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Human Cell DNA Replication Is Mediated by a Discrete Multiprotein Complex

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Abstract A discrete high molecular weight multiprotein complex containing DNA polymerase α has been identified by a native Western blotting technique. An enrichment of this complex was seen at each step in its purification. Further purification of this complex by ion-exchange chromatography indicates that the peak of DNA polymerase α activity co-purifies with the peak of in vitro SV40 DNA replication activity eluting from the column. The complex has a sedimentation coefficient of 18S in sucrose density gradients. We have designated this complex as the DNA synthesome. We further purified the DNA synthesome by electroeluting this complex from a native polyacrylamide gel. The eluted complex retains in vitro DNA synthetic activity, and by Western blot analysis, contains DNA polymerase δ, proliferating cell nuclear antigen, and replication protein A. Enzymatic analysis of the electroeluted DNA synthesome indicates that the synthesome contains topoisomerase I and II activities, and SDS–PAGE analysis of the electroeluted DNA synthesome revealed the presence of at least 25 major polypeptides with molecular weights ranging from 20 to 240 kDa. Taken together, our evidence suggests that the DNA synthesome may represent the minimal DNA replication unit of the human cell. J. Cell. Biochem. 85: 762–774, 2002.

Key words: DNA synthesome; electroelution; DNA polymerase α; low pressure liquid chromatography; SV40; in vitro

Mammalian cell DNA replication is an intricate and highly efficient process requiring the coordinated activity of a variety of enzymes and accessory proteins. The elucidation of the role played by the human DNA replication apparatus, and its components in regulating the DNA synthetic process, is crucial to our understanding of both normal and aberrant cell proliferation. Using the simian virus 40 (SV40)-based in vitro DNA replication assay system, a minimum number of proteins have been identified to be required to drive a mammalian DNA replication fork. These proteins include DNA polymerases α and δ, DNA topoisomerases I and II, DNA primase, DNA ligase I, proliferating cell nuclear antigen (PCNA), replication factor C (RFC), replication protein A (RPA), and the nucleases RNase H1 and FEN1/RTH1 [reviewed in Stillman, 1994; Hickey and Malkas, 1997; Malkas, 1998]. Increasing evidence has supported the concept that many of the enzymes and factors involved in the replication of mammalian DNA function together as an organized multiprotein complex [Baril et al., 1983; Jackson and Cook, 1986; Mathews and Slabaugh, 1986; Vishwanatha et al., 1986; Tubo and Berezney, 1987; Biswas and Biswas, 1988; Syväoja and Linn, 1989; Reddy and Fager, 1993; Maga and Hübscher, 1996; Hickey and Malkas, 1997; Malkas, 1998]. We have previously reported that a DNA replication-competent multiprotein form of DNA polymerase can be isolated from a variety of mammalian cell lines

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and tissues [Malkas et al., 1990; Wu et al., 1994; Applegren et al., 1995; Coll et al., 1996, 1997; Tom et al., 1996; Lin et al., 1997]. The multiprotein form of DNA polymerase was purified from cells using a series of purification steps, which included differential centrifugation, polyethylene glycol (PEG) precipitation, ion-exchange chromatography, and sucrose density gradient sedimentation. The sedimentation coefficient of the human cell replication-competent multiprotein form of DNA polymerase is 18S, as determined by sucrose gradient analysis [Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997]. The integrity of the multiprotein form of DNA polymerase was maintained after treatment with detergents, salt, RNase, DNase, ion-exchange chromatography using DEAE-cellulose (Whatman) or Q-Sepharose (Pharmacia), and following sedimentation through sucrose and glycerol density gradients. Our observations suggest that the co-purification of these proteins was independent of a nonspecific interaction with other cellular macromolecular components [Malkas et al., 1990; Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997]. Using enzymatic assays to functionally define the presence of specific components of this multiprotein complex, along with denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analyses, we demonstrated that DNA polymerases α, δ, and ϵ, DNA ligase I, topoisomerases I and II, DNA primase, PCNA, RFC, RPA, and DNA helicase co-purify with the replication-competent multiprotein form of DNA polymerase [Malkas et al., 1990; Wu et al., 1994; Applegren et al., 1995; Coll et al., 1996, 1997; Tom et al., 1996; Lin et al., 1997]. Most importantly, we demonstrated that this 18S form of the DNA polymerase was fully competent to support SV40 origin-specific large T-antigen-dependent DNA replication in vitro [Malkas et al., 1990; Wu et al., 1994; Applegren et al., 1995; Coll et al., 1996; Tom et al., 1996; Lin et al., 1997]. Since replication of the SV40 genome is dependent on its host's DNA replication apparatus, our observation suggests that the multiprotein form of DNA polymerase must also play a role in human cell DNA replication.

Our observations led us to propose that the replication-competent multiprotein form of DNA polymerase represented an isolated multiprotein DNA replication complex (MRC) [Malkas et al., 1990; Wu et al., 1994; Applegren et al., 1995], which we have termed the DNA synthesome [Coll et al., 1997; Lin et al., 1997]. A model was proposed for the synthesome that was based on the observed fractionation, chromatography, and density gradient sedimentation profiles for the proteins that co-purify with the replication-competent multiprotein complex [Malkas et al., 1990; Wu et al., 1994; Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997]. Recent co-immunoprecipitation studies support the model and suggest that DNA polymerases α and δ, DNA primase, and RFC, as well as PCNA, tightly associate with one another [Coll et al., 1997]. Several other synthesome components were found to interact via direct contact with only PCNA or DNA polymerase α [Coll et al., 1997].

Nondenaturing or native PAGE has been used by several groups to identify DNA replication complexes [Holler et al., 1985; Vishwanatha et al., 1986; Syväoja and Linn, 1989; Park et al., 1995; Tom et al., 1996]. In this report, we have analyzed the synthesome using native PAGE. Our data indicate that the highly purified form of the synthesome is a discrete high molecular weight protein species that fully supports large T-antigen dependent SV40 DNA replication in vitro, suggesting that the synthesome may represent the minimal replication apparatus of mammalian cells.

MATERIALS AND METHODS

Cell Culture

Suspension cultures of MCF7 cells were grown in Joklik's modified Eagle's medium supplemented with 10% fetal bovine serum. Exponentially growing cells (5×10^5 cells/ml) were harvested and washed three times with phosphate-buffered saline (PBS). The cells were then pelleted and stored at -80°C prior to initiating synthesome isolation.

Subcellular Fractionation

MCF7 cells were fractionated to the level of the previously-described sucrose interface protein fraction (P4) using our published procedures [Malkas et al., 1990; Coll et al., 1996].

Low Pressure Liquid Chromatography (LPLC)

P4 fraction (5 ml) containing approximately 30 mg of protein was loaded onto a Bio-Rad Q5 column pre-equilibrated with buffer A (50 mM

Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 0.1% glycerol, and 1 mM EDTA). The column was then washed with buffer A and eluted using a gradient of 50–500 mM KCl in buffer A. One milliliter fractions were collected. The eluted fractions were then dialyzed into buffer B (20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT), and aliquots of each fraction were stored at $-80^{\circ}C$.

Sucrose Gradient Sedimentation

A 10 ml continuous 10–30% sucrose gradient in 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 50 mM KCl, and 1 mM EDTA was formed using a Sorvall Gradient Maker. Approximately 1 mg of the pooled LPLC eluted fractions (fractions 27 and 28) containing the peak of the DNA polymerase α and in vitro SV40 DNA replication activities was loaded onto the gradient. The gradient was then centrifuged at 100,000*g* for 16 h at a temperature of 4°C. After centrifugation, 1 ml fractions of the gradient were collected. The collected fractions were then dialyzed for 2 h against two changes of buffer B. The dialyzed fractions were then stored at $-80^{\circ}C$. Marker proteins having defined sedimentation coefficient values were centrifuged alongside the gradients containing the LPLC fraction. The proteins, alcohol dehydrogenase (7S) and thyroglobulin (19S), served as marker proteins. As described previously in Malkas et al. [1990], the positions of these proteins in the density gradients were used to estimate the sedimentation coefficient of the replication-competent form of DNA polymerase α.

Electroelution of the Multiprotein Form of Human Cell DNA Polymerase From Preparative Native Polyacrylamide Gels

Four percent native polyacrylamide gels containing a 3.5% stacking gel were prepared using the Bio-Rad mini-Protean II gel apparatus, as previously described [Tom et al., 1996]. One milligram of pooled LPLC protein fraction was loaded onto the gel. Electrophoresis was initially started at 50 V until the dye front entered the 4% separating PAGE gel, at which time the voltage was increased to 90 V. Electrophoresis was continued until the dye front reached the bottom of the gel. Following electrophoresis, the 4% gel was trimmed to fit onto the Bio-Rad Mini Whole Gel Eluter, as described by the manufacturer. The gel was then soaked in 20 mM HEPES, pH 7.5, for 10 min, and layered onto the elution chamber core. The Whole Gel Eluter was assembled according to the manufacturer's instructions and elution of the resolved synthesome protein fraction from the 4% PAGE gel was performed as described in the Bio-Rad protocol provided with the apparatus. HEPES (20 mM, pH 7.5) was used as the elution buffer in these procedures. The electroelution was initiated at 60 mA for 1 h and then continued at 30 mA for an additional 2 h. The proteins bound to the cellophane membrane at the end of the elution were removed by reversing the polarity of the electroeluter cell, and continuing electrophoresis for 30 s at 100 V. The eluted fractions were harvested, with each fraction containing approximately 500 μl of liquid. The fractions were stored at $-80^{\circ}C$ until they were analyzed.

Western Blot Analysis Following Denaturing or Native PAGE

The electroeluted synthesome fractions were resolved by either 8% SDS–polyacrylamide gel or 4% native polyacrylamide gel and electrophoretically transferred to a nitrocellulose filter membrane as previously described [Applegren et al., 1995; Tom et al., 1996]. The filter membranes were then incubated with monoclonal antibody SJK 132-20 directed against human DNA polymerase α [Tanaka et al., 1982] (American Type Culture Collection, Rockville, MD), monoclonal DNA polymerase δ antibody (Transduction Laboratories, Lexington, KY), PCNA antibody (Oncogene Science, Cambridge, MA), and monoclonal RPA antibody (Oncogene Science). Following incubation with the primary antibody, the membranes were incubated with anti-mouse secondary antibody conjugated to horseradish peroxide, and the high molecular weight form of DNA polymerase α was detected using a light-enhanced chemiluminescence system (Amersham, Arlington Heights, IL). Thyroglobulin (669 kDa) and bovine serum albumin (BSA) (66 kDa) were used as molecular size markers (Sigma, St. Louis, MO).

Enzyme and In Vitro DNA Replication Assays

DNA polymerase α activity was assayed according to published procedures [Malkas et al., 1990] using an activated calf thymus DNA template (Sigma).

DNA polymerase δ activity was detected essentially as described in Han et al. [2000]. The 50-μl assay mixture contained 50 ng poly(dA)/

oligo(dT) (20:1); 0.25 μl [α-^{32}P]dTTP; 2.5 μl core buffer (containing 10 mM $MgCl_2$; 10 μM TTP; 25 mM HEPES, pH 5.9; 200 μg/ml BSA; 5% glycerol), and 30 μl of protein fraction. The reaction was carried out at 37°C for 15 min, and the whole reaction mixture was then spotted onto Whatman DE81 filters. The filters were processed to quantify the amount of radiolabeled nucleotide incorporated into DNA template [Sambrook et al., 1989; Malkas et al., 1990].

In vitro SV40 DNA replication assay was performed essentially as described in Malkas et al. [1990] and Abdel-Aziz et al. [2000] using a 25 μl assay volume. The in vitro DNA replication reaction was performed at 37°C for 4 h, and stopped by adding EDTA and SDS to a final concentration of 25 mM and 10%, respectively. The reaction mixture was then added to tubes containing 100 μg of yeast RNA in 1% SDS, and the mixture was subjected to proteolytic digestion for 1 h at 37°C with 2 μg of proteinase K. DNA replication products formed in the assay were then isolated by extracting the digestion mixture twice with phenol/chloroform/isoamyl alcohol and one time with chloroform/isoamyl alcohol. The extracted DNA was then precipitated in the presence of 2 M ammonium acetate with 2-propanol and the DNA pellet collected by centrifugation. The pellet was finally resuspended in 10 mM Tris/1 mM EDTA, and the reaction products were analyzed using a 1% alkaline agarose gel containing 50 mM NaOH/1 mM EDTA. The gels were dried, and autoradiograms of the dried gels were produced by exposing the dried gels to Kodak film at − 80°C.

Topoisomerase I activity was measured using a commercially available kit and the instructions supplied by the manufacturer (TopoGEN, OH). A 20 μl assay contains 0.2 μg supercoiled DNA with 2 U of topoisomerase I or 15 μl of electroeluted fraction 5. The reaction was incubated in 37°C for 1 h followed by digestion with 50 μg/ml proteinase K for another 30 min. Then the reaction mixture was loaded onto a 1% agarose gel made with TAE buffer followed by ethidium bromide stain. Relaxed plasmid substrate (0.1 μg) was used as a marker. Camptothecin (200 μM) is a specific topoisomerase I inhibitor and was used in the assay as a control.

Topoisomerase II activity was determined using a commercially available assay kit (TopoGEN). In a 20-μl assay, 0.15 μg of Kinetoplast DNA (KDNA) was incubated with 15 μl of electroeluted fraction 5 or 2 U of topoisomerase II enzyme at 37°C for 1 h. The reaction mixture was then resolved through a 1% agarose gel containing 0.5% ethidium bromide, both in the gel and electrophoresis buffer. Etoposide (100 μM) is a specific inhibitor for topoisomerase II and was used in the assay as a control. Linear KDNA (0.1 μg) and decatenated KDNA (0.1 μl) were used as markers.

RESULTS

Enrichment of a Discrete High Molecular Weight Form of DNA Polymerase α During Subcellular Fractionation and Purification of the Human Cell DNA Synthesome

We previously demonstrated that a readily sedimentable form of DNA polymerase α, having a uniform sedimentation coefficient of 18S in sucrose density gradients, could be isolated from human cells [Malkas et al., 1990; Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997]. Only the 18S form of DNA polymerase α was observed to be able to fully support large T-antigen dependent in vitro replication of SV40 origin containing DNA [Malkas et al., 1990; Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997]. It was proposed that this replication-competent 18S form of the DNA polymerase represented a MRC (designated the DNA synthesome) that could potentially function as an organized DNA synthetic apparatus in intact cells [Malkas et al., 1990; reviewed in Stillman, 1994; Applegren et al., 1995; Coll et al., 1996; Hickey and Malkas, 1997; Lin et al., 1997]. To begin to evaluate whether the replication-competent synthesome was a discrete multiprotein species, a methodology relying on native PAGE was developed for this analysis [Tom et al., 1996].

In this current report, nondenaturing (native) PAGE was used to determine whether we were enriching the abundance of a discrete high molecular weight form of DNA polymerase α using the subcellular fractionation procedure developed to achieve the partial purification of the DNA synthesome. MCF7 cell homogenates were fractionated according to our published procedures [Malkas et al., 1990; Coll et al., 1996], and the DNA synthesome was initially purified to the level of the DNA replication-competent P4 protein fraction, as described previously [Malkas et al., 1990; Applegren et al., 1995; Coll et al., 1996; Lin

et al., 1997]. In those reports, we demonstrated that the replication-competent P4 protein fraction contained a form of the human cell DNA polymerase α that had a sedimentation coefficient of 18S, while the replication-deficient S4 fraction lacked this form of the DNA polymerase [Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997]. To determine whether this 18S form of the DNA polymerase present in the P4 was a discrete complex, we resolved 40 μg of each of the fractions obtained during the purification of the homogenate (H) (i.e., the S1, S2, S3, NE, NE/S3, and P4 fractions) using native PAGE. The resolved protein species were electrophoretically transferred to a nitrocellulose filter membrane (see Materials and Methods section), and the nitrocellulose filter membrane was incubated with the antibody SJK 132-20, which specifically recognizes human cell DNA polymerase α. The results of these Western blot analyses are shown in Figure 1. A discrete high molecular weight form

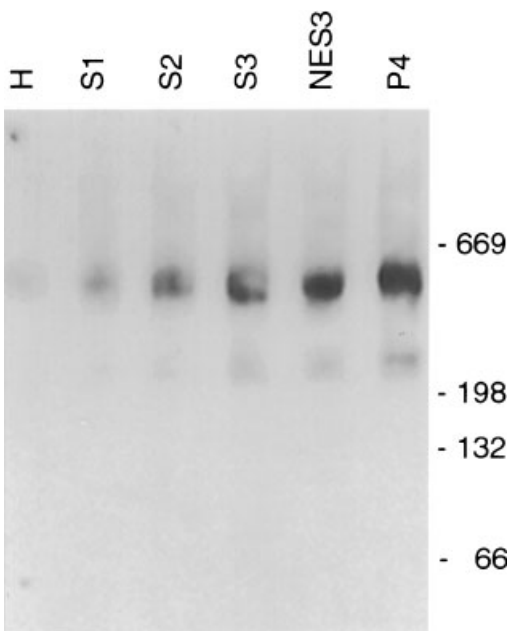

Fig. 1. Native Western blot analysis of various protein fractions obtained at different stages in the purification of the human cell DNA synthesome. Forty micrograms of protein from each purification step of the subcellular fractionation used to purify the DNA synthesome were resolved by native PAGE using a 4% polyacrylamide gel. The resolved proteins were transferred to a nitrocellulose filter membrane, and the membrane was incubated with the murine monoclonal antibody SJK 132-20 (see Materials and Methods section). The relative positions of specific molecular markers (thyroglobulin, 669 kDa), BSA trimer (198 kDa), BSA dimer (132 kDa), BSA monomer (66 kDa) resolved in the native gel system are indicated in the figure.

of DNA polymerase α with a consistent relative mobility just below that of thyroglobulin was identified in each fraction. There was an enrichment of this form of DNA polymerase α as a function of synthesome purification from the H through the P4 fraction. Our observation of the increasing abundance of the high molecular weight form of DNA polymerase α with each purification step correlated with the previously observed increase in the specific activity for synthesome-mediated T-antigen dependent in vitro SV40 DNA replication associated with each of these purification steps [Applegren et al., 1995; Lin et al., 1997].

Integrity of the High Molecular Weight Form of DNA Polymerase α Was Maintained Following LPLC

Prior to this current report, we demonstrated that both the 18S form of DNA polymerase α and in vitro SV40 origin-specific DNA replication activity were present in the human cell derived P4 fraction, and that these activities co-purified with one another following chromatography of the P4 fraction on the anion exchange resin Q-Sepharose (Pharmacia) [Malkas et al., 1990; Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997]. We now report that the integrity of the high molecular weight form of DNA polymerase α, revealed by native PAGE, was also maintained following ion exchange chromatography. To demonstrate this, the P4 protein fraction was subjected to LPLC on a Bio-Rad Q5 column as described in the Materials and Methods section. The fractions eluted from the columns were then assayed for both DNA polymerase α and in vitro SV40 DNA replication activity (see Materials and Methods section). As observed previously, the major peak of in vitro SV40 DNA replication activity (fractions 27 and 28) eluted from the column exactly coincided with the peak of DNA polymerase α activity eluting from the column (data not shown). The column fractions containing the highest levels of the co-purifying in vitro DNA replication and DNA polymerase α activities were pooled, as were column fractions surrounding the peak containing these activities. The protein species present in 20 μl of each of the pooled fractions were resolved by native PAGE using a 4% polyacrylamide gel, and the resolved protein species were electrophoretically transferred to a nitrocellulose filter membrane (see Materials and Methods section). The nitrocellulose filter membrane was then

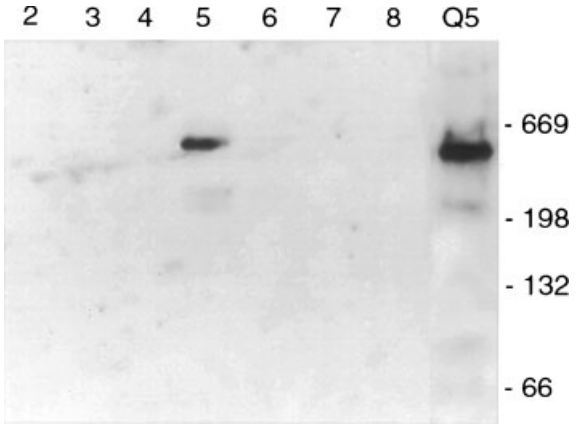

Fig. 2. Native Western blot analysis of various protein fractions obtained following sucrose gradient velocity sedimentation of the Bio-Rad Q5 column eluate. Forty microliters of each of the sucrose gradient fractions and 40 μg of Q5 column elute (pooled fractions 27 and 28) were resolved using native PAGE (4% gel), and the resolved proteins were transferred to a nitrocellulose filter membrane. The membrane was incubated with the murine monoclonal antibody SJK 132-20 (see Materials and Methods section). Numbers at the top of the figure represent sucrose gradient fractions. The relative positions of specific molecular standards are listed alongside the figure.

incubated with the anti-DNA polymerase α antibody, SJK 132-20. It was observed that, relative to the P4 fraction, the high molecular weight form of DNA polymerase α was significantly enriched in the pooled column fractions containing the peak of co-eluting in vitro DNA replication and DNA polymerase α activities (compare P4 lane in Fig. 1 with Q5 lane in Fig. 2). Our data indicate that the integrity of the discrete high molecular weight form of DNA polymerase α was maintained after low-pressure liquid chromatography. Our results also suggested a clear correlation between the ability of the chromatographically-purified P4 fraction to support in vitro replication of SV40 origin containing DNA and the presence of the discrete high molecular weight form of DNA polymerase α in the replication-competent fraction(s).

Discrete High Molecular Weight Form of DNA Polymerase α Has a Sedimentation Coefficient of 18S

The replication-competent multiprotein form of the human DNA polymerase α has been previously shown to have a sedimentation coefficient of 18S in sucrose density gradients [Applegren et al., 1995; Lin et al., 1997]. We now attempted to determine the sedimentation coefficient of the discrete high molecular weight form of the DNA polymerase α purified by LPLC. The pooled Bio-Rad Q5 column eluted fractions (fractions 27 and 28) containing the peak of the DNA polymerase α and in vitro SV40 origin-specific DNA replication activities, as well as the high molecular weight form of the DNA polymerase α, were subjected to sucrose density gradient analysis as described in the Materials and Methods section. It was observed that the discrete high molecular weight form of DNA polymerase α present in chromatographic fractions 27 and 28 had a sedimentation coefficient of 18S (Fig. 2). This sedimentation coefficient correlated exactly with that determined previously for the replication-competent multiprotein form of this DNA polymerase [Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997]. Our result suggested that the discrete high molecular weight form of DNA polymerase α identified by native PAGE analysis could, in fact, be the previously-described 18S replication-competent DNA polymerase termed the DNA synthesome [Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997].

Discrete High Molecular Weight Form of DNA Polymerase α Was Stable to Electroelution Procedures Following Native PAGE Resolution

In order to attempt to establish a relationship between the previously-described 18S replication-competent DNA synthesome [Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997] and the discrete high molecular weight form of the DNA polymerase α identified using native PAGE (Figs. 1 and 2), it was necessary to extract the high molecular weight DNA polymerase α protein species from the native gel. This was accomplished by the use of an electroelution method devised to extract the replication competent form of the DNA polymerase α from the 4% nondenaturing polyacrylamide gel (see Materials and Methods section). For this study, the pooled protein fraction derived from LPLC (Fig. 2; fractions 27 and 28) of an MCF7 cell P4 fraction containing both the major peak of DNA polymerase α and in vitro SV40 DNA replication activities was subjected to native PAGE (see Materials and Methods section). The native 4% polyacrylamide gel containing the resolved protein fraction was eluted using a Bio-Rad Whole Gel Eluter, and individual electroelution fractions were collected as described in the Materials and Methods section. The electroelution fractions found to contain significant levels

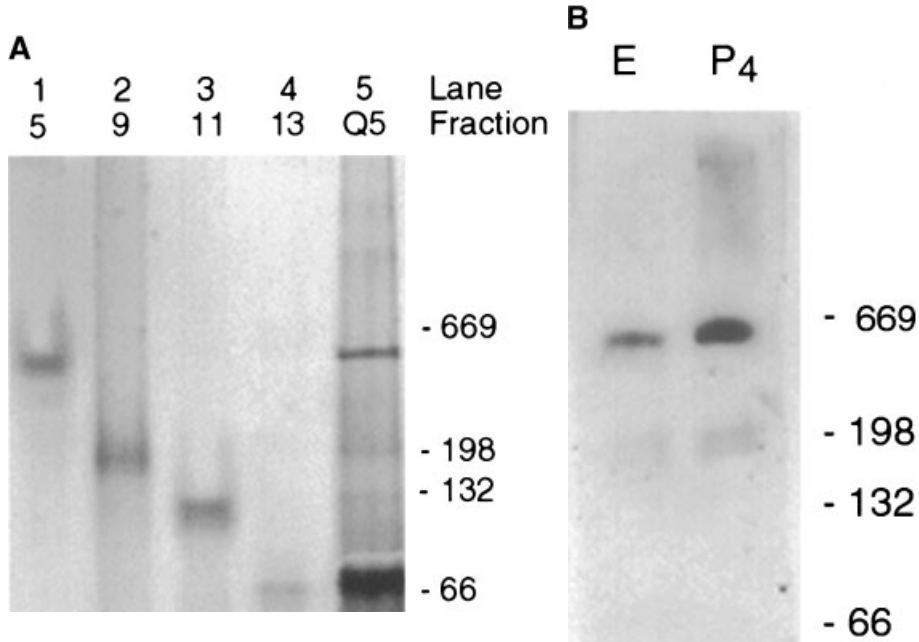

Fig. 3. Silver stain and Western blot analyses following nondenaturing PAGE of the electroeluted fractions. **A**: Silver stain of resolved protein species following nondenaturing PAGE of electroeluted fraction 5. Fifty microliters of each of the collected electroeluted fractions were resolved by native PAGE using 4% gels, and the resolved proteins were stained with silver stain (see Materials and Methods section). **Lanes 1–4** contain electroeluted fractions 5, 9, 11, and 13. One microgram of pooled Q5 column eluted fractions 27 and 28 (Fig. 2) was used for comparison of the relative mobility of the high molecular mass complexes. **B**: Western blot analysis following nondenaturing PAGE of electroeluted fraction 5. One microgram of electroeluted fraction 5 (denoted as (**E**) in the figure) and 40 μg of P4 (Fig. 1) were resolved by 4% native PAGE and transferred to a nitrocellulose filter membrane. The membrane was then incubated with antibody SJK 132-20 to identify the relative position of DNA polymerase α on the filter membrane (see Materials and Methods section). The relative positions of specific molecular standards are listed alongside the scan of the blot.

of protein (fractions 5, 9, 11, and 13) were resolved again by native PAGE followed by silver staining the gel. Figure 3A shows the different protein species resolved by the native gel. The protein species observed in lane 1 of the gel had a relative mobility consistent with that obtained for the discrete, replication-competent, high molecular weight form of DNA polymerase α (compare lane 1 with 5).

The discrete high molecular weight form of DNA polymerase α band was partitioned in the first 5–6 electroelute fractions, however, this band was greatly enriched in fraction 5 (Fig. 3A, lane 1). We did not detect this band in fractions 7–14; instead, faster migrating bands were detected with silver staining (Fig. 3A, lanes 2–4). We focused our attention on the slower migrating protein band because our previous work with DNA synthesome derived from HeLa cells [Tom et al., 1996] indicates that the faster migrating protein bands are most likely degradation products of the DNA synthesome.

To verify that the protein species eluted in fraction 5 from the native polyacrylamide gel (Fig. 3A, lane 1) was in fact the discrete high molecular weight form of DNA polymerase α, this electroeluted fraction was analyzed by native PAGE along with a P4 fraction and the resolved protein species were transferred electrophoretically to a nitrocellulose filter membrane (see Materials and Methods section). The nitrocellulose filter membrane was then subjected to Western blot analysis using anti-DNA polymerase α. It was observed that the electroeluted fraction (fraction 5) contained the discrete high molecular weight form of DNA polymerase α (Fig. 3B) found to be present in the P4 fraction (Fig. 1).

Native PAGE Purified High Molecular Weight Form of DNA Polymerase α Contains In Vitro DNA Synthetic Activity

The high molecular weight form of DNA polymerase α that was present in fraction 5 of the electroelution step (described above) was evaluated for both its ability to support T-antigen-dependent DNA replication in vitro and for the presence of specific replication

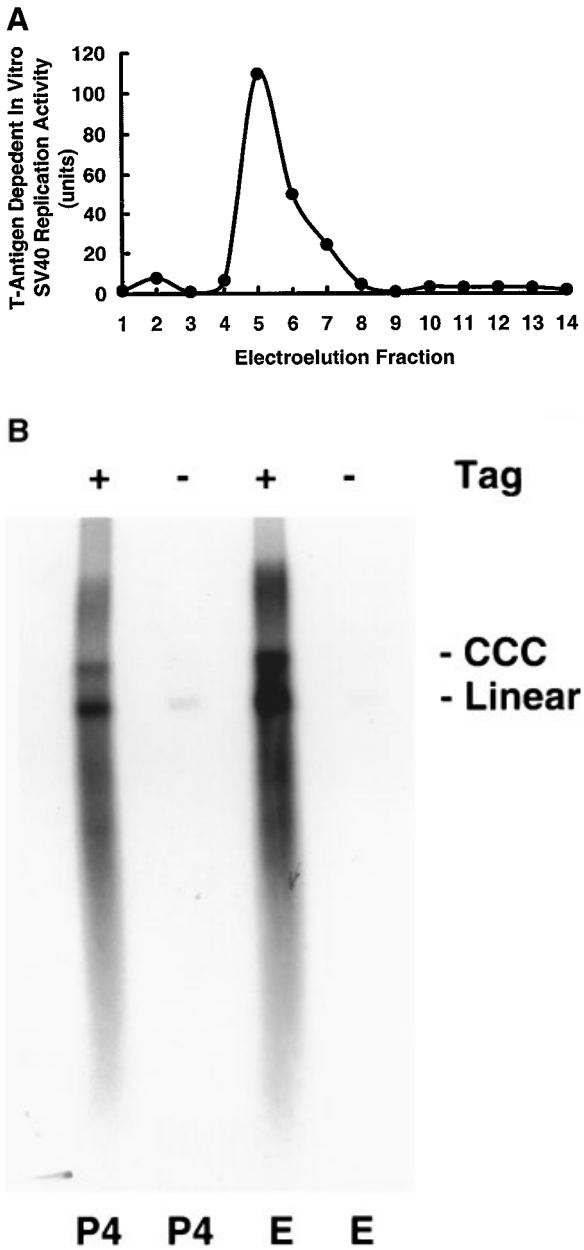

Fig. 4. T-antigen dependent in vitro SV40 DNA replication activity of the electroeluted fractions. **A**: In vitro SV40 DNA replication activity. The in vitro SV40 DNA replication assay was performed on individual electroeluted fractions as described (see Materials and Methods section). One unit of in vitro SV40 DNA synthesis activity was defined as 1 pmole of total [α-^{32}P]dNMP incorporated into SV40 origin-containing DNA per hour at 37°C. **B**: Replication products formed by electroeluted fraction 5. The replication products formed in the in vitro replication assay by electroeluted fraction 5 (**E**) and the P4 fraction were extracted and analyzed by 1% alkaline agarose gel electrophoresis (see Materials and Methods section). CCC, covalently closed circular; linear, single-stranded linear DNA; Tag, SV40 large T-antigen.

essential proteins. Each of the above-described electroeluted fractions from the native polyacrylamide gel was assayed for in vitro SV40 DNA replication activity. It was observed that the major peak of in vitro replication activity was present in electroelution fraction 5 (Fig. 4A). Our analysis of fraction 5 indicated that the level of in vitro DNA synthetic activity supported by this fraction was significantly enriched over the levels supported by the various replication-competent fractions obtained at earlier steps in the purification. Fraction 5 had a specific activity for T-antigen-dependent in vitro DNA replication that was approximately 3,696-fold higher than that observed in MCF7 cell homogenates (Table I). The ability of the high molecular weight form of DNA polymerase α to produce full-length daughter DNA molecules is shown in Figure 4B. Figure 4B also demonstrates that the in vitro replication reaction supported by the electroeluted synthesome (fraction 5) was T-antigen dependent.

The assay component requirements for in vitro SV40 DNA replication activity supported by the highly purified high molecular weight form of DNA polymerase (i.e., the DNA synthesome) present in electroelution fraction 5 are shown in Table II. It was observed that synthesome-driven in vitro SV40 DNA replication activity required the large T-antigen protein as well as an SV40 replication origin containing DNA. The replication reaction was dependent on the presence of both ribonucleotides and deoxyribonucleotides, Mg^{2+}, and an ATP regenerating system. The inhibition of DNA synthetic activity by antibodies directed

TABLE I. Specific Activity Analysis of In Vitro SV40 DNA Replication Activity as a Function of Synthesome Purification

Fractions	Specific activity (units/mg)	Purification fold
H	0.14	1.0
S1	0.22	1.6
S2	0.66	4.7
S3	0.71	5.1
NE/S3	1.03	7.3
P4	2.55	18.2
Q5	5.25	37.5
Sucrose peak	67.68	483.2
E	517.53	3696.1

In vitro DNA replication reactions were performed as described in the Materials and Methods. One unit of activity was defined as 1 pmole of total [α-^{32}P] dNMP incorporated into SV40 origin containing DNA per milligram protein per hour at 37°C.

TABLE II. Assay Component Requirements for DNA Synthesome Driven In Vitro DNA Replication

Assay components omitted or added	Relative DNA synthesis
(+) T-antigen	100
(−) T-antigen	0
(−) CPK/PC	9
(−) Mg^{2+}	0
(−)DNA	0
(−)dATP, dGTP, dTTP	0
(−) ATP, CTP, GTP, UTP	8
(+) Polymerase α antibody (1 μg)	11
(+) PCNA antibody (1.5 μg)	31

Reactions were performed as described in the Materials and Methods. Individual assay components were omitted from specific reactions as indicated. Electroeluted fraction 5 was preincubated with specific antibodies recognizing either PCNA or DNA polymerase α for 1 h at 4°C prior to the addition of the other assay components, and the reactions were performed at 37°C for 1 h (see Materials and Methods section). Relative DNA synthesis was calculated as the percentage of total [α-^{32}P]dNMP incorporated into DNA relative to a control reaction containing all of the reaction components, but lacking any neutralizing antibody.

against either the DNA polymerase α polypeptide or the DNA polymerase δ accessory protein PCNA indicates that both DNA polymerases α and δ mediate synthesome-driven in vitro SV40 DNA replication.

Discrete High Molecular Weight Form of DNA Polymerase (i.e., the DNA Synthesome) Contains DNA Replication Essential Proteins

SDS–PAGE analyses of the electroeluted DNA synthesome revealed the presence of approximately 25 polypeptides staining with silver and spanning a wide range of molecular weights (Fig. 5).

The highly purified DNA synthesome was also examined for the presence of DNA replication essential proteins using Western blot analyses and specific enzymatic activities. It was observed that the replication-competent synthesome (electroelution fraction 5) contained enzymatic activities for DNA polymerase α and δ as well as topoisomerases I and II (Fig. 6A–D). Our data indicate that these protein activities co-purify with one another and with in vitro DNA synthetic activity. Western blot analyses of the electroeluted fraction 5 also revealed the presence of PCNA, RPA, and the 125 kDa polypeptide of DNA polymerase δ in this fraction (Fig. 7). Thus, our results indicate that the PCNA and RPA proteins also co-purify with the enzymatic activities reported in Figure 6.

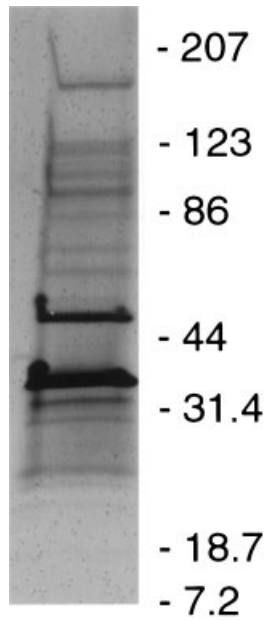

Fig. 5. SDS–PAGE analysis of the replication-competent DNA synthesome in electroeluted fraction 5. The components of the highly purified DNA synthesome were resolved using a 4–20% linear gradient of polyacrylamide (SDS–PAGE) under denaturing conditions. The resolved polypeptide bands were visualized by silver staining. The relative positions of specific molecular mass standards are listed alongside the scan of the gel.

DISCUSSION

Since the development of the in vitro SV40 DNA replication model system by Li and Kelly [1984], progress has been rapid in delineating the minimal number of enzymes and factors required for the synthesis of mammalian DNA. The majority of studies have utilized partially-purified enzymes and factors to reconstitute DNA synthetic activity [Fairman and Stillman, 1988; reviewed in Kelly, 1988; Stillman, 1989; Hurwitz et al., 1990; Tsurimoto et al., 1990]. Although some data exist on the functional association of several enzymes and factors [Mathews and Slabaugh, 1986; Vishwanatha et al., 1986; Tubo and Berezney, 1987; Biswas and Biswas, 1988; Syväoja and Linn, 1989; Reddy and Fager, 1993; Maga and Hübscher, 1996], the precise molecular interactions that occur between the proteins required to support DNA replication remain largely undefined. The ability to reconstitute DNA replication activity utilizing individual components does not exclude the existence of large multiprotein complexes containing many or all of the proteins that function in vivo to replicate DNA. Several reports have described the isolation of large

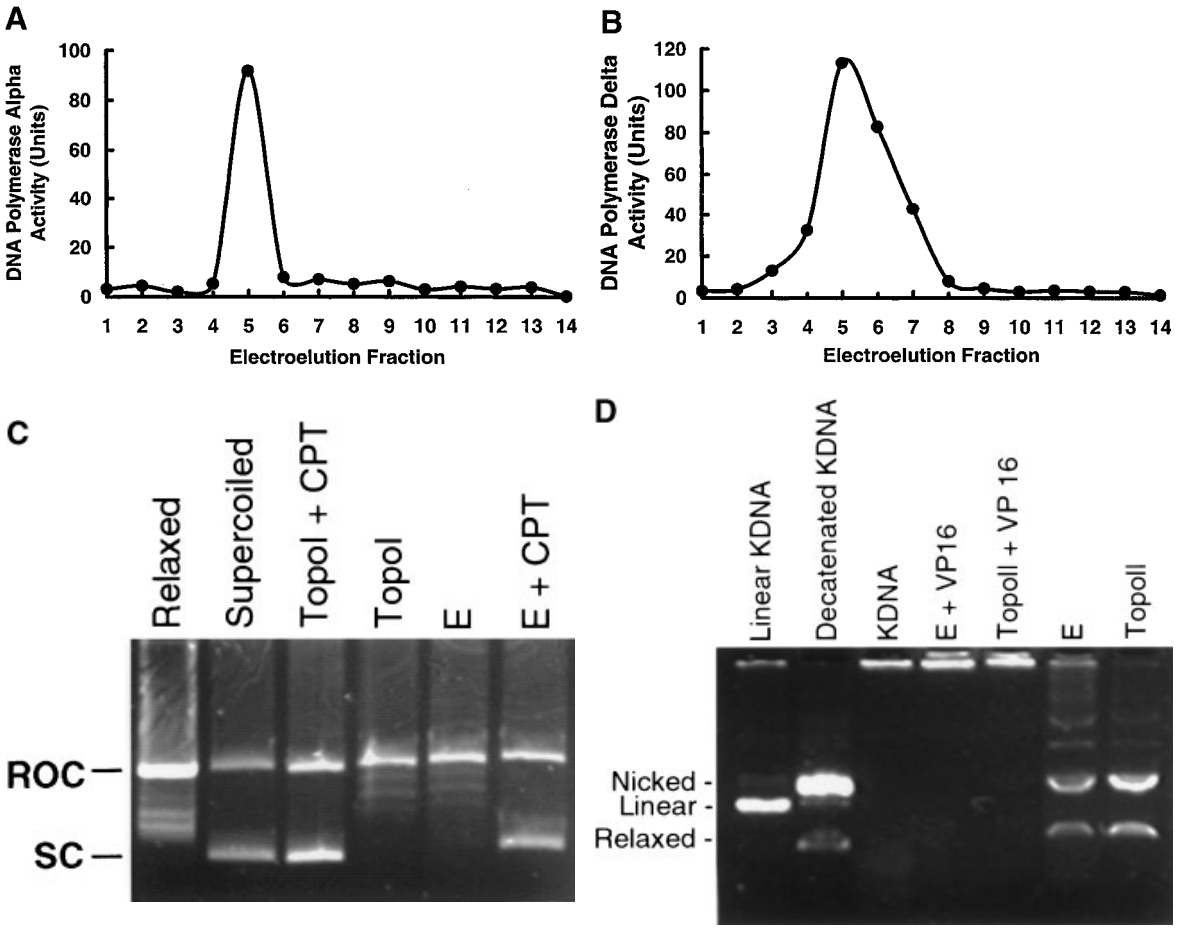

Fig. 6. Analysis of the DNA polymerases activities and replication proteins contained in the electroeluted DNA synthesome. Electroeluted fractions were assayed for (**A**) DNA polymerase α activity and (**B**) DNA polymerase δ activity, respectively, as described in the Materials and Methods section. One unit of DNA polymerase α activity equals 1 pmole of total [α-^{32}P]dNMP incorporated into an activated calf thymus DNA template per hour at 37°C. One unit of DNA polymerase δ activity equals 1 pmole of [α-^{32}P]dTMP incorporated into the DNA template per 15 min at 37°C. **C**: Topoisomerase I activity assay of the purified DNA synthesome. Agarose gel electrophoretic analysis showing the conversion of 0.2 μg of supercoiled plasmid DNA to the relaxed form by electroeluted fraction 5 (**E**). The assays were performed as described in the Materials and Methods section. Reactions containing 2 U of purified topoisomerase I were used as a positive control. Reactions containing the topoisomerase I specific inhibitor, camptothecin (200 μM), were used in the assay as a negative control. ROC, relaxed open circular; SC, supercoil. **D**: Topoisomerase II activity assay of the purified DNA synthesome. The presence of topoisomerase II in electroeluted fraction 5 (E) was assayed by decatenation of kinetoplast (KDNA) as described in the Materials and Methods section. Linear and decatenated KDNA served as reference markers, and topoisomerase II activity was inhibited in one reaction by the inclusion of 100 μM etoposide. This reaction served as a negative control.

macromolecular complexes of replication essential proteins from extracts of eukaryotic cells [Jackson and Cook, 1986; Mathews and Slabaugh, 1986; Vishwanatha et al., 1986; Syväoja and Linn, 1989; Reddy and Fager, 1993; Maga and Hübscher, 1996]. For example, a megacomplex of enzymes involved in dNTP synthesis and DNA polymerization, known as replitase, had been purified from Chinese embryo fibroblast cells [Reddy and Fager, 1993]. In other work, Jackson and Cook [1986a] reported the retention of 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 some evidence was obtained suggesting that it associates with the nuclear matrix [Jackson and Cook, 1986a]. Additionally, the isolation of 100–150S megacomplexes from regenerating rat liver that contain DNA polymerase α-primase and associate with the

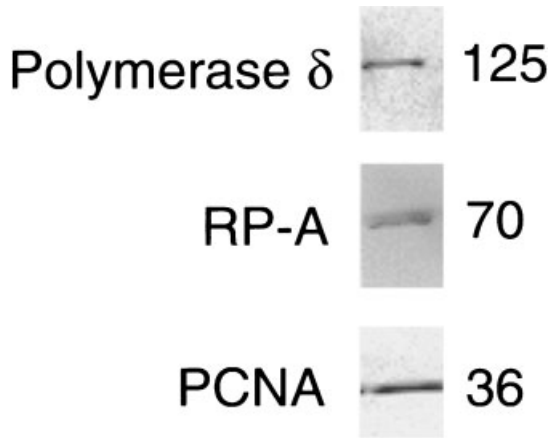

Fig. 7. Western blot analysis of DNA synthesome-associated replication essential proteins. The proteins contained in 1 μg of electroeluted fraction 5 were resolved using 8% polyacrylamide gel under denaturing conditions (see Materials and Methods section). The resolved polypeptides were transferred onto a nitrocellulose filter membrane, and the membrane was incubated with antibodies recognizing DNA polymerase δ, RPA, or PCNA (see Materials and Methods section).

nuclear matrix had been reported [Tubo and Berezney, 1987]. By using a complementary assay for RFC-dependent DNA polymerase activity on a single-primed M13 DNA template, Maga and Hübscher [1996] have isolated from calf thymus a multiprotein complex that may be active in DNA replication. Size-exclusion column chromatography yielded a molecular mass of about 900 kDa. This putative asymmetric DNA polymerase complex contained at least DNA polymerase α, DNA polymerase δ, and RFC. Previous reports have utilized native PAGE to identify large molecular weight forms associated with DNA polymerase activity. A 640-kDa DNA polymerase α containing complex [Vishwanatha et al., 1986] and a 470-kDa DNA polymerase δ-containing complex were identified by native PAGE and immunoblot. Although these complexes have shown some polymerases activities, no data have shown the ability of these complexes to support SV40 replication in vitro.

Fluorescence microscopic analysis studies of the newly-replicated DNA by Ma et al. [1998] have revealed discrete granular sites of replication. The authors suggest that each replication site contains numerous replicons clustered together. Those replication sites persist throughout the cell cycle and subsequent daughter cells [Berezney et al., 1995; Jackson and Pombo, 1998]. Furthermore, it has been demonstrated that PCNA is targeted to sites of DNA replication in the mammalian cell nucleus [Somanathan et al., 2001].

In this report, we described the identification and extensive purification of the synthesome as a functional and discrete multiprotein complex from human breast cancer MCF7 cells. Our results demonstrate that the DNA synthesome is a functional multiprotein complex that is fully competent to participate in all of the reactions required for large T-antigen-dependent SV40 in vitro replication and potentially the replication of genomic DNA in mammalian cells.

The actual mass of the replication complex should be well over 1 mDa (megadalton) by adding the mass of all the proteins required for replication. However, the current methods for determining the molecular weight of a protein, such as gel filtration, gradient centrifugation, and electrophoretic mobility, measure molecular size rather than mass [Orchard and May, 1993]. This is especially true in our case because we are measuring the mass of a protein complex rather than a single protein. Therefore, the molecular mass of the synthesome we obtained by native PAGE and sedimentation coefficient only serves as a relative reference. The identification and stoichiometry of each individual protein component of the synthesome will assist in defining the actual mass of this high molecular weight complex. Taken together, the correlation between enrichment in the abundance and activity of individual components of the DNA synthesome throughout its purification (as well as the enhancement in the inherent in vitro SV40 DNA replication activity of the complex), suggests that the DNA synthesome exists as a discrete entity within the cell, and that use of the synthesome driven assay system may enable us to accurately model in vitro many of the events that occur during intact mammalian cell DNA synthesis. We, therefore, propose that this high molecular weight DNA polymerase α containing complex, the DNA synthesome, may represent a discrete functional unit of the cellular DNA replication machinery. With respect to the regulation of the activity of the DNA synthesome, we have shown that this high molecular weight DNA polymerase α containing complex appeared to disassemble in terminally-differentiated HL-60 cells [Lin et al., 1997a], while in actively cycling cells, this complex stays together throughout the cell cycle. The data of Lin et al. [1997a] suggest that the DNA

synthesome is not assembled just prior to or during S phase and then disassembled just after S phase, but rather is disassembled only when the cells permanently leave the cell cycle. A study has also reported that the Werner Syndrome gene product, which contains a 3′-5′ exonuclease and a 3′-5′ DNA helicase domain, co purifies with this DNA replication complex previously described by us [Wu et al., 1994] and interacts with PCNA and topoisomerase I [Lebel et al., 1999]. Our demonstration that a discrete complex can be isolated and purified from asynchronous cultures of proliferating cells is consistent with this suggestion. Furthermore, the ability of the complex to support all of the reactions needed to replicate SV40 origin containing DNA in vitro, throughout each step of its purification, suggests that the interactions between the components of the DNA synthesome are specific and of sufficiently high affinity to maintain the replicative function of the complex.

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