopy to map DNA replication sites in the mammalian interphase cell nucleus after incorporation of biotinylated dUTP. Discrete replication granules were observed to be distributed throughout the nuclear interior and along the periphery. In situ prepared nuclear matrix structures also incorporated biotinylated dUTP into replication granules that were indistinguishable from those detected within the intact nucleus. The authors suggest that each replication granule may correspond to a replication cluster assembly, in which numerous tandemly arranged replicons are coordinately synthesizing DNA. Jackson and Cook [1986b] reported the retention of a large megacomplex containing DNA polymerase α and other enzymes for DNA synthesis in agarose entrapped nuclei. The megacomplex could function in the replication of endogenous chromosomal DNA, and evidence was obtained suggesting that it may associate with

the nuclear matrix [Jackson and Cook, 1986a]. Tubo and Berezney [1986a,b] also reported the isolation of large 100–150S megacomplexes containing DNA polymerase-primase activities that were associated with the nuclear matrix from regenerating rat liver. The megacomplexes appear to be composed of clusters of 10S and 17S complexes that underwent dissociation when the isolated megacomplexes were left at 4°C for an hour or more. Although more extensive investigation is required, these studies suggest that the mammalian DNA synthesizing apparatus may assemble in some fashion into megacomplexes. These megacomplexes may then associate with the components of the nuclear architecture to form a supramolecular structure for DNA synthesis.

In this report we have identified and purified a 17S multiprotein form of DNA polymerase from the murine mammary carcinoma cell line FM3A. We have also demonstrated that the integrity of the murine cell complex is maintained after treatment with detergents, high salt, nucleases, or ion-exchange chromatography. Most importantly, we have demonstrated that this complex of proteins is fully competent to replicate PyV DNA in vitro. The requirements for PyV DNA replication in vitro by the isolated mouse cell 17S multiprotein complex are comparable to the requirements that have been observed with crude extracts from permissive cells [Murakami et al., 1986; Dermody et al., 1988]. The in vitro PyV DNA replication products of the 17S multiprotein form of DNA polymerase are resistant to DPN I, which digests the methylated assay input plasmid DNA [Murakami et al., 1986], consistent with semi-conservative replication of full length daughter DNA, as has been observed for large T-antigen dependent PyV replication in crude cell-free extracts [Murakami et al., 1986].

The observation that DNA polymerase δ copurifies with the mouse cell 17S multiprotein form of DNA polymerase extends our current knowledge regarding the proteins present within the mammalian *M*ultiprotein DNA *R*eplication *C*omplex (MRC). In a previous publication on the human (HeLa) cell MRC [Malkas et al., 1990b] we interpreted our results on the inhibition of the MRCs in vitro SV40 DNA replication activity by BuPdGTP [Byrnes, 1985; Lee et al., 1985] and the anti-polymerase α antibody 132.20 [Tanaka et al., 1982] as indicative that the MRC lacked DNA polymerase δ. However, in the Western blot experiments we report here DNA polymerase δ is identified as a component of the 17S form of the mouse MRC. We have also found DNA polymerase δ to be associated with the HeLa MRC (results to be published elsewhere). We now interpret our earlier results on the HeLa MRC in vitro SV40 DNA replication inhibition by BuPdGTP and anti-polymerase α antibody as indicative of the absolute requirement for DNA polymerase α in the initiation events of DNA synthesis by the MRC, as suggested by the work of Wold et al. [1989] and Denis and Bullock [1993] using reconstituted DNA replication systems.

Topoisomerase II and a DNA helicase activity are also associated with the replication competent mouse cell 17S MRC. The MRC's topoisomerase II would presumably function in the role of facilitating the segregation of daughter DNA molecules, as has been described by Yang et al. [1987] using a reconstituted in vitro SV40 DNA replication system. The DNA helicase activity associated with the mouse MRC could promote the melting of parental DNA strands to permit access of the elongation components of the MRC to the DNA. However, we have yet to establish that the MRC associated DNA helicase functions in the in vitro replication of PyV origin containing DNA in the presence of the PyV large T antigen.

Based on the fractionation and column chromatographic profiles for the individual proteins observed to be present in the mouse cell 17S MRC, we propose a model to represent the mammalian MRC (Fig. 10). The majority of DNA polymerases α and δ, primase, DNA helicase, DNA ligase I, and topoisomerase II are observed to co-fractionate from cells, and to co-elute following column chromatography. This would suggest that these proteins are "tightly" associated within the MRC. We propose that these proteins form the "core" of the MRC. It is interesting to note that these proteins also function primarily in the elongation phase of DNA synthesis [Kornberg and Baker, 1992]. Unlike the proteins that compose the MRC core, PCNA is observed to be more "loosely" associated with the MRC, based on our observations that although PCNA co-fractionates and co-elutes, following column chromatography, with the MRC core proteins it can also be found in fractions that do not support in vitro DNA synthesis. Because of these observations, PCNA, which functions as an accessory factor for DNA polymerase δ [Tan et al., 1986], is represented in our model as associated

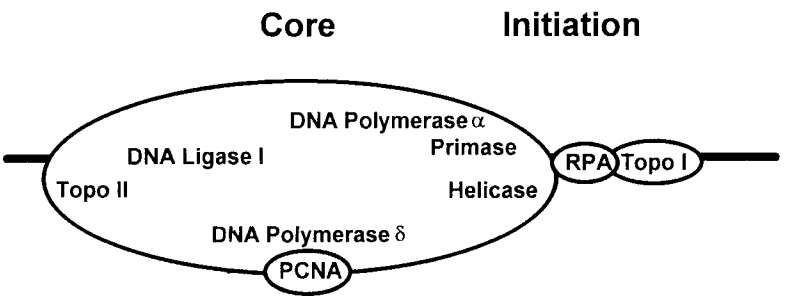

Fig. 10. A proposed model for the organization of the identified protein components of the mammalian MRC.

with the MRC, but not as a member of the MRC core complex.

The role played by large T antigen in the initiation of in vitro SV40 DNA replication has been recently reviewed [Borowiec et al., 1990; Fanning and Knippers, 1992]. Briefly, the large T antigen binds sites within the SV40 origin and melts the early palindrome region within the DNA region. The addition of topoisomerase I and RP-A facilitates the further melting of the SV40 DNA. A similar mechanism can be envisioned for the initiation events occurring in in vitro PyV DNA replication. We have observed that topoisomerase I and RP-A (data not shown), like PCNA, do not "tightly" associate with the MRC components that compose the MRC core. We, therefore, propose that topoisomerase I and RP-A constitute the MRC's "initiation" components. Together, the core and initiation components would constitute the mammalian MRC.

Since PyV is extensively dependent on the mouse cell DNA synthetic machinery for its own DNA replication, our results indicate that the isolated murine multiprotein complex may play a role not only in PyV DNA synthesis but in mammalian cell DNA replication as well. The further study of the murine cell multiprotein complex for DNA synthesis should be expected to yield a clearer understanding of the role of this complex in mediating the many reactions required to carry out mammalian DNA replication. This seems especially true since the MRC was isolated as an intact and functional entity from the cell and, therefore, presumably contains all of the components required to successfully replicate DNA. In addition, because the process of DNA replication is an important regulatory point for modulating cell proliferation, the elucidation of the role played by the mammalian multiprotein complex, and its components, on this regulatory process can be anticipated to help further our understanding of both normal and aberrant cell proliferation. It is, therefore, our goal to fully characterize this complex with respect to its structural components and activity, and relate this information to the higher level of organization within the cell. A variety of studies are underway to accomplish this goal.

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