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ARTICLES

Identification of Multiprotein Complexes Containing DNA Replication Factors by Native Immunoblotting of HeLa Cell Protein Preparations With T-Antigen-Dependent SV40 DNA Replication Activity

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Abstract Increasing evidence has supported the concept that many of the enzymes and factors involved in the replication of mammalian DNA function together as a multiprotein complex. We have previously reported on the partial purification of a multiprotein form of DNA polymerase from human HeLa cells shown to be fully competent to support origin-specific large T-antigen-dependent simian virus 40 (SV40) DNA replication in vitro. In an attempt to more definitively identify the complex or complexes responsible for DNA replication in vitro, partially purified human HeLa cell protein preparations competent to replicate DNA in vitro were subjected to native polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. The Native Western blots were probed with a panel of antibodies directed against proteins believed to be required for DNA replication in vitro. Apparent complexes of 620 kDa and 500 kDa were identified by monoclonal antibodies directed against DNA polymerase α and DNA polymerase δ, respectively.

To detect epitopes possibly unexposed within the native multiprotein complexes, blots were also analyzed following denaturation in situ following treatment with detergent and reducing agent. The epitope or access to the epitope recognized by the monoclonal antibody against DNA polymerase α was destroyed by exposure of the blots to denaturing conditions. In contrast, an epitope present on a very large complex of approximately 1000 kDa was recognized by a monoclonal antibody against proliferating cell nuclear antigen only following treatment of the native immunoblots with denaturing agents. Identification of these complexes will allow their further purification, characterization, and elucidation of their role in the replication of DNA.

Key words: native immunoblotting, SV40, DNA replication, in vitro, multiprotein complexes, in situ denaturation

The evidence supporting the role of multiprotein complexes in the replication of mammalian DNA has grown over the years [Malkas et al., 1990b; Wu et al., 1994; Applegren et al., 1995; reviewed in Mathews and Slabaugh, 1986; Malkas et al., 1990a; Reddy and Fager, 1993]. Previously we reported on a replication-competent multiprotein form of DNA polymerase isolated from human (HeLa) cells [Applegren et al., 1995; Malkas et al., 1990b] and murine (FM3A) cells [Wu et al., 1994]. The multiprotein form of DNA polymerase was isolated from the cells using a series of centrifugation, polyethylene glycol precipitation, ion-exchange chromatography, and density gradient sedimentation steps. The sedimentation coefficient of the HeLa cell multiprotein complex is 18-21S, as determined by sucrose and glycerol gradient analyses, while that of the FM3A cell complex is 17S. The integrity of the multiprotein complex is maintained after treatment with detergents, salt, RNase, DNase, and after chromatography on DE52-cellulose or Q-Sepharose, indicating 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 com-

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plex of proteins is fully competent to replicate DNA in vitro. The HeLa cell complex supports in vitro SV40 DNA synthesis, while the FM3A cell complex replicates polyomavirus origin containing DNA. This result implies that all of the cellular activities required for large-T-antigen-dependent in vitro papovavirus DNA synthesis are present within the isolated multiprotein form of the DNA replication apparatus. This is particularly important in view of the fact that papovavirus is completely dependent on the host cell's DNA synthetic machinery for its own DNA replication. Our results, therefore, suggest that the isolated multiprotein complex mediates not only papovavirus DNA synthesis, but it may also function in mammalian cell DNA replication as well. Using SDS-PAGE and immunoblot analysis, we have shown that DNA polymerase δ, DNA ligase I, topoisomerase II, RF-C, RP-A, and proliferating cell nuclear antigen cosediment in sucrose velocity analyses [Malkas et al., 1990b; Wu et al., 1994; Applegren et al., 1995]. Using functional assays, we have demonstrated that helicase, DNA polymerase α, primase and topoisomerase I also cosediment in sucrose velocity sedimentation analyses [Wu et al., 1994; Applegren et al., 1995]. Although these results are suggestive that these enzymes and factors form a functional multiprotein complex involved in DNA replication, they do not rule out the possibility of fortuitous copurification or the presence of multiple forms of multiprotein complexes.

Previous reports have utilized native polyacrylamide gel electrophoresis to identify large molecular weight forms associated with DNA polymerase activity [Vishwanatha et al., 1986; Syraoja and Lin, 1989; Holer et al., 1986]. However, no data have been published on the positive activity of these complexes or the protein preparations from which they were purified for SV40 origin-specific T-antigen-dependent DNA replication. In order to more definitively identify the presence of multiprotein complexes in replication-competent human (HeLa) cell preparations and characterize the components of these complexes, we analyzed human HeLa cell protein preparations by a simplified native gel electrophoresis protocol followed by immunoblotting. A panel of antibodies directed against components believed required for origin-specific T-antigen-dependent DNA replication in vitro were used to probe the immunoblots. In this report we show that several apparent large-molecular-weight complexes can be identified by this approach.

MATERIALS AND METHODS

Cell Culture

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 subcellular fractionation.

Fractionation and Chromatographic Procedures

HeLa cells were fractionated essentially as described by Malkas et al. [1990b] with some variation as outlined in Figure 1. In brief, frozen cell pellets (approximately 15 g wet weight) were quickly thawed and resuspended in 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; and 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 (S-1) fractions were each collected separately. Mitochondria were removed from the S-1 fraction by centrifugation at 27,000 g for 15 min, and the resultant supernatant was designated an S-2 fraction. The S-2 fraction was then subjected to centrifugation at 100,000*g* for 60 min to remove microsomes. The post-microsomal supernatant (S-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 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 1 hr at 4°C. The extracted nuclei were centrifuged for 60 min at 100,000*g* and the supernatant (NE) was collected. The NE and S-3 fractions were pooled and made 2 M

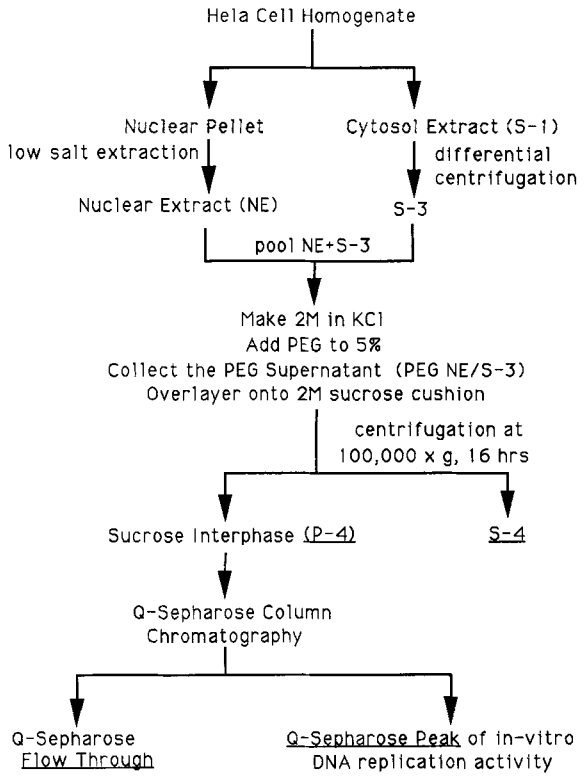

Fig. 1. The flow diagram of the isolation scheme used to purify the human cell multiprotein DNA replication complex (MRC). A detailed description of the isolation scheme is presented in Materials and Methods.

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 hr at 4°C. PEG precipitated material was pelleted by centrifugation for 30 min at 16,000*g*, and the supernatant (PEG NE/S-3) was collected. The PEG NE/S-3 was dialyzed (to remove the PEG) for 2 hrs against one change 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 dialysate was clarified by centrifugation for 10 min at 13,000*g*. The clarified PEG NE/S-3 fraction was layered over a 2 M sucrose cushion and subjected to centrifugation at 100,000 g for 16 hrs at 4°C. The material above the sucrose interphase was collected and designated the S-4 fraction. The sucrose interphase fraction was collected and designated the P-4 fraction. Q-Sepharose chromatography of the P-4 fraction was carried out as described by Malkas et al. [1990b].

The protein fractions designated Q-Sepharose peak were used in the experiments described in this report. The replication-competent multiprotein DNA replication complex was previously shown to reside in the P-4 and Q-Sepharose peak [Malkas et al., 1990b]. The S-4 and Q-Sepharose flow-through fractions do not support in vitro DNA replication [Malkas et al., 1990b].

Native Polyacrylamide Gel Electrophoresis, Western Blotting, and In Situ Denaturation of Western Blots

Forty micrograms of Q-Sepharose peak protein were resolved using a 4% native polyacrylamide gel and 3.5% stacking gel, essentially as described by Laemmli [1970] with the exclusion of sodium dodecyl sulfate (SDS) in the polyacrylamide gel, running, and sample buffers. 2-Mercaptoethanol was excluded from the sample buffers. All samples were maintained at 4°C prior to and electrophoresed at 4°C. Proteins were electrophoretically transferred to nitrocellulose at 12 V for 12–16 hrs at 4°C, essentially as described by Towbin et al. [1979]. Hybridoma supernatant of a monoclonal antibody prepared against the C-terminal peptide of DNA polymerase δ [Yang et al., 1992] was the generous gift of Dr. Marietta Lee, and it was used without dilution to probe the native immunoblots. A purified mouse monoclonal antibody IgG2a directed against human proliferating cell nuclear antigen (PCNA) was purchased from Oncogene Science, (Cambridge, MA) and used at a final dilution of 1 μg/ml. A mouse hybridoma that produced monoclonal antibody SJK 132-20 directed against human DNA polymerase α [Tanaka et al., 1982] was obtained from the American Type Culture Collection, Rockville, Maryland. IgG was purified by chromatography on a protein A-linked agarose column purchased from Repligen Inc. and used at a final concentration of 5 μg/ml. Immunodetection was carried out using a light-enhanced chemiluminescence (ECL) detection system according to manufacturer's instructions (Amersham, Arlington Heights, IL). Thyroglobulin (669 kDa) and bovine serum albumin (66 kDa) were obtained from Sigma (St. Louis, MO; Gel Filtration Molecular Weight Markers, MW-GF-1000) and used as molecular size markers. Bovine serum albumin dimer (132 kDa) and trimer (198 kDa) were reproducibly seen in native polyacrylamide gels and used as molecular size markers.

Native immunoblots previously probed with antibody were stored moist at 4°C prior to further treatment. In situ denaturation was car-

ried out by exposure of immunoblots to 100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris-HCl (pH 6.7), at 50°C for 30 min. Following two 10-min washes in phosphate-buffered saline (PBS), blots were blocked and treated as per ECL manufacturer's instructions (Amersham, Arlington Heights, IL).

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 (12.5 μl) contained 80 mM Tris-HCl (pH 7.5); 7 mM $MgCl_2$; 1 mM DTT; 0.5–7 μg of Q-Sepharose peak protein; 0.5–2 μg of purified SV40 large T-antigen; 25 ng of plasmid pSVO+ containing an insert of SV40 replication-origin DNA sequences [Stillman et al., 1985]; 100 μM each of dTTP, dATP, and dGTP; 200 μM each rCTP, rGTP, and UTP; 4 mM ATP; 25 μM [^{32}P]dCTP; 40 mM creatine phosphate; and 0.5 ng of creatine kinase. The standard reaction was incubated for 2 hrs at 37°C. 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].

RESULTS

Purification of Replication-Competent HeLa Cell Proteins

Homogenates of human HeLa cells were purified from a low-salt nuclear extract post-microsomal supernatant fraction (NE/S-3) by poly(ethylene glycol) precipitation of the combined NE/S-3 fraction, followed by discontinuous gradient centrifugation, and Q-Sepharose chromatography [Malkas et al., 1990b] (Fig. 1). Virtually all of the large T-antigen-dependent SV40 in vitro replication activity in the combined NE/S-3 fraction partitions with the readily sedimentable form of DNA polymerase and the P-4 fraction [Malkas et al., 1990b]. The readily sedimentable form of DNA polymerase in the P-4 fraction was further purified by chromatography on Q-Sepharose. The Q-Sepharose column was eluted with a linear gradient of KCl (50–500 mM), and the peak of activity for SV40-origin/T-antigen-dependent DNA replication was used for these studies.

HeLa Cell Proteins Purified by Q-Sepharose Chromatography Stimulate Large T-Antigen-Dependent DNA Replication

HeLa cell proteins purified by Q-Sepharose chromatography were tested for their ability to stimulate large T-antigen-dependent SV40 origin-specific DNA replication. Various amounts of the Q-Sepharose purified proteins were added to a standard reaction containing large T-antigen, pSVO+, a plasmid containing the SV40 origin of replication, phosphocreatine, phosphocreatine kinase, ribonucleotides, and dNTPs. The requirement for each of these components was established and reported earlier [Applegren et al., 1995]. Replication of DNA was assessed after a 2-hr incubation by determining ^{32}P incorporation in comparison to control reactions without added T-antigen (Materials and Methods). A protein-concentration-dependent level of ^{32}P incorporation (Fig. 2) was stimulated by amounts of Q-Sepharose purified protein ranging from approximately 0.5 μg to 7 μg. After a 2-hr incubation, the large majority of ^{32}P DNA incorporation is T-antigen dependent.

Demonstration of the origin specificity, semiconservative replication products produced in this assay with Q-Sepharose purified HeLa cell

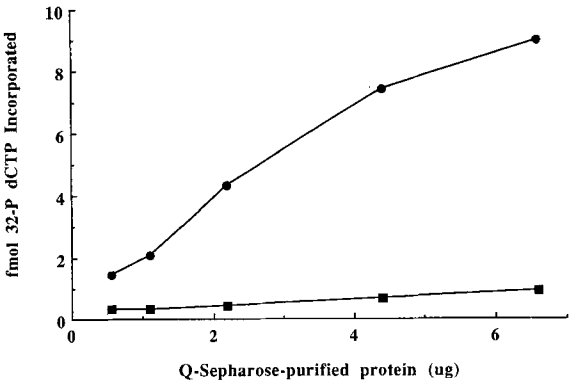

Fig. 2. T-antigen dependence and Q-Sepharose peak protein dependence of DNA synthesis in the SV40 replication assay. SV40 in vitro replication activity was assayed in the presence (filled circle) or absence (filled square) of T-antigen and varying amounts of the Q-Sepharose purified HeLa cell proteins as described in Materials and Methods.

proteins has been published previously [Malkas et al., 1990a,b].

Native Polyacrylamide Gel Electrophoresis of Q-Sepharose Purified HeLa Cell Proteins

In native polyacrylamide gel electrophoresis (native-PAGE), proteins are separated on the basis of charge, shape and size characteristics [Garfin, 1990]. Some proteins do not resolve well or migrate away from the resolving gel, depending on the pH of the gel and the running buffer [Andrews, 1986; Hames, 1981; Allen et al., 1984]. Thus, prior to conducting our native immunoblotting experiments, it was necessary to determine the profile and resolution of the Q-Sepharose purified proteins in our standard native-PAGE running conditions. We utilized a simple buffer system essentially identical to that used for SDS-PAGE with the exclusion of sodium dodecyl sulfate and reducing agent in all buffers. Forty migrograms of Q-Sepharose purified HeLa cell protein was electrophoresed through a 4% native polyacrylamide gel and stained with Coomassie brilliant blue. Under our standard conditions (Materials and Methods), a wide size range of proteins resolve in this system (Fig. 3). Based on the adequate resolution of proteins visible by Coomassie staining, native immunoblotting experiments were conducted.

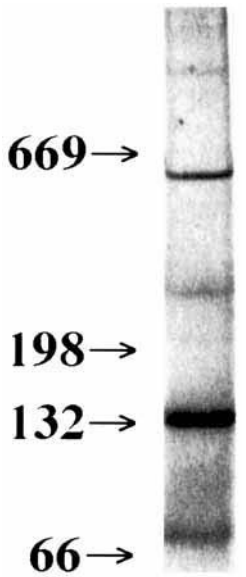

Fig. 3. Native polyacrylamide gel analysis of Q-Sepharose purified HeLa cell proteins. Q-Sepharose purified HeLa cell protein (40 μg) was electrophoresed through a 4% native polyacrylamide gel and stained with Coomassie brilliant blue as described in Materials and Methods. Molecular size standards in kilodaltons are indicated.

Immunoblotting of Q-Sepharose Purified HeLa Cell Proteins

Forty μg of Q-Sepharose purified HeLa cell protein was resolved through a 4% native polyacrylamide gel and electrophoretically transferred to nitrocellulose. A panel of antibodies against DNA replication enzymes and factors was used to probe the nitrocellulose blots. Among the antibodies tested, monoclonal antibodies directed against human DNA polymerase α and DNA polymerase δ detected bands of 620 kDa and 500 kDa, respectively (Fig. 4, panels 1 and 2). Free proliferating cell nuclear antigen (PCNA) was detected as a band migrating below bovine serum albumin monomer by a monoclonal antibody directed against PCNA (Fig. 4, lane 3). In order to search for epitopes unexposed on the surface of the native multiprotein complexes, following the initial probing of native immunoblots with antibody, the blots were treated with sodium dodecyl sulfate and 2-mercaptoethanol (Materials and Methods) for 30 min at 50°C. This treatment was carried out to simultaneously strip the blot of bound antibody and allow the possibility of denaturation in situ on the surface of the blot. Following this treatment, the immunoblots were reprobed with monoclonal antibodies directed against DNA polymerase α, DNA polymerase δ, and PCNA (Fig. 5).

The epitope recognized by the monoclonal antibody directed against DNA polymerase α was no longer accessible to binding following denaturation and reprobing of the immunoblots. The 620-kDa complex detected in native immunoblots could no longer be detected on the identical blot following denaturation in situ (Fig. 5, lane 1), despite long overexposures of film following ECL treatments. The 500-kDa complex detected by a monoclonal antibody against DNA polymerase δ could be detected in both native immunoblots and immunoblots following in situ denaturation (Fig. 5, lane 2).

Denaturation of native immunoblots exposed an epitope recognized by a monoclonal antibody directed against PCNA present on a large complex migrating between thyroglobulin marker and thyroglobulin dimer sized approximately at 1000 kDa, which was not detected on the same blot prior to denaturation (Fig. 5, lane 3).

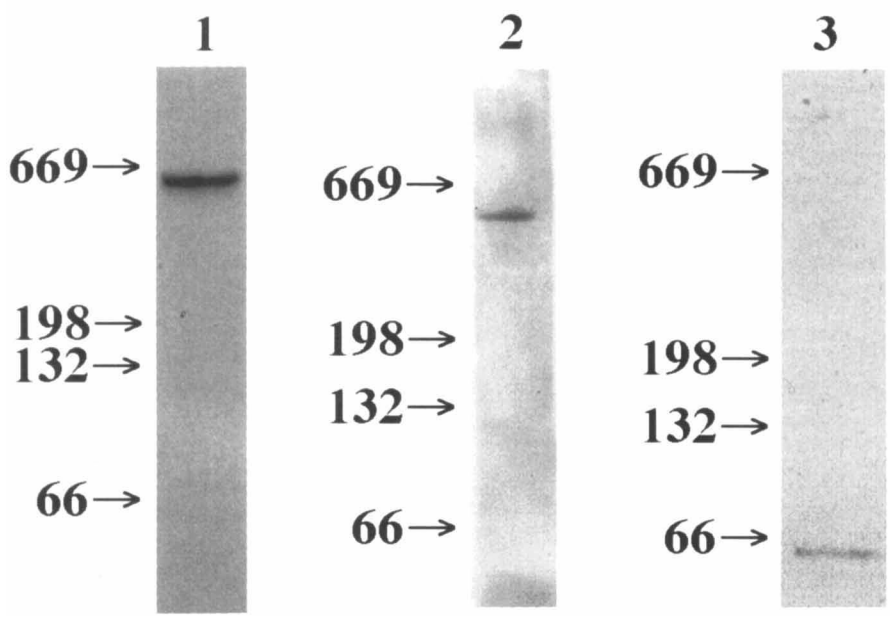

Fig. 4. Native Western blot analysis of Q-Sepharose purified HeLa cell proteins. Forty μg of Q-Sepharose purified HeLa cell proteins were resolved through a 4% native polyacrylamide gel and electrophoretically transferred to nitrocellulose as described in Materials and Methods. The Native Western blots were probed with monoclonal antibodies directed against DNA polymerase α **(lane 1)**, DNA polymerase δ **(lane 2)**, and proliferating cell nuclear antigen **(lane 3)**. Following incubation in primary antibody (Materials and Methods), blots were incubated in species-specific secondary antibody conjugated to horseradish peroxidase (HRP). Light-enhanced chemiluminescence detection of the immobilized protein was accomplished using the ECL Western blotting detection system (Amersham). Molecular size standards in kilodaltons are indicated.

DISCUSSION

The introduction of an in vitro SV40 DNA replication system provided a mechanism for studying the enzymes and factors required for replication of mammalian DNA [Li and Kelly, 1984]. Utilizing this in vitro system, many enzymes and factors have been identified that appear to be required for origin-specific T-antigen-dependent replication. These include DNA polymerase α-primase complex; DNA polymerase δ; PCNA; RP-A; topoisomerases I and II; and RF-C [reviewed in Kelly, 1988; Hurwitz et al., 1990; Stillman, 1989]. The possible interaction of many of these components into multiprotein complexes has been reported for many years [reviewed in Malkas et al., 1990a; Reddy and Fager, 1993].

In 1990, Malkas et al. purified a replication-competent form of DNA polymerase from human HeLa cells that was recently sized by sucrose velocity sedimentation at 18S [Applegren et al., 1995]. SDS-PAGE Western blot analysis showed the presence of DNA polymerase δ, topoisomerase I, RF-C, RP-A, DNA ligase I, and PCNA in both the Q-Sepharose purified and sucrose velocity sedimented protein samples [Applegren et al., 1995; Malkas et al., 1990a,b]. By functional assays, the presence of primase, DNA polymerase α, topoisomerase II, and helicase was also demonstrated in both Q-Sepharose purified and sucrose velocity sedimented protein samples [Applegren et al., 1995]. These results supported the concept that all of these DNA replication enzymes and factors form a multiprotein complex that efficiently carries out the replication of mammalian DNA. A similar profile of replication proteins competent to replicate polyoma DNA in vitro was found to co-purify from mouse FM3A cells using a similar fractionation protocol [Wu et al., 1994]. A model was proposed for the complex of co-sedimented enzymes and factors called the **m**ultiprotein DNA **r**eplication **c**omplex or MRC [Wu et al., 1994; Applegren et al., 1995].

The studies described in this paper were designed to identify potential candidates for the MRC in Q-Sepharose purified HeLa cell proteins using native gel immunoblotting. This approach was selected to allow the positive identification of large protein complexes associated with DNA replication enzymes and factors. Initial screening experiments showed the majority of anti-

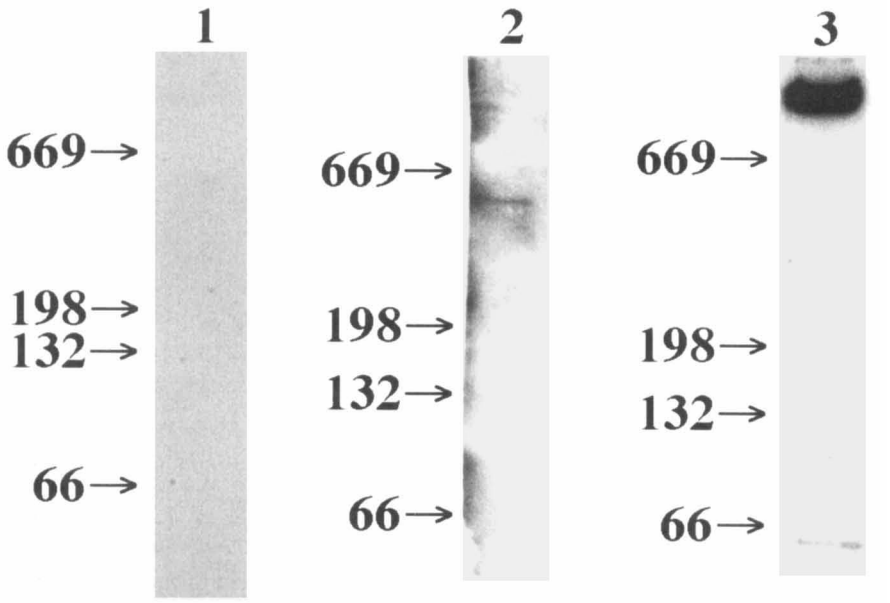

Fig. 5. In situ denaturation and reprobing of native Western blots. Native Western blots previously probed with antibody were treated with 2% sodium dodecyl sulfate and 100 mM 2-mercaptoethanol and, following two washes in phosphate-buffered saline, blocked and processed as for standard Western blots (Amersham). Native Western blots, stripped and denatured in situ, were probed with DNA polymerase α **(lane 1)**, DNA polymerase δ **(lane 2)**, and proliferating cell nuclear antigen **(lane 3)**. Detection of bound antibody was accomplished as described earlier (Fig. 4 and Materials and Methods). Molecular size standards in kilodaltons are indicated.

bodies tested, including those directed against topoisomerase I, DNA ligase I, RP-A, RF-C, and topoisomerase II, detected no weak bands in the native gel immunoblots (data not shown).

The majority of these antibodies have been used previously to document the presence of these DNA replication enzymes and factors in SDS-PAGE immunoblots of Q-Sepharose purified proteins from HeLa cells [Applegren et al., 1995]. These findings suggest that the epitopes recognized by these antibodies are inaccessible or poorly accessible in native proteins. This is not surprising since significant regions of proteins in a large multiprotein complex might be buried within the complex.

In order to address this possibility, immunoblots were exposed to denaturing conditions in an attempt to denature proteins in situ on the surface of the blot. This was accomplished by treating immunoblots with detergent and reducing agents commonly used for stripping standard SDS-PAGE immunoblots prior to reprobing (Materials and Methods).

The potential usefulness of this treatment was demonstrated by the detection of a very large complex of 1000 kDa recognized by mouse monoclonal antibody against human PCNA only in blots denatured in situ. In situ denaturation also appears to destroy access to certain epitopes as demonstrated by the loss of signal following in situ denaturation and probing of immunoblots with a monoclonal antibody against human DNA polymerase α. This loss of signal apparently reflects a mechanical obstruction of the relevant epitope following in situ denaturation rather than destruction of a conformational epitope since this monoclonal antibody functions in standard SDS-PAGE immunoblots [Wu et al., 1994]. The inability to detect other proteins involved in DNA replication on native immunoblots even after in situ denaturation suggests that this treatment is only partially effective in unmasking internally shielded epitopes in multiprotein complexes. Despite these limitations, in situ denaturation of native immunoblots should be a generally applicable technique for study of internal protein structures in multiprotein complexes from other systems.

The finding of three different apparent multiprotein complexes in this study raises interesting questions regarding the possible relationship and role of each of these molecules in the replication of DNA. The 620-kDa complex detected by a monoclonal antibody against DNA polymerase α is similar in molecular weight to a

640-kDa multiprotein form of DNA polymerase α called polymerase α2 identified by Vishwanatha et al. [1986]. In addition to DNA polymerase α, this complex was also found to contain primase, a single-strand-specific exonuclease, diadenosine 5′,5′′′-P¹,P⁴-tetraphosphate binding activities and accessory primer recognition proteins C1 and C2. We do not believe the 620-kDa complex identified in this study is identical to the polymerase α2 complex for the following reasons. Purified polymerase α2 complex has no activity in the in vitro SV40 DNA replication assay [Baril et al., 1988]. The 620-kDa complex identified in this study has thus far only been identified in HeLa cell preparations with high activity in the in vitro DNA replication assay. Lastly, a monoclonal antibody produced against, and reactive with, the polymerase α2 complex in native immunoblots does not bind to the 620-kDa complex (R. Hickey, T. Tom, unpublished data). The relationship of the 620-kDa complex identified in this study and the polymerase α2 complex is unknown. The inactivity of the polymerase α2 complex in the in vitro SV40 DNA replication assay suggests this complex may be a degradation product or partial assembly or disassembly product of a functional complex. Alternatively, the polymerase α2 complex may participate in DNA repair functions distinct from chromosomal replication functions.

Numerous molecular weight forms have been identified by immunoblotting experiments to be associated with DNA polymerase δ [Lee, 1988; Lee et al., 1981, 1984]. A large-molecular-weight form of DNA polymerase δ from HeLa cells estimated to have a molecular weight of 470 kDa in native polyacrylamide gels was identified by Syraoja and Linn [1989]. The relationship of the 500-kDa complex identified in this study to the 470-kDa DNA polymerase of species described earlier is unknown.

This study was designed to evaluate the usefulness of native gel immunoblotting as a technique to identify potential large-molecular-weight complexes containing proteins involved in DNA replication. No attempt was made to more precisely size the complexes in this system since, in native polyacrylamide gels, mobility is influenced not only by size, but by shape and charge, and this may produce an inaccurate size estimate. With this fact in mind, we cannot exclude the possibility that each of the identified molecules may not be multiprotein complexes, but highly asymmetric molecules. However, this seems unlikely due to the magnitude of size of the molecules identified. Further, in the case of PCNA, the free form is visible on native Western blots of the Q-Sepharose purified HeLa cell proteins. The ability to identify these molecules in a simple, reproducible system is a more useful aspect of this study which will allow the further purification and characterization of multiprotein DNA replicative complexes.

The function and relationship of each of the complexes identified is unknown. Each of these complexes could represent functional intermediates involved in DNA replication. Alternatively, these complexes could represent multimers of one another or completely unrelated complexes that cooperate with one another during DNA replication. Determination of whether any of these complexes actually functions to replicate DNA in vitro must await further purification and characterization.

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