Whole Gel Eluter Purification of a Functional Multiprotein DNA Replication Complex*

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Introduction

The process of DNA replication is an important regulatory point for modulating cell proliferation. The elucidation of the role played by the human DNA replication apparatus, and its components, in this regulatory process is anticipated to further our understanding of both normal and cancer cell proliferation. The concept that many enzymes and factors involved in the replication of mammalian DNA function together as an organized multiprotein complex has been supported by increasing evidence [reviewed in reference 1]. We have previously reported that a highly purified multiprotein form of DNA polymerase can be isolated from a variety of mammalian cell types and tissues.[2–6] We have shown that this multiprotein form of DNA polymerase, designated the DNA synthesome, is fully competent to support origin DNA sequence specific large T-antigen-dependent papovavirus DNA replication *in vitro*.[2–6] The DNA synthesome was purified from cells using a series of steps which included centrifugation, polyethylene glycol precipitation, ion-exchange chromatography, and density gradient sedimentation. The integrity of the DNA synthesome has been shown to be maintained after treatment with detergents, salt, RNase, DNase, chromatography on DEAE-cellulose (Whatman) and Q-Sepharose (Pharmacia), and following sedimentation in sucrose and glycerol density gradients, indicating that the ready co-purification of the proteins with one another was independent of nonspecific interaction with other cellular macromolecular components.[2–4] Native polyacrylamide gel electrophoresis (PAGE) of the synthesome from HeLa cells revealed the presence of several high molecular weight multiprotein species.[7] One of these complexes was readily recognized in western blot analysis by a monoclonal antibody against the DNA replication essential protein DNA polymerase α.[7] This DNA polymerase α containing complex was shown to have a high specific *in vitro* simian virus 40 (SV40) origin dependent DNA replication activity. We have recently found that Bio-Rad's Whole Gel Eluter can greatly aid in the purification of the DNA synthesome. What follows is the description of the conditions we developed for the isolation of the DNA synthesome using the Whole Gel Eluter.

Materials and Methods

CELL CULTURE

Suspension cultures of HeLa cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of calf and fetal bovine serum. Exponentially growing cells were harvested and washed three times with phosphate-buffered saline (PBS). The cells were then pelleted by low-speed centrifugation. The cell pellets were stored at -80 °C prior to initiating subcellular fractionation.

SYNTHESOME PURIFICATION PROCEDURES

The DNA synthesome was purified prior to the Whole Gel Eluter step essentially as described by Malkas *et al.*[2] and as outlined in Figure 1.

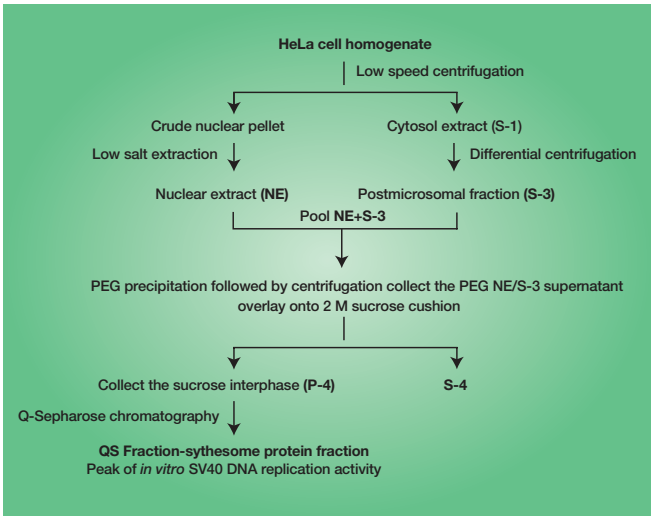

Fig. 1. *The flow diagram of the isolation scheme used to purify the mammalian cell DNA synthesome.*

NATIVE PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS AND ELECTRO-ELUTION

Four percent native polyacrylamide gels, 1.5 mm thick, and containing a 3.5% stacking gel were prepared with a 1.5 mm preparative comb using the Mini-PROTEAN® II gel apparatus (Bio-Rad). Sodium dodecyl sulfate (SDS) was excluded from these gels, as well as from the running and sample buffers. Five milligrams of the synthesome protein fraction, purified as described in Figure 1, was loaded onto the gel. Electrophoresis was initially started at 50 volts until the dye front entered the 4% separating PAGE gel, at which time the voltage was increased to 90 volts. Electrophoresis was continued until the dye front reached the bottom of the gel. Following electrophoresis, the PAGE gel was trimmed to fit onto the Mini Whole Gel Eluter as described in the Whole Gel Eluter instruction manual. The gel was then soaked in 20 mM HEPES, pH 7.5, for 10 minutes and layered onto the elution chamber core. The Whole Gel Eluter was assembled as described in the instruction manual, and elution of the resolved synthesome protein fraction from the 4% PAGE gel was carried out as described in the protocol provided with the apparatus. Twenty millimolar HEPES, pH 7.5, was used as the elution buffer in these procedures. The electro-elution was initiated at 60 mA for 1 hour, and then continued at 30 mA for an additional 2 hours. The proteins bound to the cellophane membrane at the end of elution were removed by reversing the polarity of the eluter cell and continuing electrophoresis for 30 seconds at 100 volts. The eluted fractions were harvested, with each fraction containing approximately 500 µl of liquid. These fractions were then analyzed by native PAGE and the presence of several enzymatic activities determined in order to assess whether the Whole Gel Eluter could be used for the purification of an intact and functional DNA synthesome.

NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS, SILVER STAINING

Fifty microliters of the electro-eluted fractions were resolved using a 4% native polyacrylamide gel. SDS was excluded from the gel, running buffer, and sample buffer. The gel was electrophoresed at 90 volts until the dye front reached the bottom of the gel. Following electrophoresis, the gel was silver stained using the Silver Stain Plus kit (Bio-Rad).

DNA POLYMERASE α ASSAY

DNA polymerase α activity was assayed with an activated calf thymus DNA template (Sigma Co.) according to published procedures,[2] using [^{32}P]dTTP in place of [^{3}H]dTTP in the assay.

DNA POLYMERASE δ ASSAY

DNA polymerase δ activity was detected essentially as described in reference 8, with some modification. The 50 µl assay mixture contained 50 ng poly(dA)/oligo(dT) (20:1), 0.25 µl [^{32}P]dTTP, 2.5 µl core buffer (10 mM $MgCl_2$, 10 µM TTP; 25 mM HEPES, pH 5.9, 200 µg/ml bovine serum albumin, 5% glycerol). The reaction was carried out at 37 °C for 15 minutes and the whole reaction mixture were spotted onto Whatman DE81 filters. The filters were then processed to quantify the amount of radiolabeled nucleotide incorporated into the DNA template.[10]

IN VITRO SV40 REPLICATION ASSAY

The assay was performed essentially as described in Malkas *et al*.[2]

Results

THE WHOLE GEL ELUTER CAN RAPIDLY AND EFFICIENTLY ELECTRO-ELUTE HIGH MOLECULAR WEIGHT PROTEIN COMPLEXES FROM NATIVE PAGE GELS

We have previously found that when the replication-competent DNA synthesome protein fraction, purified from human cells as outlined in Figure 1, was subjected to native PAGE, several distinct high molecular weight protein species were observed.[7] An example of a silver stained native PAGE resolution of the DNA synthesome protein fraction is shown in Figure 2, lane 5. We have recently identified the specific high molecular weight protein band that contains the fully functional DNA synthesome (Tom *et al.*, to be published elsewhere). The high molecular weight DNA synthesome protein band is indicated by an arrow in Figure 2, lane 5. The initial identification of the discrete DNA synthesome protein band in the native PAGE (Tom *et al.*, to be published elsewhere) required the time consuming procedure of first cutting the gel into small pieces that were then each individually placed into dialysis tubing and the synthesome protein subsequently electro-eluted from the gel using standard methods.[10] This tedious process of obtaining highly purified forms of the DNA synthesome from native PAGE has recently been greatly facilitated by the incorporation of the Whole Gel Eluter into the purification protocol.

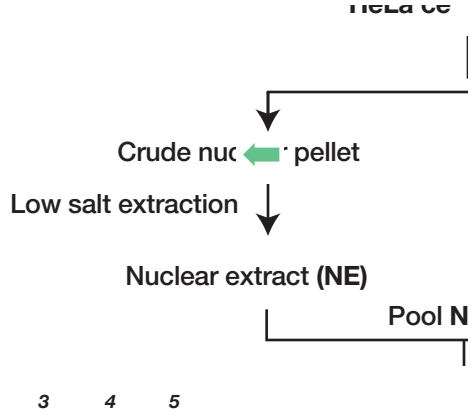

Fig. 2. *Native polyacrylamide gel analysis of electro-eluted fractions. The collected electro-elution fractions number 5, 9, 11, 13, were found to contain the most significant levels of protein when compared to the other electro-elution fractions. These fractions were then again subjected to native PAGE. The resolution of fractions 5, 9, 11, 13 is shown in lanes 1-4, respectively. Lane 5 contain the pattern of resolution of the QS protein fraction in a native PAGE.*

The DNA synthesome protein fraction was derived using the procedure outlined in Figure 1. The protein fraction was then resolved by native PAGE as described in the Methods. The native gel containing the resolved synthesome protein fraction was eluted using the Whole Gel Eluter as described in the Methods, and individual eluted fractions were collected. The electro-elution fractions found to contain significant levels of protein were resolved again by native PAGE. This native gel was then silver stained. Figure 2 clearly shows that the Whole Gel Eluter could be successfully used to elute the different protein complexes resolved by the native gel (Figure 2, lane 1–4).

THE DNA SYNTHESOME IS STILL FUNCTIONAL AFTER ITS ELECTRO-ELUTION WITH THE WHOLE GEL ELUTER

To determine if the incorporation of the Whole Gel Eluter into the synthesome purification protocol affected the function of the DNA synthesome, the electro-elution fractions were assayed for DNA polymerases α and δ and *in vitro* SV40 DNA replication activities. The results of these assays are shown in Figure 3, panels A–C. It was observed that the major peaks for the DNA polymerases α and δ and *in vitro* replication activities were all in electro-elution fraction 5. Fraction 5 was observed to also contain the high molecular weight protein band (Figure 2, lane 1) that has been recently identified as the replication-competent DNA synthesome (Figure 2, lane 5) (Tom *et al.*, to be published elsewhere). All together these results indicate that the Whole Gel Eluter can be used successfully in the purification of protein complexes.

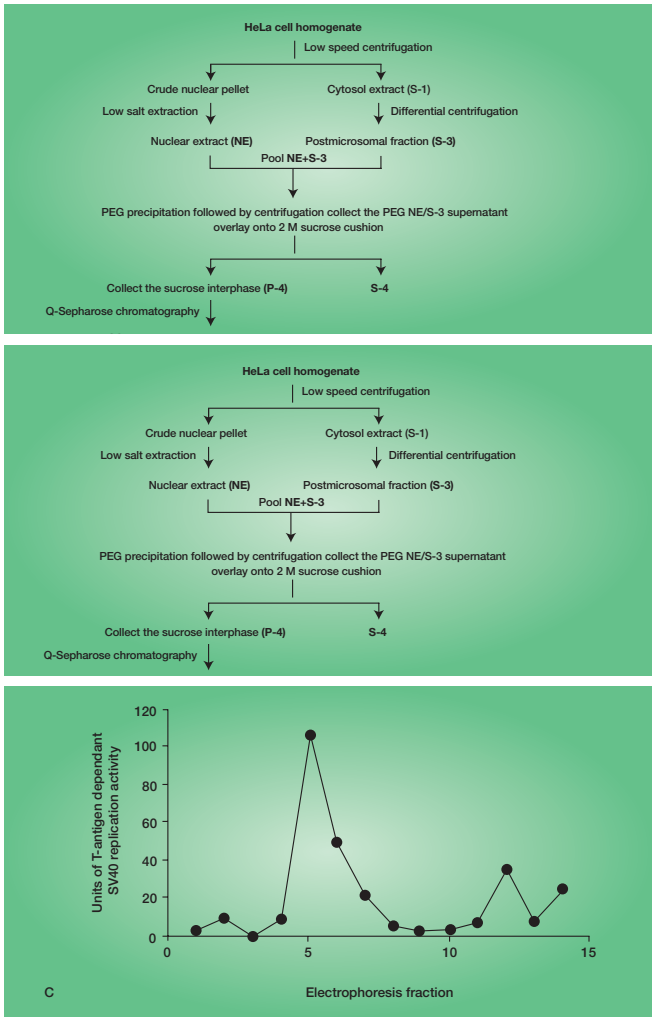

Fig. 3. *DNA replication and polymerase α and δ enzymatic activities of the electro-elution fractions. A. DNA polymerase α activity. One unit of DNA polymerase α activity equals one picomole of total dNMP incorporated into DNA for 30 minutes at 37 °C. B. DNA polymerase δ activity. One unit of DNA polymerase δ activity equals one picomole of dTMP incorporated into the template per 15 minutes at 37 °C. C.* in vitro *SV40 origin-dependent DNA replication activity. One unit of activity equals one picomole of total dNMP incorporated into DNA per hour at 37 °C.*

Discussion

The evidence supporting the role of multiprotein complexes in the replication of mammalian DNA has grown over the years.[1] The identification and characterization of the DNA replication machinery from the mammalian cell has fundamental relevance to a wide spectrum of basic and applied sciences. Once isolated and characterized, multiprotein DNA replication complexes may provide insight into the mechanisms involved in regulation of the cell cycle, apoptosis, and carcinogenesis. The data presented in this report show that the Whole Gel Eluter can be successfully used in the purification of an intact and functional DNA synthesome. The ease of use of the Whole Gel Eluter, combined with the excellent recovery of the synthesome from native gels, has saved the laboratory time which we have used more effectively toward the further characterization of the synthesome. We are also currently determining whether the lower molecular complex species identified by native PAGE (Figure 2, lanes 2–4) represent either subassemblies of the synthesome or denaturation products of the replication complex.

The Whole Gel Eluter can also be envisioned to be of potential use in the isolation of transcription and protein synthesis complexes resolved by native PAGE. From our own experience with the apparatus, we found that both the type of elution buffer and the buffer pH are critical features for the elution of a functioning protein complex. We found it necessary to switch from a Tris-based elution buffer to a HEPES-based system in order to significantly increase our recovery of a functional synthesome complex. However, despite the time and effort required to optimize the conditions needed to electro-elute the synthesome from native PAGE using the Whole Gel Eluter, this laboratory feels that the apparatus has greatly facilitated the isolation of a highly purified form of the DNA synthesome, and we have now incorporated the use of the apparatus as part of our standard synthesome purification protocol.

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