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Further Characterization of the Human Cell Multiprotein DNA Replication Complex

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Abstract Evidence for multiprotein complexes playing a role in DNA replication has been growing over the years. We have previously reported on a replication-competent multiprotein form of DNA polymerase isolated from human (HeLa) cell extracts. The proteins that were found at that time to co-purify with the human cell multiprotein form of DNA polymerase included: DNA polymerase α, DNA primase, topoisomerase I, RNase H, PCNA, and a DNA-dependent ATPase. The multiprotein form of the human cell DNA polymerase was further purified by Q-Sepharose chromatography followed by glycerol gradient sedimentation and was shown to be fully competent to support origin-specific and large T-antigen dependent simian virus 40 (SV40) DNA replication in vitro [Malkas et al. (1990b): Biochemistry 29:6362–6374].

In this report we describe the further characterization of the human cell replication-competent multiprotein form of DNA polymerase designated MRC. Several additional DNA replication proteins that co-purify with the MRC have been identified. These proteins include: DNA polymerase δ, RF-C, topoisomerase II, DNA ligase I, DNA helicase, and RP-A. The replication requirements, replication initiation kinetics, and the ability of the MRC to utilize minichromosome structures for DNA synthesis have been determined. We also report on the results of experiments to determine whether nucleotide metabolism enzymes co-purify with the human cell MRC. We recently proposed a model to represent the MRC that was isolated from murine cells [Wu et al. (1994): J Cell Biochem 54:32–46]. We can now extend this model to include the human cell MRC based on the fractionation, chromatographic and sedimentation behavior of the human cell DNA replication proteins. A full description of the model is discussed. Our experimental results provide further evidence to suggest that DNA synthesis is mediated by a multiprotein complex in mammalian cells.

Key words: mammalian cell, DNA replication, multiprotein complex, simian virus 40, HeLa cells

The precise mechanisms involved in the regulation of the eukaryotic cell DNA synthesizing machinery are poorly understood. In vitro DNA replication systems have identified several mammalian enzymes and proteins required for DNA synthesis [reviewed in Kelly, 1988; Challberg and Kelly, 1989; Stillman, 1989; Hurwitz et al., 1990; Malkas et al., 1990a; Stillman et al., 1992]. In particular, the employment of a simian virus 40 (SV40)-based cell-free DNA replication system [Li and Kelly, 1984] has identified DNA polymerase α, DNA primase, DNA polymerase δ, proliferating cell nuclear antigen (PCNA), RP-A (aka RF-A or HSSB), topoisomerases I and II, and the RF-C or Activator 1 (A-1) protein complex as actively participating in the synthesis of DNA in vitro. Although these proteins have been identified as playing a role in DNA replication, their functional organization allowing for the efficient replication of DNA has not been well defined.

Evidence for multiprotein complexes playing a role in DNA replication has been growing over the years [reviewed in Mathews and Slabaugh, 1986; Malkas et al., 1990a; Reddy and Fager, 1993]. We have previously reported on a replication-competent multiprotein form of DNA polymerase isolated from human (HeLa) cell extracts that was observed to sediment at 18-21S

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during velocity sedimentation analyses [Malkas et al., 1990b]. The proteins found to co-purify with the human cell multiprotein form of DNA polymerase include: DNA polymerase α, DNA primase, topoisomerase I, RNase H, PCNA, and a DNA-dependent ATPase [Malkas et al., 1990b]. The multiprotein form of the human cell DNA polymerase was further purified by Q-Sepharose chromatography and glycerol gradient sedimentation and was shown to be fully competent for supporting origin-specific and large T-antigen dependent SV40 DNA replication in vitro [Malkas et al., 1990b]. This laboratory has also recently identified and characterized a 17S multiprotein form of DNA polymerase from murine (FM3A) cells [Wu et al., 1994] that is capable of supporting DNA replication using the polyomavirus (PyV)-based in vitro DNA synthesis system [Murakami et al., 1986; Dermody et al., 1988]. Proteins and enzymatic activities identified to co-purify with the mouse cell multiprotein form of DNA polymerase include: DNA polymerases α and δ, DNA primase, PCNA, DNA ligase I, DNA helicase, and DNA topoisomerases I and II. It was also demonstrated that the integrity of the murine cell multiprotein form of DNA polymerase was maintained after treatment with detergents, salt, RNase, or DNase, suggesting that the association of the proteins with one another was independent of nonspecific interactions with other cellular macromolecular components. It was proposed that the isolated mouse cell multiprotein form of DNA polymerase represented a mammalian multiprotein DNA replication complex (MRC). We introduced a model to represent the MRC based on the fractionation and chromatographic profiles of the individual proteins found to co-purify with the complex [Wu et al., 1994].

In this report we describe the further characterization of the human (HeLa) cell replication-competent multiprotein form of DNA polymerase or MRC. Our experimental results provide further evidence to suggest that DNA synthesis is mediated by a multiprotein complex in mammalian cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Harvest

Suspension cultures of HeLa 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, 137 mM 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). The cell pellets were stored at −80°C prior to initiating the isolation of the MRC.

Cell Fractionation and Column Chromatographic Procedures

HeLa cells were fractionated essentially as described by Malkas et al. [1990b] and as outlined in Figure 1. Sucrose gradient centrifugation was performed as in Wu et al. [1994]. The protein fractions designated P-4, Q-Sepharose peak, S-4, Q-Sepharose flow-through, and Sucrose gradient peak were used in the experiments described in this report. The replication-competent MRC was previously shown to reside in the P-4, Q-Sepharose peak and Sucrose gradient peak protein fractions [Malkas et al., 1990a,b]. The S-4 and Q-Sepharose flow-through fractions do not support in vitro DNA replication [Malkas et al., 1990b].

Enzyme Assays

DNA polymerase α activity was assayed according to published procedures [Lamothe et al., 1981; Vishwanatha et al., 1986] with activated and primed, single-stranded DNA templates. One unit of DNA polymerase α activity equals 1 nmol of total TMP incorporated into DNA per hour at 35°C.

DNA primase activity was assayed by measuring the incorporation of [^{3}H]dATP into acid-insoluble material in the presence of poly(dC) as template [Vishwanatha and Baril, 1986]. One unit of DNA primase activity equals 1 nmol of dAMP incorporated into acid-insoluble material per hour at 30°C.

DNA helicase activity assays were performed as described in our previously published procedures [Wu et al., 1994] with the following modification: the addition of 0.5 mg/ml heparan sulfate in the assay.

Topoisomerase I activity was assayed as described in Hickey et al. [1988] for the relaxation of pSVO$^+$ plasmid DNA [Stillman et al., 1985].

Thymidine kinase (TK) activity was measured as described by Postel and Levine [1975]. Protein fractions were incubated with 25 μl of a TK-enzyme assay mix (TK-EAM, consisting of 110 mM Tris-HCl, pH 7.8, 7 mM $MgCl_2$, 4 mM

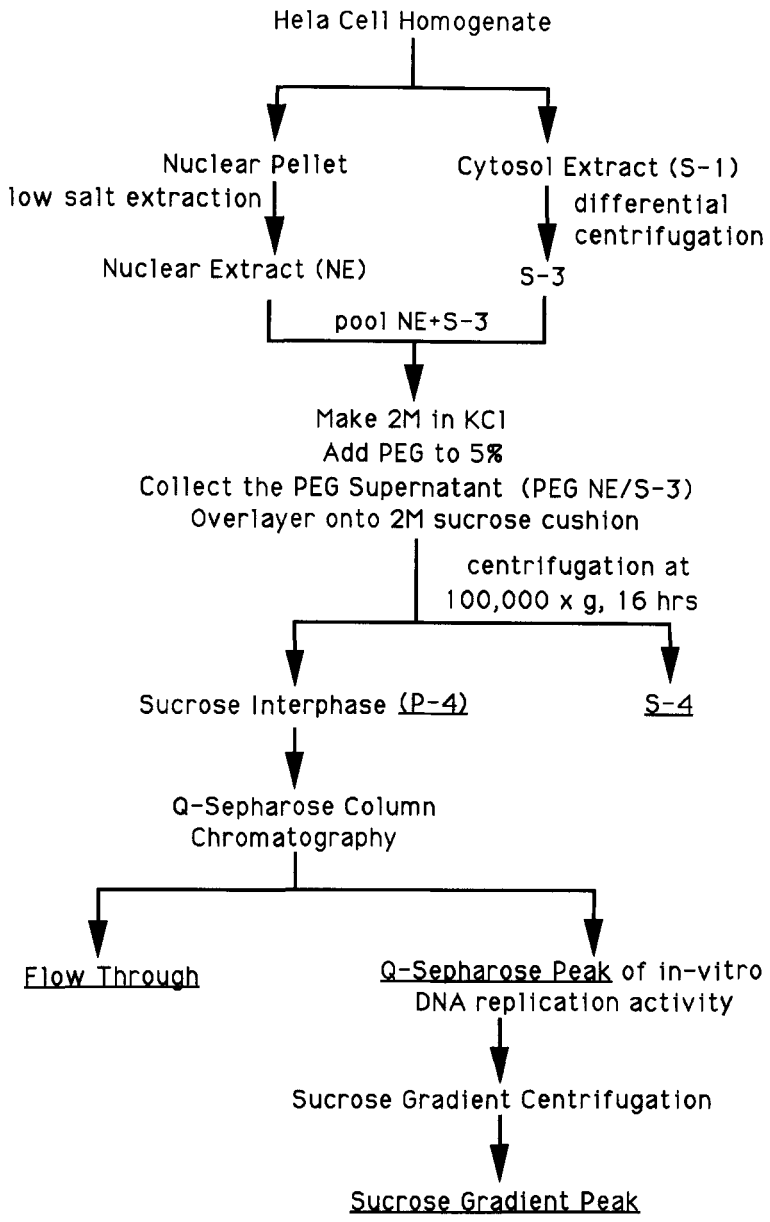

Fig. 1. The flow diagram of the isolation scheme used to purify the human cell multiprotein DNA replication complex (MRC). A description of the isolation scheme is presented in Experimental Procedures.

DTT, 12 mM ATP, and 2.5 μCi ^{3}H-thymidine [^{3}H-TdR] [20 Ci per mmol, New England Nuclear, Boston, MA]) in a total assay volume of 50 μl for 20 min at 37°C. The reaction was stopped by boiling the reaction mix for 2 min. The reaction mix was spotted onto Whatman DE-81 filter discs and dried under an infrared heat lamp. The filter discs were then washed four times in 1 mM ammonium formate, followed by two washes in methanol. The filters were dried and the levels of radioactivity determined by liquid scintillation spectrometry. Several different protein fraction concentrations were initially assessed to determine the amount of protein required to produce a linear correspondence between reaction rate and protein concentration. One unit of TK activity equals 1 nmol of ^{3}H-TdR converted to ^{3}H-thymidine monophosphate (^{3}H-TMP) by enzyme incubated at 37°C for 20 min.

Dihydrofolate-reductase-methotrexate binding activity (DHFR-^{3}H-MTX) was measured es-

sentially as described by Johnson et al. [1978]. Protein fractions were incubated for 10 min at room temperature with 50 μl of a DHFR-^{3}H-MTX binding assay mix (DHFR-^{3}H-MTX-BAM consisted of 0.01 M phosphate buffer, pH 6.0, 0.15 M KCl, 1 mg/ml BSA, 3×10^{-4} M NADPH, and 4×10^{-8} M ^{3}H-MTX [18 Ci/mmol, Amersham, Arlington Heights, IL]) in a total assay volume of 100 μl. After incubation, the samples were washed twice in 300 μl of a charcoal-dextran suspension (1 g acid-washed charcoal and 0.01 g Dextran T-2000, 0.25 g BSA, 30 ml H_2O, pH adjusted to 6.2 with phosphate buffer). The samples were then centrifuged for 5 min at 2,000 rpm. The supernatant was poured into scintillation vials containing 10 ml Safety-Count (Research Products International Corporation, Mt. Prospect, IL) and the level of radioactivity determined. One unit of DHFR-^{3}H-MTX binding activity equals 1 nmol of ^{3}H-MTX bound by enzyme incubated at room temperature for 10 min.

Immunodetection of DNA Ligase I, Topoisomerase II, DNA Polymerase δ, RF-C, and RP-A

Thirty micrograms of each of the various protein fractions were resolved using denaturing polyacrylamide gel electrophoresis [Laemmli, 1970]. Western blot analysis of these proteins was performed using an ECL detection system (Amersham). To detect the DNA polymerase δ polypeptide a monoclonal antibody prepared against the C-terminal peptide of the DNA polymerase δ [Yang et al., 1992] was used. The anti-polymerase δ antibody was used at a 1:1,000 dilution. The anti-DNA ligase I antibody was a gift from Dr. Alan Tomkinson and used at a dilution of 1:1,000. The anti-topoisomerase II antibody was purchased from TopoGEN, Inc., and used at a 1:1,000 dilution. The anti-RF-C monoclonal antibodies (mAb-11 and mAb-19), which recognize the 140 kDa subunit of the RF-C protein complex, and the anti-RP-A antibody (p34-20) that recognizes the 34 kDa subunit of RP-A were a kind gift from Dr. Bruce Stillman. The anti-RF-C antibodies were used simultaneously in immunoblots at dilutions of 1:500. The anti-RP-A antibody was used at a dilution of 1:500 in the immunoblots. The appropriate species-specific horseradish peroxidase conjugated secondary antibodies were used in the immunoblots.

Purification of SV40 Large T-antigen

SV40 large T-antigen was purified from 293 cells infected with recombinant adenovirus vector Ad-SVR284 via immunoaffinity chromatography of the clarified cell lysate using an immobilized protein A matrix to which was covalently coupled a monoclonal antibody from hybridoma Pab 419 reactive for SV40 large T-antigen [Simanis and Lane, 1985].

In Vitro SV40 DNA Replication Assay

Assay reaction mixtures (25 μl) contained 80 mM Tris-HCl, pH 7.5; 7 mM $MgCl_2$; 1 mM DTT; 3–20 μg of protein fraction; 0.5–1.0 μg of purified SV40 large T-antigen; 50 ng of plasmid pSV0$^+$ containing an insert of SV40 replication-origin DNA sequences [Stillman et al., 1985]; 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 standard reaction was incubated for 2 h at 35°C or for the length of time indicated in Results. The replication assay reaction products were processed using DE81 filter binding to quantify the amount of radiolabel incorporated into the replication products [Sambrook et al., 1989]. One unit of SV40 replication activity is equal to the incorporation of 1 pmol of dNMP into SV40 DNA per 2 h under the standard assay condition.

In the experiments using SV40 origin-containing minichromosomes 50 ng of nucleosome assembled or protein-free (naked) pSV0$^+$ DNA were utilized in the in vitro DNA replication assays.

Assembly of SV40 Origin-Containing Minichromosomes

Histone octamers were isolated from HeLa cells using the procedure of Stein [1989]. HeLa cells were lysed in a buffer containing 1% Triton X-100, 0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 3 mM $CaCl_2$. The nuclei were collected by centrifugation at 1,500*g*. The nuclei were then washed twice with the lysis buffer, and then twice more with the same buffer without Triton X-100. The nuclei were then resuspended at a concentration of 2×10^8 nuclei/ml in 0.7 M NaCl, 50 mM sodium phosphate, pH 6.8. To deplete the chromatin of H1, the lysed nuclei were stirred for 30 min at 4°C, and then 4 g DNA grade hydroxyapatite (Bio-Rad, Richmond, CA) were added to make a paste. The volume was

then increased 10-fold with 0.7 M NaCl, 50 mM sodium phosphate, pH 6.8, and the suspension was then stirred for 10 min at 4°C. The chromatin-bound hydroxyapatite was collected by centrifugation at 2,000*g* for 5 min. The H1 removal step was repeated six additional times. Histone octamers were isolated from the chromatin by extraction of the hydroxyapatite with 10 volumes of 2.5 M NaCl, 50 mM sodium phosphate, pH 6.8, with stirring for 15 min at 4°C, followed by centrifugation at 2,000*g* for 5 min. The extraction was carried out twice, and the supernatants were combined. A Centriprep-10 concentrator (Amicon, Danvers, MA) was used to bring the histone octamer concentration in the extract to 2–5 mg/ml. The concentrated octamer sample was then dialyzed against 2 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

Nucleosome assembly reactions were carried out using a modification of the procedure of O'Neill et al. [1992]. The following conditions were chosen because they yielded a 50% level of assembly. $pSV0^+$ DNA (6 μg) in 2 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, was mixed with 9 μg histone octamer in equal volume of the same buffer, and the mixture was incubated at ambient temperature for 30 min. The sodium chloride concentration was lowered stepwise from 2 to 0.12 M. To determine the extent of nucleosome assembly on the $pSV0^+$ DNA the reconstitution mixtures were assayed using micrococcal nuclease digestion followed by treatment with topoisomerase I. An aliquot of the nucleosome assembly reaction was digested with 0.15 U of micrococcal nuclease (Sigma Chemical Co., St. Louis, MO) for 4 min at 37°C. The digestion products were precipitated and analyzed on a 4% polyacrylamide gel containing 0.1% SDS, 36 mM Tris-HCl, 30 mM sodium phosphate, 10 mM EDTA, pH 7.8. Another aliquot of the assembly reaction was assayed using topoisomerase I, and products of this assay were characterized by electrophoresis in a 1% agarose gel containing 50 mM Tris-borate, 1 mM EDTA (TBE). Minichromosomes were also digested with *Sfi* I (20 U/μg DNA) or *Hind* III (20 U/μg) in 60 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0, for 60 min; conditions that produced completed digestion on non-assembled DNA. The micrococcal nuclease assay produced monomer length DNA of 150 bp. Complete resistance of the minichromosome to topoisomerase was also observed. Approximately 50% of the minichromosomes were resistant to digestion with either *Sfi* I or *Hind* III.

RESULTS

A readily sedimentable complex of proteins for DNA synthesis was further purified from a combined low-salt nuclear extract-postmicrosomal supernatant fraction (NE/S-3) of HeLa cell homogenates [Malkas et al., 1990b] (Fig. 1). This was accomplished by poly(ethylene glycol) precipitation of the NE/S-3 fraction, followed by discontinuous gradient centrifugation, and Q-Sepharose chromatography. Essentially all of the large T-antigen dependent SV40 in vitro replication activity in the combined NE/S-3 fraction partitions with the readily sedimentable multiprotein form of DNA polymerase in the P-4 fraction [Malkas et al., 1990b] (Fig. 1). The readily sedimentable form of DNA polymerase in the P-4 fraction was further purified by chromatography on Q-Sepharose (Fig. 1). The Q-Sepharose column was eluted with a linear gradient of KCl (50–500 mM) and the peak of SV40-origin/T-antigen-dependent replication activity eluted from the column at a KCl gradient concentration of 0.25 M. Replication activity eluting in this peak fraction coincided with a peak of DNA polymerase activity eluting from the column [Malkas et al., 1990b].

Velocity gradient sedimentation analysis of the Q-Sepharose fraction that contained the peak of in vitro DNA replication was performed as described in Wu et al. [1994]. The sucrose gradient fractions were assayed for the activities of DNA polymerase α, DNA primase, and in vitro DNA synthesis (Fig. 2A–C). The DNA polymerase, DNA primase, and in vitro DNA synthesis activities present in the Q-Sepharose fraction were observed to co-sediment in the sucrose gradient analysis. We then initiated studies to further characterize the human cell replication-competent MRC.

Human Cell DNA Replication Proteins That Co-Purify With the MRC

It has been demonstrated that DNA polymerase δ [Byrnes et al., 1976; Lee et al., 1984] is required for the synthesis of SV40 origin-containing DNA in vitro [Prelich et al., 1987]. We report here that the DNA polymerase δ polypeptide co-purifies with the human MRC. Immunoblot analyses of the P-4, Q-Sepharose peak, S-4, Q-Sepharose flow-through, and Sucrose gradient peak fractions (Fig. 3) were performed using

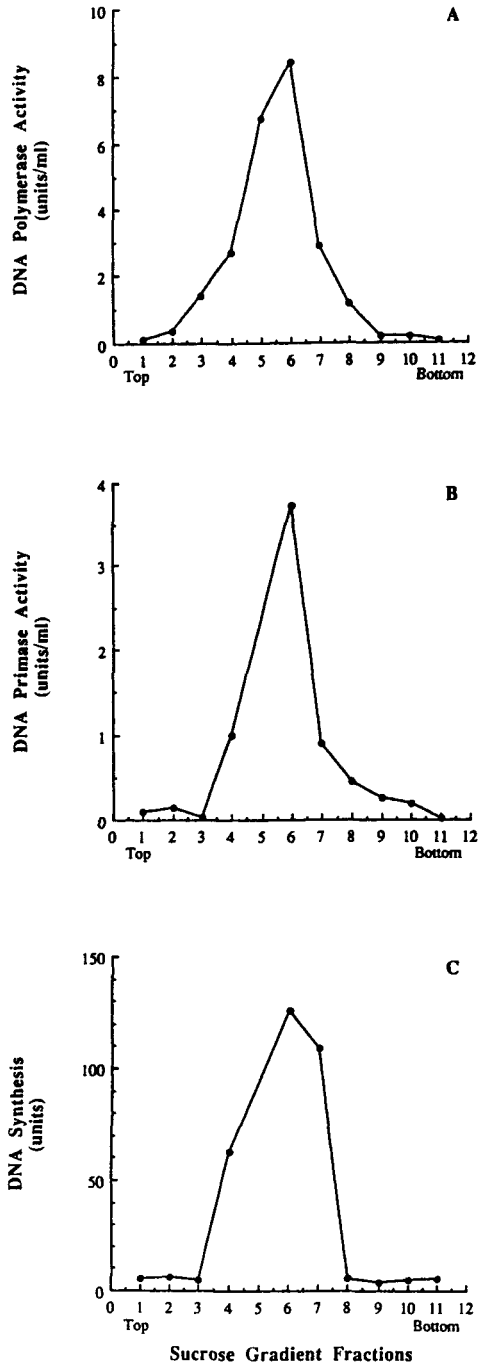

Fig. 2. Velocity gradient sedimentation analysis of the (A) DNA polymerase, (B) DNA primase, and (C) in vitro SV40 DNA replication activities eluted from Q-Sepharose. A Q-Sepharose column fraction (0.5 ml) combining the peak of in vitro SV40 DNA replication activity was layered onto a 10 ml 10–30% sucrose gradient containing 0.5 M KCl. The conditions for centrifugation and gradient fractionation were performed as described in Wu et al. [1994]. DNA polymerase, DNA primase, and DNA synthesis assays were performed as described in Experimental Procedures. The units of activity are as defined in Experimental Procedures.

an antibody prepared against the C-terminal peptide fragment of DNA polymerase δ (Experimental Procedures). DNA polymerase δ was found to exclusively co-purify with the replication competent form of DNA polymerase-primase in the P-4, Q-Sepharose peak, and Sucrose gradient peak fractions (Fig. 3a). The DNA polymerase δ polypeptide was not detectable in the replication poor S-4 and Q-Sepharose flow-through fractions.

Immunoblot analysis also demonstrates that like DNA polymerase δ, the DNA ligase I polypeptide co-purifies with the MRC-enriched P-4, Q-Sepharose peak, and Sucrose gradient peak fractions and not in the S-4 and Q-Sepharose flow through fractions (Fig. 3b). Similarly, topoisomerase II polypeptide was observed to co-purify with DNA polymerase δ and DNA ligase I in the P-4, Q-Sepharose peak, and Sucrose gradient peak protein fractions (Fig. 3c).

The DNA replication protein RF-C [Tsurimoto and Stillman, 1989; Lee et al., 1991] was also found to co-purify with MRC-enriched protein fractions (Fig. 3d). This was determined by immunoblot analyses using a monoclonal antibody that recognizes the 140 kDa subunit of the RF-C protein complex (Experimental Procedures). RF-C polypeptide was observed to partition exclusively with the P-4, Q-Sepharose peak, and Sucrose gradient peak fractions but was not observed in the S-4 or Q-Sepharose flow-through fractions.

Immunoblot analyses were also performed to determine whether RP-A [Wobbe et al., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988] associates with MRC-enriched protein fractions (Fig. 3e). Monoclonal antibody to the 34 kDa subunit of the RP-A protein was used in these studies (Experimental Procedures). These experiments show that RP-A fractionates with both the P-4 and Q-Sepharose peak protein fractions. RP-A was also found in the S-4, Q-Sepharose flow-through, and Sucrose gradient peak fractions. These results suggest that only a fraction of the total cellular RP-A co-purifies with the replication-competent MRC.

Topoisomerase I activity had been previously shown to fractionate with both the S-4 and P-4 fractions [Hickey et al., 1988]. We now show that topoisomerase I activity (Experimental Procedures) co-elutes with the replication-competent MRC following Q-Sepharose chromatography of the P-4 fraction (Fig. 4). Furthermore, topoisomerase I activity is seen to co-sediment

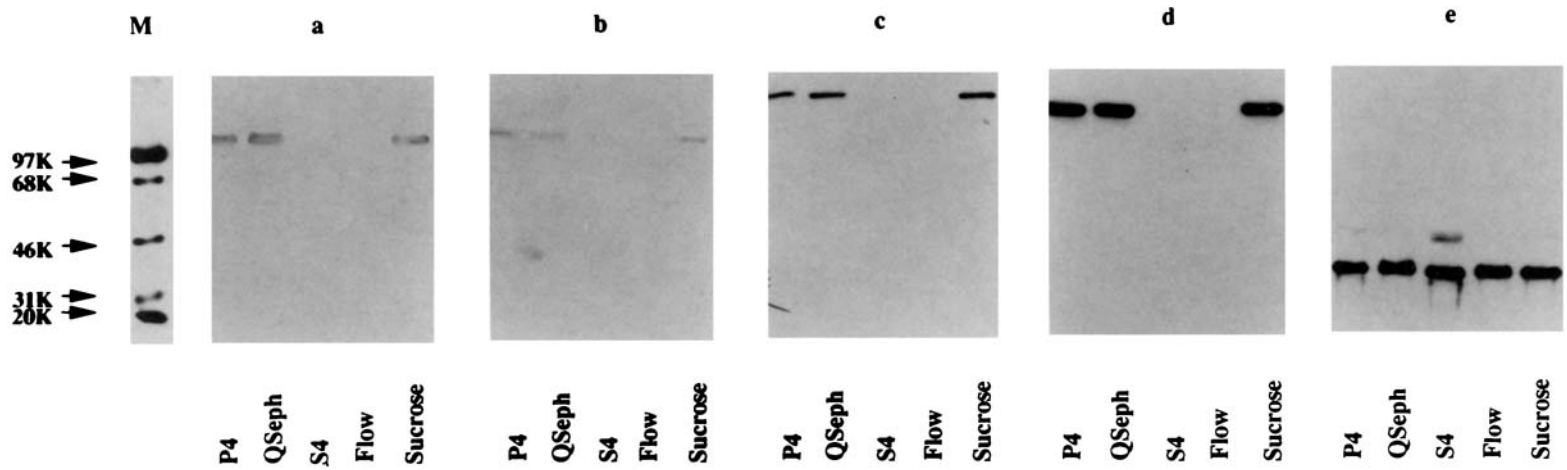

Fig. 3. Immunoblot analyses for the presence of various DNA replication proteins in S-4, P-4, Q-Sepharose flow-through, Q-Sepharose peak, and Sucrose gradient peak fractions. Thirty micrograms of each protein fraction were size fractionated on 8% polyacrylamide gels. The proteins were transferred to nitrocellulose filter membranes. The polypeptides DNA polymerase δ (**a**), DNA ligase I (**b**), topoisomerase II (**c**), RF-C (**d**), and RP-A (**e**) were visualized by sequentially incubating the membranes with the appropriate primary antibodies (see Experimental Procedures), followed by species-specific secondary antibodies conjugated to horseradish peroxidase (HRP). Light-enhanced chemiluminescence detection of the immobilized protein was accomplished using the ECL Western blotting detection system (Amersham). The resolution profile of the ECL protein molecular weight markers is shown in the panel designated M.

with the human cell MRC in the Sucrose gradient peak fraction (Fig. 4). Supercoiled plasmid DNA incubated in the absence of protein is shown in lane 1. A conversion of supercoiled plasmid DNA into relaxed form II DNA was observed following its incubation with Q-Sepharose peak (lane 2), Q-Sepharose flow-through (lane 3), and Sucrose gradient peak (lane 4). Furthermore, the topoisomerase I activity in Sucrose gradient peak was inhibited by camptothecin, a specific inhibitor of topoisomerase I (lane 5) [Hsiang et al., 1985]. Like RP-A, it appears that only a fraction of the total cellular topoisomerase I co-purifies with the MRC-enriched protein fraction.

A DNA helicase activity had been found to co-purify with the mouse cell MRC [Wu et al., 1994]. In order to establish whether a helicase activity was also associated with the human cell MRC-enriched protein fractions, DNA helicase assays were performed (Experimental Procedures) on the P-4, Q-Sepharose peak, Sucrose gradient peak, Q-Sepharose flow-through, and S-4 fractions (Fig. 5). DNA helicase activity was observed in both the P-4 and S-4 fractions. Further purification of the MRC enriched P-4 fraction through a Q-Sepharose column followed by sucrose gradient sedimentation revealed a significant enrichment of helicase activity. These data indicate that a DNA helicase activity readily co-purifies with the human cell MRC.

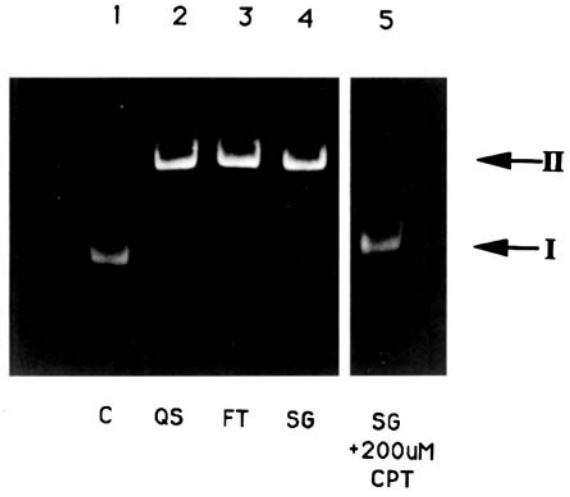

Fig. 4. Assay for the presence of topoisomerase I activity. This figure shows the degree of conversion of a supercoiled Form I plasmid DNA to a relaxed open circle Form II DNA following the incubation of 2.4 μg of supercoiled plasmid DNA (pSVO+) with 4 μg of Q-Sepharose peak, Q-Sepharose flow-through, and Sucrose gradient peak fractions. 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 the fraction. The reaction was incubated at 37°C for 20 min, and then stopped by the addition of 1% SDS. The DNA topoisomers were resolved by electrophoresis in a 1.2% agarose gel containing TAE buffer (40 mM Tris acetate, 2 mM EDTA). The resolved DNA topoisomers were visualized by staining the gel with a solution of 1 μg/ml ethidium bromide. Lane 1 shows the position of the supercoiled (Form I) pSVO+ DNA in the absence of the protein fraction. Lane 2 shows the conversion of the Form I DNA to the relaxed Form II by the topoisomerase I activity present in the Q-Sepharose peak fraction. Lane 3 represents conversion to relaxed Form II DNA found in the Q-Sepharose flow-through fraction. The degree of relaxation to Form II DNA by the topoisomerase I activity found to cosediment in the Sucrose gradient peak fraction is shown in Lane 4. Lane 5 demonstrates the inhibition of the Sucrose gradient peak topoisomerase I activity by 200 μM camptothecin.

Requirements for Human Cell MRC-Driven DNA Replication

Several requirements for human cell MRC-mediated DNA replication in vitro have been determined (Table I). Individual assay components were omitted from the DNA replication reactions in order to assess whether they were required for MRC-driven in vitro SV40-origin/T-antigen-dependent DNA synthesis. It was observed that a renewable source of ATP, supplied by phosphocreatine kinase and phosphocreatine, is essential for MRC DNA synthetic activity. In addition, the MRC replication activity is dependent on the presence of Mg^{++}, ribonucleotide and deoxyribonucleotide triphosphates, and DNA. We have also observed that the human cell MRC-mediated DNA replication reaction is much more dependent on the presence of ribonucleotide- and deoxynucleotide-triphosphates than that reported for synthesis by crude cell extracts [Li and Kelly, 1984; Stillman and Gluzman, 1985]. The reduced dependence of the in vitro SV40 DNA replication activity of the cell extracts on exogenously added ribonucleotide- and deoxynucleotide-triphosphates may possibly be due to significantly higher concentrations of these DNA precursors in the extracts. The almost absolute dependence of the MRC on the addition of the precursors to the synthesis assay mixtures suggests that these nucleotide triphosphates originally present in the cell extracts are lost as the MRC is purified from the crude cell extracts.

The addition of PCNA antibody to the replication reactions was observed to inhibit the DNA synthesis activity of the MRC. These results indicate that PCNA is required for SV40 replication activity mediated by the MRC. In addition, the human cell MRC-driven, T-antigen dependent, in vitro DNA replication reaction is inhib-

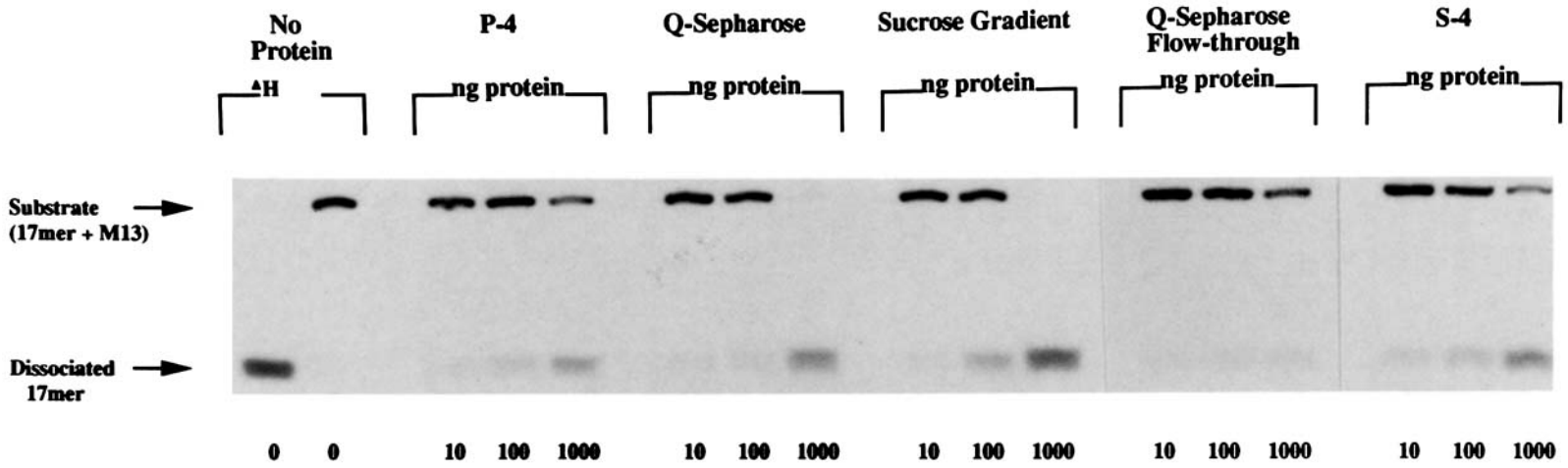

Fig. 5. DNA helicase activity. The DNA bands observed in the gel lanes migrate through a 12% polyacrylamide gel at the position expected for a single-stranded M13 DNA to which a ^{32}P-radiolabeled 17-mer oligonucleotide has been hybridized or to the position expected for an unwound ^{32}P-radiolabeled 17-mer. The fraction concentrations used in the helicase assay are indicated as nanograms of protein. The conditions for the DNA helicase assays are described in Experimental Procedures.

TABLE I. Requirements for Human Cell MRC Driven DNA Replication*

Assay component omitted or added	Relative DNA synthesis
(+) T-Antigen	100
(−) T-Antigen	0
(−) CPK/PC	4
(−) Mg++	0
(−) DNA	0
(−) dATP, dGTP, dTTP	0
(−) ATP, CTP, GTP, UTP	7
(+) ddTTP/dTTP	
100 μM/20 μM	118
500 μM/20 μM	110
(+) Aphidicolin	
1.2 μM	21
10.8 μM	0
(+) PCNA antibody, 1.5 μg[a]	23

*Complete reaction mixtures (25 μl) contained final concentrations of 80 mM Tris-HCl, pH 7.5; 7 mM $MgCl_2$; 1 mM DTT; 50 μg of protein fraction; 0.5 μg of purified SV40 large T-antigen; 30 ng of plasmid pSVO+ containing an insert of SV40 replication-origin DNA sequences; 100 μM each dTTP, dATP, dGTP; 200 μM each rCTP, rGTP, UTP; 4 mM ATP; 25 μM [^{32}P]dCTP (approximately 1,000 cpm/pmol); 40 mM creatine phosphate; 1 μg of creatine kinase. The standard reaction was incubated for 2 h at 35°C and the replication assay reaction products were processed using DE81 filter binding to quantify the amount of radiolabel incorporated into the replication products (see Experimental Procedures).

[a]The MRC was preincubated in the presence or absence of the PCNA antibody (Oncogene Science, Manhasset, NY; clone PC-10) for 1 h at 4°C prior to addition of the other assay components. Reactions were then incubated for 2 h at 35°C and processed as above.

ited by aphidicolin at 1.2 μM, but is resistant to inhibition by dideoxythymidine triphosphate (ddTTP). Both DNA polymerases α and δ have been shown to be specifically inhibited by aphidicolin [Edenberg et al., 1978; Ohashi et al., 1978; Goscin and Byrnes, 1982] and to be resistant to ddTTP [Kornberg and Baker, 1992]. The response of the human cell MRC to these polymerase inhibitors and the requirement for PCNA suggests that the observed in vitro replication activity of the MRC is mediated by both DNA polymerase α and δ.

Reduction of DNA Replication Initiation Lag Time Upon Purification of the MRC

Studies were conducted to investigate the kinetics of DNA replication initiation by the MRC as a function of its purity. In vitro SV40 DNA replication assays (Experimental Procedures) using protein fractions from different steps in the MRC purification scheme were performed. The protein fractions used in these studies were the crude S-1, the MRC-enriched P-4, and the Q-Sepharose peak fractions (Fig. 1). Aliquots of the reactions were removed at various time points during the in vitro DNA replication assays, and the level of T-antigen dependent radionucleotide incorporation into DNA was determined (Experimental Procedures). It was observed that the crude S-1 fraction initiated DNA replication after approximately a 10-min lag time (Fig. 6A). This result is comparable to those reported by others using crude human cell extracts [Stillman and Gluzman, 1985]. Further purification of the MRC from the crude S-1 fraction considerably altered the kinetics of replication initiation. The P-4 fraction readily initiated replication within 5 min after the start of the assay (Fig. 6B). As the replication-competent MRC in the P-4 fraction was purified further, following Q-Sepharose chromatography, an even greater reduction in the lag time for replication initiation was observed (Fig. 6B). The Q-Sepharose peak fraction required only a 1-min incubation prior to the rapid onset of DNA replication. These results indicate that a significant reduction in the kinetics of DNA replication initiation occurs as the MRC is purified from a crude extract. Incubation of assay mixtures lacking T-antigen resulted in virtually no detectable incorporation of radiolabeled nucleotide into DNA over the course of the entire assay.

Replication of pSVO$^+$ Minichromosomes by the MRC

The human cell MRC was assayed for its ability to utilize nucleosome-assembled pSVO$^+$ DNA as a DNA replication template. The MRC-enriched P-4 fraction (Fig. 1) was used in these studies. In vitro SV40 DNA replication assays were performed using either nucleosome-assembled or naked pSVO$^+$ DNA (Experimental Procedures). Replication was assayed over a 2 h time period, and little incorporation of radiolabeled nucleotide was observed with either type of DNA template in the absence of the large T-antigen (Fig. 7). At 30 min the level of replication of the assembled with respect to the non-assembled DNA was 90.0%, at 60 min the level was 83.2%, and at 120 min the level was 77.8%. Analysis of replication of similarly assembled minichromosomes produced a relative level of 87.3 ± 4.1% at 60 min, and 75.6 ± 9.8% at 120 min. This difference in the efficiency of replication was most likely due to the presence of nucleosomes on the pSVO$^+$ DNA, which decreased the rate at which the MRC could replicate this DNA. The results of these experiments

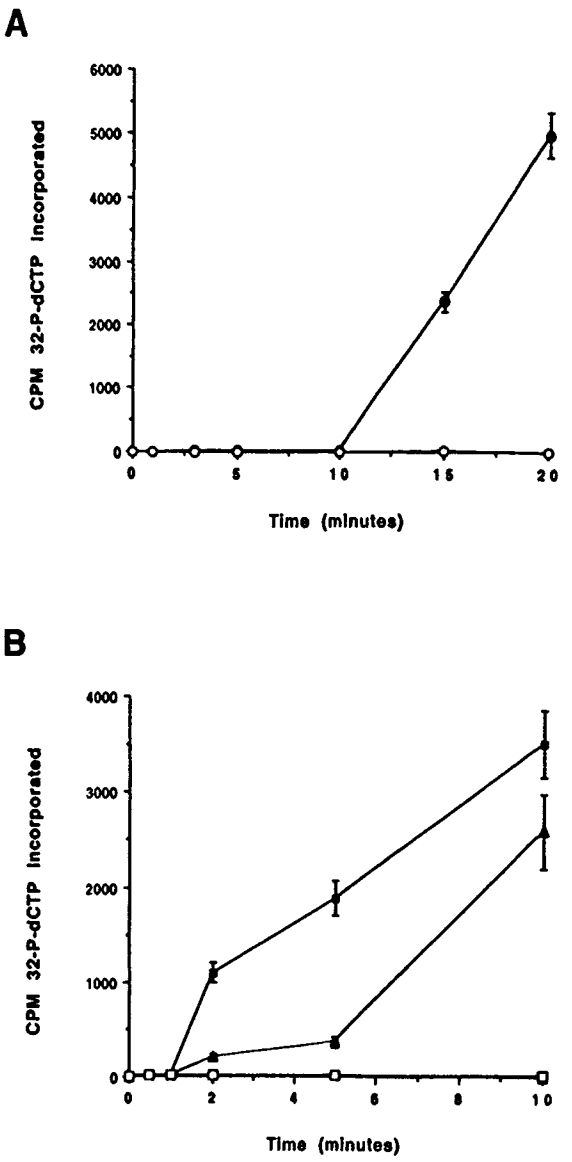

Fig. 6. Reduction of DNA replication initiation lag time upon purification of the MRC. This figure shows the time-dependent initiation of DNA synthesis at 35°C in 50 μl reaction mixtures containing 60 ng pSVO+ DNA and 50 μg of the S-1, P-4, or the Q-Sepharose peak fraction. These reactions were performed in the presence or absence of 1 μg SV40 T-antigen. The level of radiolabeled nucleotide incorporated into DNA was determined at various times during the assay incubation (see Experimental Procedures). **A:** An in vitro DNA replication time course using the crude S-1 fraction in the presence of T-antigen (●) or its absence (○). The time course experiments performed using Q-Sepharose peak in the presence of T-antigen (■) or its absence (□) and P-4 in the presence of T-antigen (▲) or its absence (△) protein fractions are represented in **B.** The range of values depicted were derived from three separate experiments.

suggest that the presence of nucleosomes on the parental DNA does not abolish the MRC's DNA synthetic activity, and thus the MRC is capable of replicating DNA that is assembled into a chromatin form.

TK and DHFR Do Not Co-Chromagraph With the Human Cell MRC

Evidence has accumulated to suggest a physical and/or functional interaction between deoxynucleotide metabolism enzymes and DNA replication proteins. In mammalian cells there have been reports that several deoxynucleotide metabolism proteins, among them thymidine kinase (TK) and dihydrofolate reductase (DHFR), interact with DNA replication proteins [reviewed in Mathews and Slabaugh, 1986; Malkas et al., 1990a; Reddy and Fager, 1993].

We wanted to determine whether TK and DHFR, which are key nucleotide metabolism enzymes, co-purified with the human cell MRC. We assayed the S-4, P-4, Q-Sepharose flow-through, and Q-Sepharose peak fractions (Fig. 1) for TK enzymatic activity and DHFR-^{3}H-MTX binding activity (Experimental Procedures) (Table II). It was observed that 70% of the TK activity partitioned with the MRC-enriched P-4 fraction. It was also observed that approximately equal amounts of DHFR-^{3}H-MTX binding activity fractionate with both the S-4 and P-4 fractions. However, the TK and DHFR-^{3}H-MTX binding activities that partition with the P-4 fraction were not observed to co-purify with the MRC following chromatography on Q-Sepharose. Essentially all of the TK and DHFR-^{3}H-MTX binding activities were observed to elute in the Q-Sepharose flow-through fraction. These results indicate that the TK and DHFR enzymes partition with the human cell MRC in the P-4 fraction but are separated from the MRC following Q-Sepharose column chromatography.

DISCUSSION

We have previously described the isolation of a DNA replication-competent multiprotein form of DNA polymerase from human cells [Baril et al., 1988; Hickey et al., 1988; Malkas et al., 1990b] and more recently from murine cells [Wu et al., 1994]. In this report we have shown that measurable replication activity by the human cell MRC depends on the in vitro assay requirements described in Table I. These requirements for in vitro DNA replication by the purified MRC are comparable to those observed in vivo [DePamphilis and Bradley, 1986] and with crude extracts prepared from cells that are permissive for SV40 DNA synthesis [Ariga and Sugano, 1983; Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe et al., 1985; Decker et al., 1987].

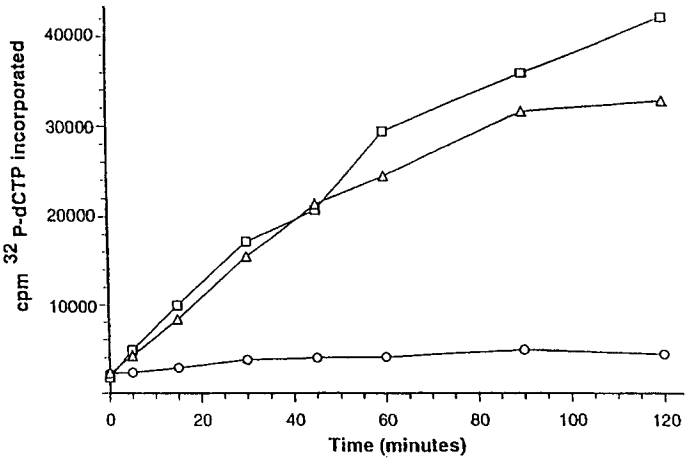

Fig. 7. Replication of pSVO+ minichromosomes. In vitro SV40 DNA replication assays (see Experimental Procedures) were performed with the HeLa P-4 fraction in reaction mixtures containing large T-antigen and 50 ng nucleosome-assembled pSVO+ DNA (△), or large T-antigen and 50 ng naked pSVO+ DNA (□), or 50 ng naked pSVO+ DNA in the absence of large T-antigen (○). The assays were incubated at 35°C for a total of 120 min. At 0, 15, 30, 45, 60, 90, and 120 min, 5 μl aliquots were removed and analyzed for incorporation of radioactive deoxyribonucleotide into DNA (see Experimental Procedures).

In the Western blot experiments we report here, DNA polymerase δ co-purifies with the human cell MRC. We have also previously reported that DNA polymerase δ co-purifies with the mouse MRC [Wu et al., 1994]. In addition we have shown that antibody against PCNA inhibits its MRC-driven replication activity (Table I). PCNA has been identified as an auxiliary protein for polymerase δ that switches it from a nonprocessive to a processive polymerase [Prelich et al., 1987; Tan et al., 1986] and was also shown to be required for in vitro SV40 DNA synthesis in a reconstituted replication system [Prelich et al., 1987; Prelich and Stillman, 1988]. All of these results together suggest that there are at least two DNA polymerases associated with the replication-competent mammalian MRC. It is conceivable that these polymerases function synchronously within the MRC, where it has been proposed that the DNA polymerase-α-primase complex is necessary for conducting both replication initiation events and lagging strand synthesis, and the DNA polymerase δ executing leading strand synthesis [Tsurimoto et al., 1990; Hurwitz et al., 1990; Tsurimoto and Stillman, 1991].

TABLE II. Fractionation and Chromatographic Profiles of TK and DHFR-^{3}H-MTX Binding Activities

Fraction	Activity[a] TK	Activity[a] DHFR-^{3}H-MTX binding
S-4	62.5	143.8
P-4	143.5	132.1
Q-Sepharose flow through	139.2	125.8
Q-Sepharose peak	0.96	0

[a]One unit of TK activity equals 1 nmol of ^{3}H-TdR converted to ^{3}H-thymidine monophosphate by enzyme incubated at 37°C for 20 min. One unit of DHFR-^{3}H-MTX binding activity equals 1 nmol of ^{3}H-MTX bound by enzyme incubated at room temperature for 10 min (see Experimental Procedures).

Further support that the mammalian cell MRC may function as a dipolymerase system is suggested by our observation that RF-C also co-purifies with the human cell MRC. Results reported by Tsurimoto and Stillman [1989, 1991] suggest that RF-C facilitates the coordinated synthesis of both the leading and lagging strands during DNA replication. These authors suggested that RF-C may act as a connector or hinge between DNA polymerases α and δ. The presence of RF-C, PCNA, DNA polymerases α and δ, and DNA primase suggests that the isolated MRC may act as a coordinated dipolymerase replication complex.

As determined by immunoblot assays, the other proteins observed to co-purify with the human cell MRC include: RP-A, a single-strand DNA-binding protein complex [Wobbe et al., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988]; topoisomerase II, which assists in the segregation of daughter DNA molecules [Yang et al., 1987]; and DNA ligase I, which covalently links matured Okazaki fragments

[Barker et al., 1987; Smith et al., 1989]. Our results regarding the co-purification of DNA ligase I with the human cell multiprotein form of DNA polymerase are further supported by the recent reports of Li et al. [1994] and their demonstration of DNA ligase I activity in the Q-Sepharose peak fraction containing the human cell MRC.

A DNA helicase activity also co-purifies with the human cell replication-competent MRC. The DNA helicase activity associated with the human MRC may promote the melting of parental DNA strands to permit access of the elongation components of the MRC to the DNA. However, work is underway to establish whether the MRC-associated DNA helicase functions in the in vitro replication of SV40 origin-containing DNA in the presence of the SV40 large T-antigen.

In intact cells SV40 uses host cell DNA topoisomerases for viral DNA replication [Snapka, 1986; Snapka et al., 1988; Avemann et al., 1988; Hsiang et al., 1985; Porter and Champoux, 1989; Shin and Snapka, 1990; Parker and Champoux, 1993]. DNA topoisomerases I and II have also been suggested to play a role in in vitro SV40 DNA replication by Yang et al. [1987]. In a previous report [Hickey et al., 1988] as well as in this report we have presented evidence indicating that topoisomerase I co-purifies with the human MRC. We also observed that the readily extractable topoisomerase II isolated from HeLa cells exclusively co-sediments with other essential replication proteins in the P-4 fraction. The topoisomerase II enzyme associated with the P-4 fraction also exclusively co-purified with the replication proteins in the "peak in vitro replication activity" fractions eluting from the Q-sepharose column as well as the sucrose gradient peak. These results are strongly suggestive of a tight association of topoisomerase II with the MRC containing the DNA polymerases α and δ. Because topoisomerase II has been recognized to be an essential nuclear matrix associated protein [Cockerill and Garrard, 1986], it is possible that one of the roles of topoisomerase II in the MRC may be to promote the association of the MRC with the nuclear matrix.

The number of reports regarding the isolation of large complexes of proteins for DNA synthesis continues to grow [reviewed in Mathews and Slabaugh, 1986; Malkas et al., 1990a; Reddy and Fager, 1993]. Many of these reports indicated that nucleotide metabolism enzymes co-purified with the complexes. Some of the nucleotide metabolism enzymes that have been identified with the complexes are thymidine kinase, thymidylate synthetase, dihydrofolate reductase, nucleoside-diphosphokinase, and DNA methylase. We have observed that a fraction of two of these nucleotide metabolism enzymes, thymidine kinase and dihydrofolate reductase, co-fractionate with the replication-competent MRC in the readily sedimentable P-4 fraction. However, these two enzymes do not co-purify with the MRC following chromatography of the P-4 fraction on Q-Sepharose. In addition, the absence of these enzymes does not seem to affect the function of the MRC in the Q-Sepharose peak fraction for SV40 in vitro DNA replication. Although these nucleotide metabolism activities do not co-purify with the MRC following column chromatography, it does not rule out the possibility that the MRC may in fact be linked in some manner with nucleotide biosynthetic enzymes in intact cells.

The actual DNA template replicated within cells is chromatin, and not a purified (naked) DNA. Chromatin contains DNA associated with histone proteins, around which the DNA is wrapped to form nucleosomes. Therefore, we have also evaluated the ability of the MRC to utilize as a substrate for replication an SV40 origin containing DNA template assembled into chromatin. For assembly of the SV40 DNA into chromatin we utilized a procedure that required only the addition of purified histone octamers to the DNA template [Stein, 1989; O'Neill et al., 1992]. In this way, we could directly determine whether the purified MRC could replicate through a chromatin template without concern that proteins (e.g., transcription factors) which are present in crude assembly extracts may be facilitating the process of replication. Our analysis of minichromosome replication by the MRC varied in two ways from the experiments reported by Cheng and Kelly [1989], Ishimi et al. [1991], and Ishimi [1992]. In our experiments an organized complex of proteins was used for the replication assay instead of either crude extracts or a reconstituted DNA replication system; and second, minichromosomes were assembled with histone octamers, circumventing the need for nucleosome assembly factors. With this purified system, we found that the MRC could replicate the chromatin template. The amount of replication of the minichromosome by the MRC was 77.8% that of a protein-free template at 120 min after the initiation of replication. This extent of replication is consistent with what was observed previously for replica-

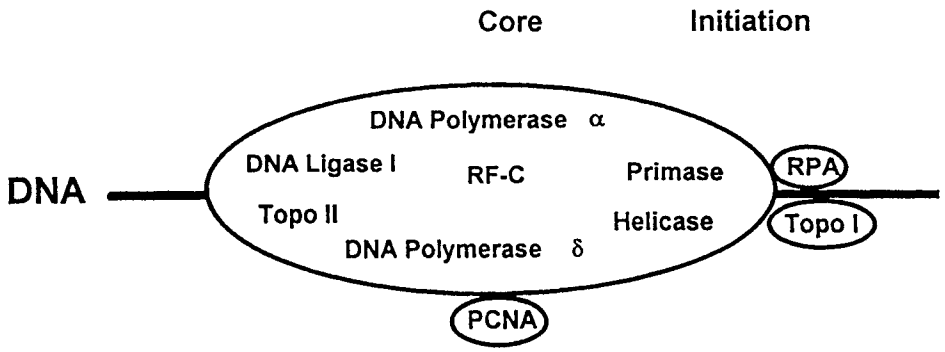

Fig. 8. A proposed model for the organization of the identified protein components of the mammalian cell MRC. The full description of the model is discussed in the text.

tion of SV40 minichromosomes using HeLa cell extracts [Cheng and Kelly, 1989; Ishimi, 1992] or purified replication factors [Ishimi et al., 1991] when the level of nucleosome assembly of the minichromosomes was equivalent to that of our study. In both the Cheng and Kelly [1989] and Ishimi [1992] studies, it was shown that at high histone to DNA ratios the replication efficiency of minichromosomes was greatly reduced. In our study, the histone to DNA ratio was about half of that shown to dramatically inhibit replication in the Cheng and Kelly [1989] and Ishimi [1992] studies. Our estimate of nucleosome assembly is based on micrococcal nuclease and topoisomerase studies as well as the observation that the restriction enzymes Sfi I and Hind III digest only 50% of the minichromosome template under conditions where protein free DNA is completely digested (data not shown). In our study the observed decrease in the efficiency of replication of the minichormosome as compared to the protein-free template is most likely a reflection of the MRC encountering nucleosomes as it replicates the chromatin template. The observation that there is only a 25% decrease in the efficiency of replication of the minichromosomes over the entire course of the assay suggests that the MRC is able to efficiently replicate this template despite the presence of the nucleosome structure.

We have previously reported for the mouse cell MRC [Wu et al., 1994] that DNA polymerases α and δ, DNA primase, DNA ligase I, and DNA topoisomerase II were observed to readily co-purify. We proposed that these proteins formed the "core" component of the MRC. The model proposed for the mouse cell MRC can now be extended to include the human cell MRC (Fig. 8). In this report we have demonstrated that the majority of human cell DNA polymerases α and δ, primase, DNA helicase, DNA ligase I, and topoisomerase II co-purify together following Q-Sepharose chromatography and sucrose gradient centrifugation. Our results suggest that these proteins may be "tightly" associated within the human cell MRC. From our results presented in this report on the fractionation, chromatographic and sedimentation behavior of RF-C, we suggest that RF-C is also a member of the MRC core component. RF-C is clearly present in only the replication-competent P-4, Q-Sepharose peak, and Sucrose gradient peak fractions. It is undetectable in the replication incompetent S-4 and Q-Sepharose column flow-through fractions. It is interesting to note that those replication proteins we have identified as being in the core component of the MRC 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. This is based on our observations that, although PCNA co-fractionates and co-elutes following column chromatography with the MRC core component proteins, it can also be found in protein fractions that do not support in vitro DNA synthesis [Malkas et al., 1990b]. 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 with the MRC, but not as a member of the MRC core component. Co-immunoprecipitation and chemical cross-linking studies are underway to provide more direct evidence for the physical association of the replication proteins that compose the MRC.

The role played by large T-antigen in the initiation of in vitro SV40 DNA replication has

been reviewed [Borowiec et al., 1990; Fanning and Knippers, 1992]. Briefly, the large T-antigen binds to sites within the SV40 origin and melts the early palindrome region within the origin region. The addition of topoisomerase I and RP-A facilitates the further melting of the SV40 DNA. We have observed that topoisomerase I and RP-A, like PCNA, do not "tightly" associate with the MRC proteins that compose the MRC core component. We, therefore, propose that topoisomerase I and RP-A constitute the MRC's "initiation" component. Together, the core and initiation components would constitute the mammalian cell MRC.

This report represents the next step in the process required to accurately describe the cellular DNA synthetic apparatus. It is a demonstration that the proteins recognized to be essential for in vitro SV40 origin dependent DNA synthesis can be isolated from the cell as an intact complex. Furthermore, we have shown that the human cell MRC closely resembles the murine cell MRC [Wu et al., 1994] in both its protein composition and its fractionation and chromatographic profile. In addition, our data regarding both the human and murine MRC supports the dipolymerase model proposed from replication reconstitution studies [Tsurimoto et al., 1990; Hurwitz et al., 1990; Tsurimoto and Stillman, 1991].

Because the process of DNA replication is an important regulatory point for modulating cell proliferation, the elucidation of the role played by the mammalian cell multiprotein replication complex, and its components, in 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 multiprotein replication complex with respect to its structural components and activity, and relate this information to the higher order of structure present within the cell nucleus. A variety of studies are currently underway in our laboratories to accomplish these goals.

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