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Cofractionation of HeLa Cell Replication Proteins With *Ors*-Binding Activity

Marcia T. Ruiz, Christopher E. Pearson, Torsten Nielsen, Gerald B. Price, and Maria Zannis-Hadjopoulos

McGill Cancer Centre, Department of Medicine, McGill University, Montréal, Québec H3G 1Y6, Canada

Abstract *Ors* (origin enriched sequence) 8 is a mammalian autonomously replicating DNA sequence previously isolated by extrusion of nascent monkey (CV-1) DNA in early S phase. A 186 bp fragment of *ors* 8 has been identified as the minimal sequence required for origin function, since upon its deletion the in vivo and in vitro replication activity of this *ors* is abolished. We have fractionated total HeLa cell extracts on a DEAE-Sephadex and then on a Affi-Gel Heparin column and identified a protein fraction that interacts with the 186 bp fragment of *ors* 8 in a specific manner. The same fraction is able to support the in vitro replication of *ors* 8 plasmid. The ors binding activity (OBA) present in this fraction sediments at approximately 150 kDa in a glycerol gradient. Band-shift elution experiments of the specific protein-DNA complex detect by silver-staining predominantly two protein bands with molecular weights of 146 kDa and 154 kDa, respectively. The fraction containing the OBA is also enriched for polymerases α and δ, topoisomerase II, and replication protein A, (RP-A). © 1995 Wiley-Liss, Inc.

Key words: *ors*, replication origin, replication proteins, purification, HeLa cells, in vitro replication

The mechanism of regulation of chromosomal DNA replication in higher eukaryotic cells is poorly understood. In the yeast *S. cerevisiae*, a set of autonomously replicating sequences (ARS) are available that are able to confer a high frequency of transformation on plasmid DNA [Stinchcomb et al., 1979] and act as origins of replication in the chromosome [Huberman et al., 1987, 1988; Brewer and Fangman, 1987; Huberman et al., 1988; Dubey et al., 1991]. Sequence analyses of the ARS revealed a common 11 base pair sequence, referred to as the ARS core consensus sequence (ACS) [Broach et al., 1983; Newlon, 1988]. A detailed study of *ARS1* and *ARS121* has led to the identification of sequences necessary for the autonomous replication function [Marahrens and Stillman, 1992; Walker et al., 1991]. Proteins that are able to interact with these sequences have been identified. The OBF1 phosphoprotein (also known as ABF1 and BAF1) recognizes one of the auxiliary elements present in both *ARS1* and *ARS121* that lies within a 3′ region next to the ACS [Eisenberg et al., 1988; Francesconi and Eisenberg, 1989, 1991; Buchman et al., 1988, 1990; Sweder et al., 1988; Diffley and Stillman, 1988, 1989; Rhode et al., 1989; Halfter et al., 1989a,b]. A single-stranded DNA binding protein that recognizes the T-rich strand of the ACS has been reported [Hofmann and Gasser, 1991; Schmidt et al., 1991; Kuno et al., 1990]. Estes et al. [1992] were able to reconstitute in vitro a specific and stable multiprotein-DNA complex at the *ARS121* nuclear origin, while Bell and Stillman [1992] reported the purification of a multiprotein origin recognition complex (ORC) that is able to specifically recognize the double-stranded (ds) *ARS1* ACS in an ATP-dependent fashion. The genes encoding two of the subunits of the ORC have been isolated [Li and Herskowitz, 1993; Foss et al., 1993]. The functional characterization of both components supports the evidence that ORC plays an in vivo role in chromosomal replication, likely acting as an initiator protein in yeast [Bell et al., 1993].

The SV40 in vitro replication system, which mimics the in vivo SV40 DNA replication [Li and Kelly, 1984, 1985; Stillman and Gluzman, 1985; Wobbe et al., 1985], is a very useful model in the study of eukaryotic DNA replication. In this system, as in vivo, only a single viral pro-

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Address reprint requests to Maria Zannis-Hadjopoulos, McGill Cancer Centre, 3655 Drummond Street, Montréal, Québec H3G 1Y6 Canada.

tein, the SV40 large T-Antigen (T-Ag), is required for viral DNA replication, with all the other necessary factors being supplied by the host cell. This in vitro system has proven to be useful in the biochemical analysis of the proteins involved in the replication process. Fractionation of human cell extracts has identified several proteins that are necessary for complete in vitro SV40 DNA replication, which are believed to participate in the same process in the cell. Reconstitution experiments have demonstrated that, in the presence of ATP, only three proteins—the viral T-Ag, the single-stranded DNA-binding protein (RP-A or human SSB), and the DNA polymerase α—primase complex—are essential for origin recognition, unwinding of the double-stranded DNA, and synthesis of the first nascent RNA/DNA strands [Wobbe et al., 1987; Ishimi et al., 1988; Tsurimoto et al., 1990; Borowiec et al., 1990; Matsumoto et al., 1990; Weinberg et al., 1990, Bullock et al., 1991; Erdile et al., 1991; Murakami et al., 1992]. In mammalian cells, the protein(s) involved in chromosomal origin recognition and initiation of DNA replication have not been identified.

Our laboratory has isolated and cloned early-replicating origin-rich sequences (*ors*) from synchronized CV-1 (monkey) cells [Kaufmann et al., 1985]. These DNA sequences are capable of transient autonomous replication in vivo, upon transfection in monkey (CV-1 and COS-7) and human (HeLa) cells [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991], and in an in vitro replication system that uses HeLa cell extracts [Pearson et al., 1991]. Both in vivo and in vitro replication initiates in the *ors* and is semiconservative, bidirectional, sensitive to the presence of aphidicolin, and independent of SV40 T-Ag [Frappier and Zannis-Hadjopoulos, 1987; Pearson et al., 1991, 1994].

Among the functional *ors*, *ors* 8 has been analyzed extensively both in vivo [Frappier and Zannis-Hadjopoulos, 1987; Mah et al., 1993; Todd et al., 1994] and in vitro [Pearson et al., 1991, 1994]. Recently, an internal fragment (186 bp) of *ors* 8 was identified by deletion analysis as essential for its autonomous replication function [Todd et al., in press].

In this paper, we present evidence of a specific interaction between the 186 bp fragment of *ors* 8 and proteins present in HeLa cell extracts. Based on this, the purification of protein(s) that interact specifically with this fragment was initiated. The purified fractions obtained at each step of the purification were tested for their ability to bind the 186 bp fragment in a specific manner and to support in vitro replication of *ors* 8. The purification scheme is representative of three replicates, all of which gave the same results. A partially purified protein fraction was obtained that is able to preferentially recognize the 186 bp fragment of *ors* 8 and support the in vitro replication of an *ors* 8 plasmid template. This ors binding activity (OBA) sediments at approximately 150,000 in a glycerol gradient. In the pool containing the OBA, the cellular replication proteins DNA polymerase α, polymerase δ, topoisomerase II, and RP-A were also detectable.

MATERIALS AND METHODS

Cells and Plasmids

HeLa S3 cells were maintained in suspension in Eagle's minimal essential medium for suspension (SMEM) with 10% fetal calf serum. pBR322 and *ors* 8 plasmids were propagated in *Escherichia coli* HB101, as previously described [Pearson et al., 1991]. *Ors* plasmids are comprised of CV-1 monkey DNA sequences inserted into the *NruI* site of pBR322 [Kaufmann et al., 1985; Zannis-Hadjopoulos et al., 1985].

Preparation of DNA Fragments and End-Labeling

Ors 8 (GenBank accession number M26221) [Rao et al., 1990] plasmid DNA was used as template in PCR reactions to amplify the *ors* 8 fragment, as described previously [Mah et al., 1993]. Briefly, the *ors* 8 portion of the plasmid was amplified using external (pBR322-specific) primers (+ and −, sequence of nucleotide positions 954–968 of the top pBR322 strand and 994–979 of the bottom pBR322 strand, respectively). The amplified *ors* 8 was then digested with *NdeI* and *RsaI* to obtain the 186 bp subfragment. The digestion products were separated by 4% polyacrylamide gel electrophoresis (PAGE), the 186 bp band was excised, and the DNA was eluted by isotachophoresis [Ofverstedt et al., 1984]. A nonspecific competitor fragment, pBRfg, was prepared by PCR amplification of a pBR322 DNA sequence (nucleotides 860–1065 bp).

Prior to end-labeling, the 186 bp fragment was dephosphorylated at the 5′ ends with calf intestine alkaline phosphatase (New England Biolabs, Beverly, MA) and then end-labeled with

γ^{32}P-ATP (Amersham, Canada, Oakville, Ontario, Canada) using T4 Kinase (BRL, Gaithersburg, MD). Free nucleotides were removed by passage of the end-labeled DNA through Nick columns (Pharmacia, Piscataway, NJ).

Preparation of Cell Extracts

Extracts from HeLa S3 cells, adapted for suspension culture, were prepared as described before [Pearson et al., 1991]. For chromatographic purposes, nuclear and cytosolic extracts (total cell extracts) were mixed and dialyzed against buffer A (26 mM Hepes, pH 7.8, 82 mM potassium acetate, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 1.0 mM PMSF, 10 mM $Na_2S_2O_4$, 4% glycerol).

Column Chromatography

Total cell extracts (75 ml, 534 mg of protein) were applied to a DEAE-Sephadex A-50 column (Pharmacia) (75, 2.5 × 15.3) that had been previously equilibrated in buffer A. The flowthrough (FT) was collected, the column was further washed with three column volumes of buffer A, and the bound protein was eluted with a linear salt gradient of potassium acetate (0.082–1 M) in buffer A. The fractions collected from the elution gradient were pooled in four different pools (A, B, C, and D) according to their salt concentration, and each pool was individually dialyzed against buffer A to the same conductivity (see also Fig. 3). Pool B was then dialyzed against buffer B (0.01 M KH_2PO_4, pH 7.4, 0.15 M NaCl, 2.5 mM EDTA, 1.0 mM PMSF, 2 μg/ml aprotinin, 0.1 μM pepstatin A, 5% glycerol) and loaded (30 ml, 60.9 mg of protein) onto an Affi-Gel Heparin column (Bio-Rad, Hercules, CA) (44, 2.5 × 9). The FT was recovered, and the proteins bound to the matrix were eluted with a linear salt gradient of NaCl (0.15–1 M) in buffer B. The protein peak that resulted from this elution was concentrated, dialyzed against buffer B, and named pool E (see Fig. 3).

Glycerol Gradient Centrifugation

The procedure was essentially as described by Malkas et al. [1990]. A 10–40% glycerol gradient in buffer B was formed over a 0.5 ml cushion of 2 M sucrose. An aliquot of 100 μl (100 μg of protein) of pool E was loaded onto the gradient and spun for 16 h at 150,000*g* at 4°C in a SW50.1 rotor. After centrifugation, fractions of 100 μl were collected from the bottom of each tube. A set of molecular weight standard proteins—thyroglobulin 669,000 (19 S), aldolase 158,000 (7.3 S), hemoglobin 64,500 (4.46 S), and chymotrypsinogen A 25,000 (2.54 S)—was run in parallel gradients.

Gel Mobility Shift Assays

Gel mobility shift (DNA binding) assays were typically performed in 20 μl volume by incubating 5 μg of the total cell extracts, or other indicated amounts of protein from the different purification steps, with 0.2–1 ng (1.7–8.3 fmols) of end-labeled 186 bp DNA fragment. Reactions were carried out in binding buffer (10 mM Tris-HCl, pH 7.5, 80 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1% Triton X-100, 4% glycerol), in the presence of 1 μg of double-stranded (ds) poly(dI-dC) (Pharmacia), used as nonspecific competitor. After incubation for 30 min on ice, the reaction mixture was analyzed by 4% polyacrylamide gel electrophoresis (PAGE); the gel was then dried and exposed for autoradiography.

Band-Shift Elution of Proteins Bound to the 186bp Fragment

Band-shift reactions were performed and visualized as above. The various protein-DNA complexes were excised from the gel, and the proteins and the DNA were eluted by isotachophoresis and analyzed by SDS-PAGE (8%) [Laemmli, 1970], using the Bio-Rad minigel casting system. Control reactions, which were performed in the presence of all the components except DNA (protein control), as well as the unbound DNA alone, were run in parallel. The proteins were visualized by silver-staining of the gel, as described by Morrissey [1981].

Immunodetection Experiments

Western blotting experiments were performed essentially as described [Burnette, 1981]. Polyacrylamide gel electrophoresis in denaturing conditions was performed as previously described [Laemmli, 1970], using 20 μg per lane of each of the various protein fractions obtained during the purification. Proteins were electrotransfered to Immobilon-P (Millipore, Bedford, MA) and probed with the different primary antibodies. Except for topoisomerases I and II, a secondary rabbit antimouse antibody (Organon Teknika, Durham, NC) was also used. The blots were then developed by incubation with

I^{125} conjugated protein A (Amersham). The membranes were exposed to a Fuji imaging plate and visualized in a Fujix BAS 2000 Analyzer.

To detect the RP-A protein a monoclonal antibody against its 70 kDa [Kenny et al., 1990] subunit was used (kindly provided by Dr. M. Wold, University of Iowa, Iowa City, IA). The antipolymerase δ monoclonal antibody [Yang et al., 1992] was a gift from Dr. M. Lee (University of Florida, Miami, FL) and Dr. L. Malkas (University of Maryland, Baltimore, MD). Monoclonal antibodies against polymerase α (SJK 287-38 and SJK 237; ATCC, Rockville, MD) were kindly provided by Dr. E. Faust, Lady Davis Institute (Jewish Hospital, Montreal) and Dr. L. Malkas, respectively. The anti-RP-C monoclonal antibody (RFC 19, directed against the 140 kDa subunit) (personal communication) was a gift from Dr. B. Stillman (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). A human polyclonal antibody against topoisomerase I (TopoGen), a rabbit polyclonal antibody against human topoisomerase II (TopoGen), and an anti-PCNA monoclonal antibody (Boehringer Mannheim, Mannheim, Germany) were also used.

In Vitro DNA Replication

In vitro replication activities were measured essentially as previously described [Pearson et al., 1991], with the following modifications. Reactions were carried out in a total volume of 50 μl containing 4 μg or 8 μg of protein, either from total cell extracts or from the different pools obtained at each purification step, and 50 ng or 100 ng of *ors* 8 or pBR322 plasmid DNAs as template. The reaction products were electrophoresed on 1% agarose gels at 2.5 v/cm in TAE buffer.

Protein concentration was determined by the method of Bradford [1976] and the Nucleic Acid Soft-Pac module from a DU-65 Spectrophotometer (Beckman, Mississauga, Ontario, Canada).

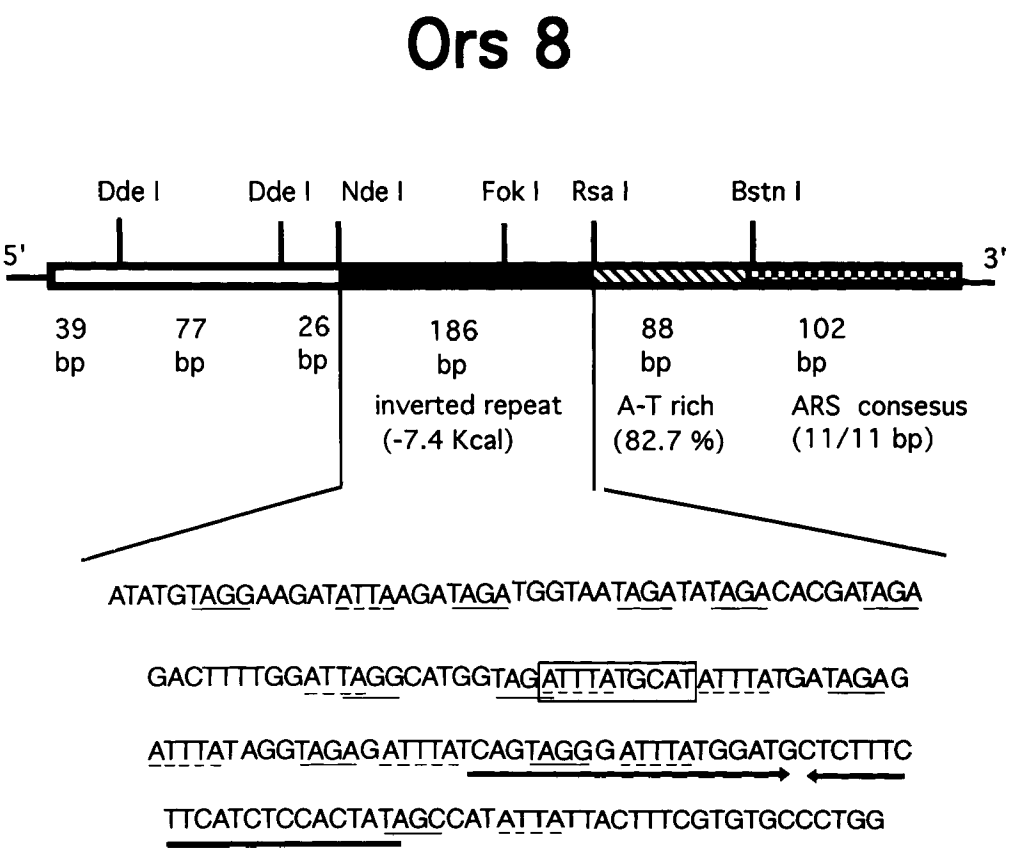

Fig. 1. *Ors* 8 map with sequence of the 186 bp fragment underneath. The 186 bp fragment contains repeats of TAGA, TAGG, TAGC (underlined). The ATTA and ATTTA motifs are commonly found in matrix attachment sites, at origins of replication, and at the recognition sites for homeotic proteins (dashed line). An imperfect inverted repeat (12 bp × 13 bp, 19 bp intervening sequence) is represented by inverted arrows. The OCT-1 binding site, ATTTATGCAT, containing three mismatches, is boxed.

RESULTS

Ors 8, an autonomously replicating monkey DNA sequence activated at the onset of S phase [Kaufmann et al., 1985; Zannis-Hadjopoulos et al., 1985], has sequence characteristics that are frequently present at origins of replication, such as inverted repeats (IRs), AT-rich regions, consensus sequence for matrix or scaffold attachment (MAR/SAR), and transcriptional regulatory elements (summarized in Fig. 1). The minimal sequence required for *ors* 8 function is an internal region of 186 bp [Todd et al., in press], which contains an imperfect inverted repeat sequence (12 bp × 13 bp, 19 bp intervening sequence), an imperfect (7 of 10 bp) recognition site for OCT-1 [Herr et al., 1988], and ATTA/ATTTA motifs that are frequently present in matrix attachment sites (MAR), in recognition sequences for the homeotic proteins, and in origins of replication [Boulikas, 1992, 1993] (Fig. 1). Deletion of this fragment eliminates the replication activity of *ors* 8, both in vivo and in vitro [Todd et al., in press].

Specific Ors Binding Activity (OBA) in HeLa Cell Extracts

Binding activity for the 186 bp fragment of *ors* 8 in total HeLa cell extracts was detected by the gel retardation assay in the presence of excess nonspecific competitor poly (dI-dC) (Fig. 2). Three major protein-DNA complexes (1, 2, and 3) were detectable.

The specificity of the binding activity for the 186 bp fragment was tested in a series of competition binding assays using either unlabeled 186 bp fragment or a 206 bp fragment of pBR322 (pBRfg), with no sequence homology to the 186 bp fragment, as specific and nonspecific competitors, respectively (Fig. 2). Complex 1 formation appeared to be due to nonspecific binding, as it was abolished by the presence of fiftyfold molar excess of either the nonspecific or specific competitor. This complex appeared to be unstable, as it was not detected in all band-shift experiments (data not shown; see Fig. 5). Complex 2 was specifically competed by fiftyfold molar excess of cold 186 bp fragment but was not competed by equal or higher amounts (>500-fold molar excess) of pBRfg. In contrast, complex 3 was intermediate in binding efficiency compared to complexes 1 and 2. Complex 3 was competed by tenfold molar excess of cold 186 bp fragment and was also competed by 250-fold molar excess of pBRfg. Finally, a much less prominent, slower migrating complex was also detectable above complex 3, which was competed by tenfold molar excess of cold 186 bp fragment and not by the nonspecific competitor pBRfg.

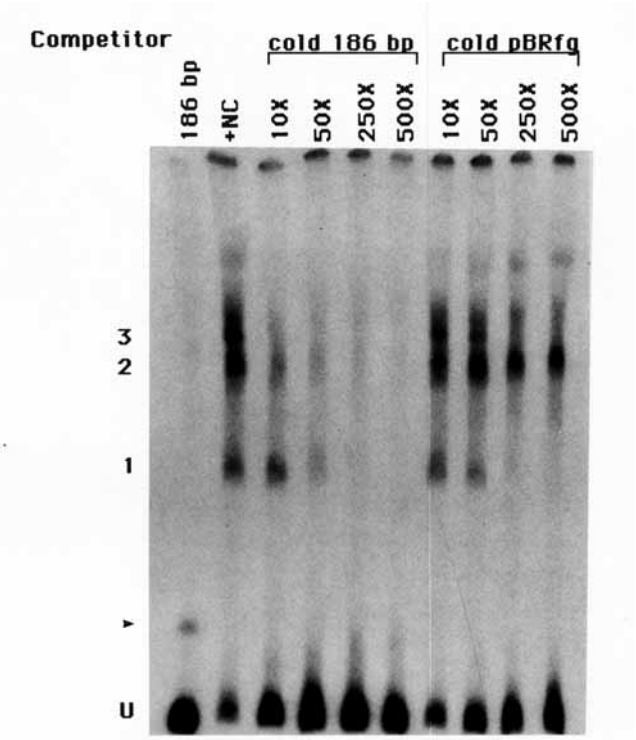

Fig. 2. Competition of DNA band-shift complexes in total cell extracts. Band-shift reactions containing 1 ng of radioactively labeled 186 bp fragment from *ors* 8 and 7 μg of protein from the total extracts (NC) were performed in the presence of increasing amounts of specific (186 bp) and nonspecific (pBRfg) cold competitors. The unbound DNA (U) and the most prominent protein-DNA complexes (1, 2, and 3) are labeled. The arrowhead indicates the 186 bp secondary structure (see text for details).

The slower migrating band (arrowhead) seen in the lane loaded with the unbound 186 bp DNA fragment (U) resulted from the formation of a secondary structure induced in the process of purification of the fragment (data not shown); this phenomenon has also been previously reported by others [Svaren et al., 1987] for fragments containing inverted repeats. This anomalously migrating minor band was also bound; however, its binding was not competed by either the specific or nonspecific competitors, as would be expected for binding that is structure-specific [Frappier et al., 1987].

Localization of the OBA and In Vitro Replication Activity in Fractionated Extracts

In order to purify the observed specific *ors* binding activity, total HeLa cell extracts were

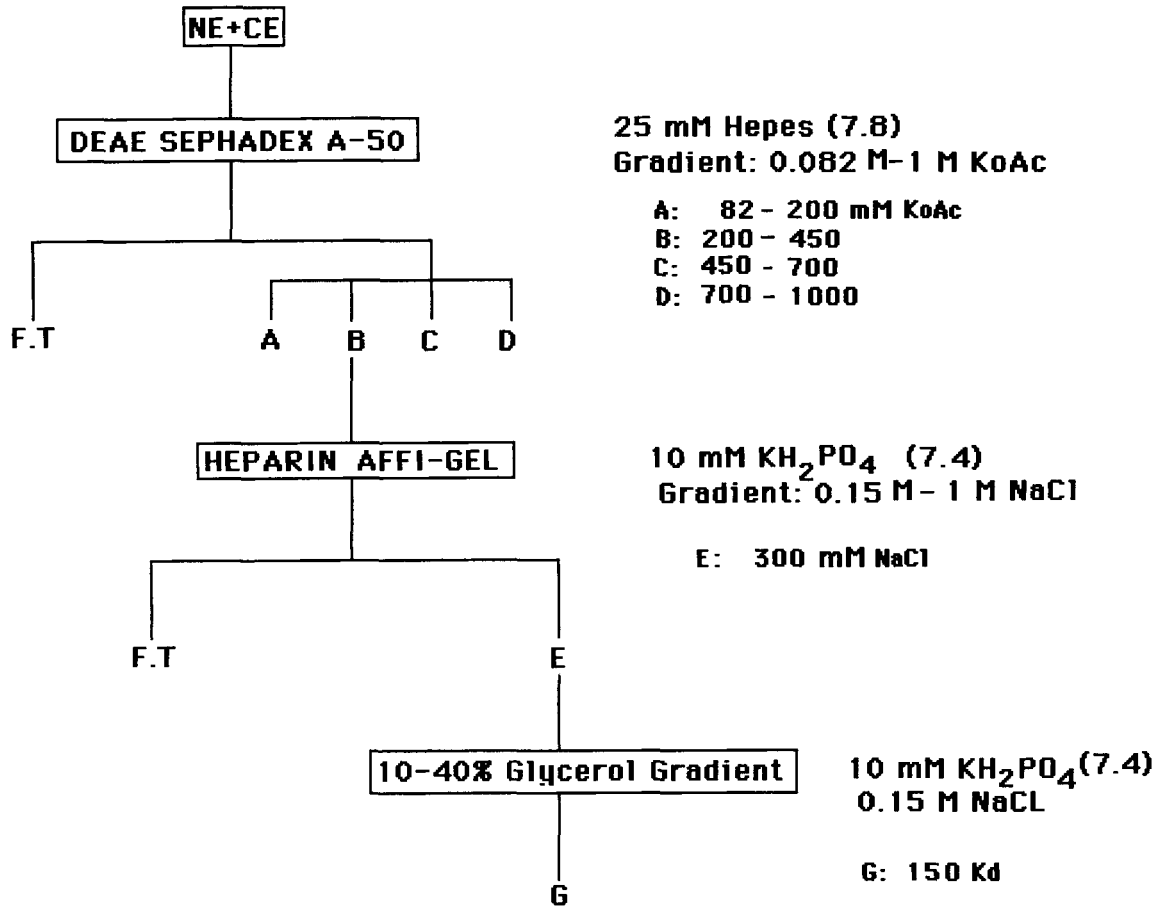

Fig. 3. Fractionation protocol of the OBA details of the fractionation steps are given in Materials and Methods. Pools obtained in each purification step are indicated to the right in the figure.

fractionated initially through a DEAE-Sephadex A-50 column into five components (Fig. 3); the flowthrough (FT) fraction was collected immediately after applying the sample onto the column, while the proteins retained by the matrix were eluted with a linear salt gradient (0.082–1 M potassium acetate), as described in Methods (Figs. 3, 4). The protein elution (Fig. 4A) and the DNA binding activity (Fig. 4B) profiles of every second fraction were assessed. A peak of binding activity for the 186 bp fragment of *ors* 8 was eluted in the gradient fractions 71–95 (Fig. 4B), giving rise to distinct protein-DNA complexes. Some, albeit different, binding activity could also be observed in the FT fraction. Fractions from the gradient were then pooled according to their salt content into four different protein pools, named A, B, C, and D (Fig. 3).

The four pools were dialyzed to the same salt content (82 mM potassium acetate) and were assayed for DNA binding activity to the 186 bp fragment of *ors* 8 (Fig. 5); total cell extract (NC) was used as control. All binding assays were performed using 5 μg and 10 μg of total protein. A very strong OBA could be detected in both the FT fraction and in pool B (Fig. 5A). The OBA in pool B is similar to that seen in the total cell extracts (NC) and is enriched for complex 2 (arrowhead), which is specifically competed by the 186 bp fragment (see Fig. 2). The FT fraction does not give rise to this complex, and, unlike pool B, its binding activity produces a smear on the gel rather than a single, well-defined protein-DNA complex. In pool A, a weaker binding activity is detected, which gives rise to less well-defined complexes and is clearly different from the pattern observed with either pool B or FT. Finally, pool C showed a very weak binding activity for the 186 bp fragment, while pool D had no DNA binding activity, even when very large amounts of protein (10 μg) were used.

In order to correlate the observed OBA with in vitro replication activity [Pearson et al., 1991],

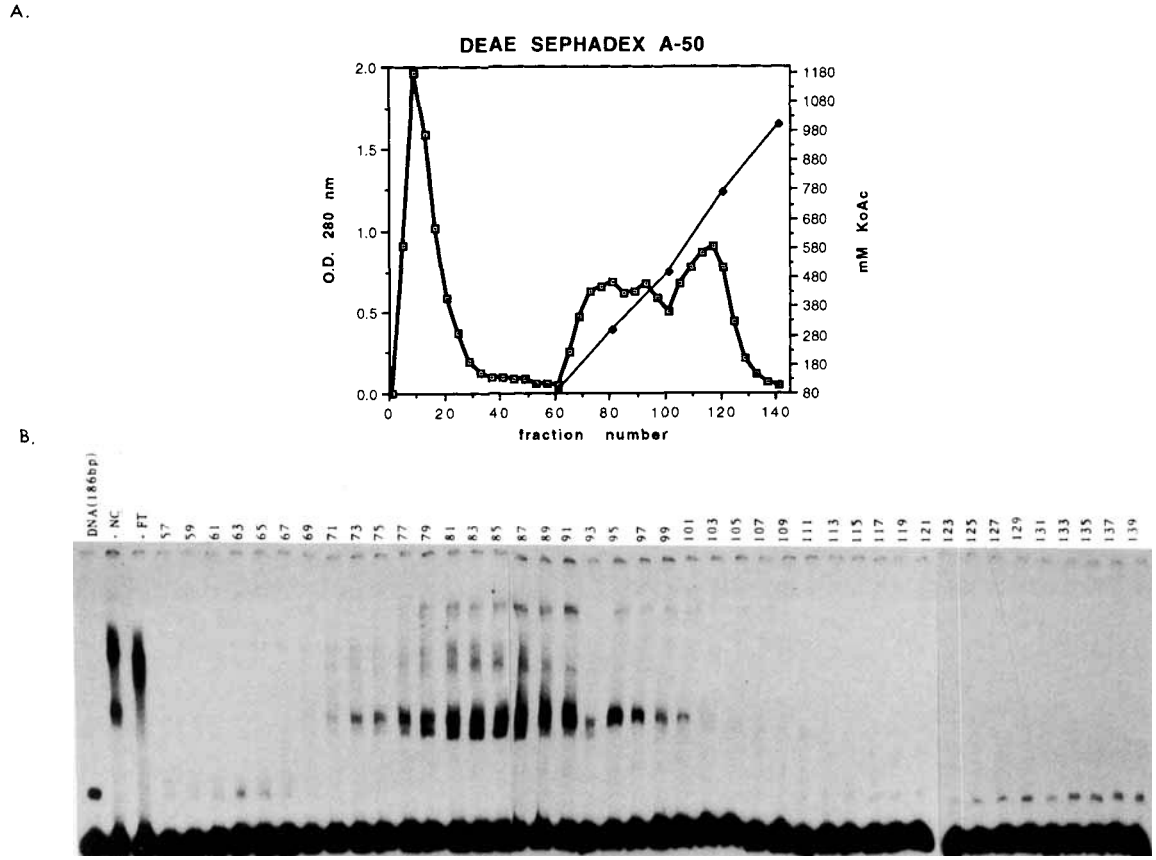

Fig. 4. DEAE-Sephadex A-50 fractionation. **A:** Profile of DEAE-Sephadex A-50 column. OBA was fractionated on DEAE-Sephadex as described in Materials and Methods. The protein absorbance at 280 nm (dotted squares) and the potassium acetate molarity (solid squares) are shown. **B:** The flowthrough pool and every second fraction eluted with the potassium acetate gradient were tested with the 186 bp fragment in gel retardation assay. The DNA probe alone and in the presence of total extracts (NC) is shown.

we assessed the ability of the FT fraction and pools A, B, C, and D, to support in vitro DNA replication of *ors* 8 plasmid (Fig. 5B). The FT fraction was as capable of sustaining in vitro replication of *ors* 8 as were the total cell extracts (NC), giving rise to supercoiled (form I), relaxed circular (form II), and linear (form III) *ors* 8 DNA products in addition to the slower migrating replicative forms. The four pools (A–D) were less efficient in supporting the in vitro replication of *ors* 8, and there was variability among them; of the four, pool B appears to be the most efficient at replicating *ors* 8 in vitro.

In view of its strong OBA, giving rise primarily to specific complex 2, and its ability to replicate *ors* 8 in vitro, pool B was used in the subsequent purification steps.

Heparin Affi-Gel and Glycerol Gradient

Pool B from DEAE column was loaded onto a Affi-Gel Heparin column. The FT fraction was recovered, and the proteins bound to the matrix were eluted with a linear salt gradient of NaCl (0.15–1 M) (Figs. 3, 6A). The OBA was eluted at 0.3 M NaCl as a single, broad peak (fractions 41–51) (Fig. 6B, Gradient), while it was absent from the FT fractions (Fig. 6B). Similar complexes (lanes 40–51) to those generated by pool B before (lane B) and after dialysis (lane B dial.) against buffer B were enriched in this step.

The fractions from the Heparin column that were enriched for OBA were pooled (pool E) (see Fig. 3). Pool E was subsequently sedimented onto a preformed 10–40% glycerol gradient (Fig. 7A) that had been calibrated with a set of known molecular weight markers. The gradient was fractionated, band-shift reactions were performed on alternate fractions (Fig. 7B), and the peak OBA was detected in fraction 26–27, corresponding to a molecular weight of approximately 150,000. Fractions 21–27, which were enriched for OBA, were pooled (pool G) (see Fig.

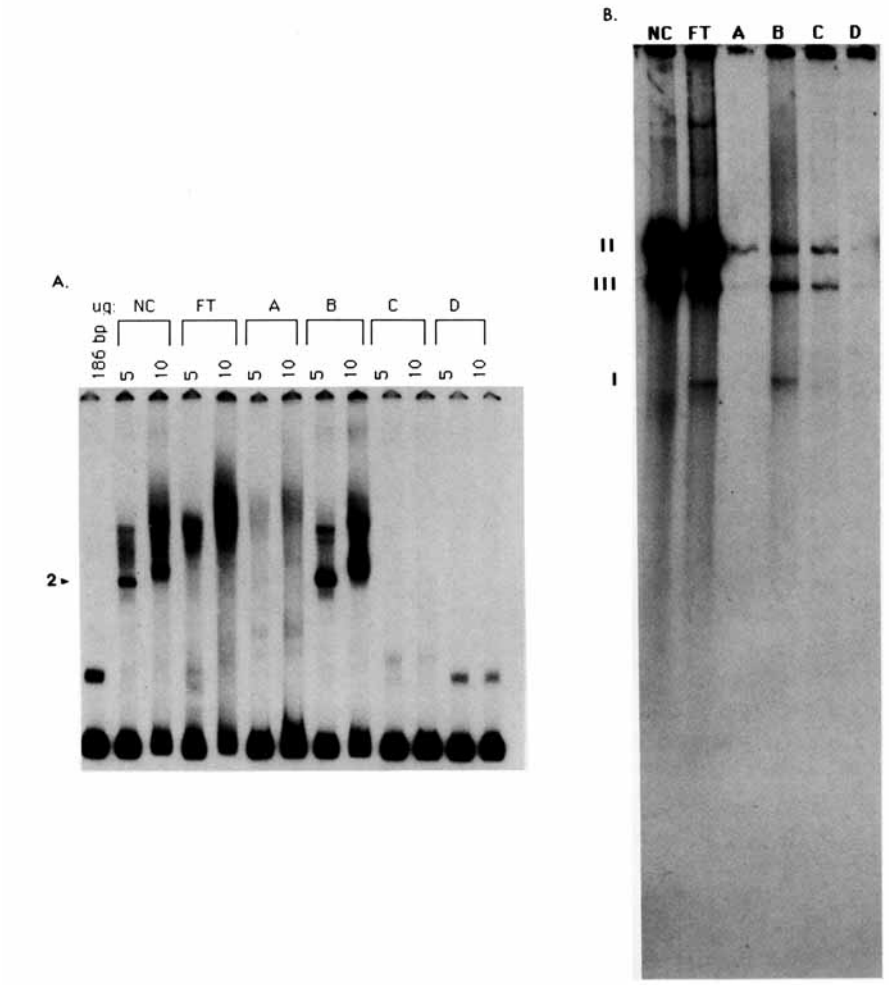

Fig. 5. Correlation between OBA and in vitro replication activity in the fractionated extracts. **A:** Protein (5 and 10 μg) from total cell extracts (NC) and from pools FT, A, B, C, and D prepared from the DEAE-Sephadex A-50 column were assayed for binding activity with 1 ng of the 186 bp fragment. **B:** Eight micrograms of protein from total cell extracts (NC) and from pools FT, A, B, C, and D was tested for in vitro replication activity in the presence of 100 ng of the *ors* 8 template. The migration position of forms I, II, and III on 1% agarose gel is shown.

3). To demonstrate that the recognition of the 186 bp fragment of *ors* 8 by the proteins present in pool G is specific, we performed competition binding assays. All visible complexes were specifically competed by cold 186 bp fragment but not by the nonspecific cold pBRfg fragment (data not shown).

OBA Is Enriched Through Purification

In order to monitor the extent of the enrichment in OBA through the purification procedure, a binding assay was performed using increasing amounts (50, 500, and 1,000 ng) of protein from each different pool (i.e., total extracts (NC) and pools B, E, and G) (Fig. 8). A complex detectable as a doublet, which migrates similarly to complex 2 (Figs. 2, 5), was enriched as the purification proceeded from total cell extracts (NC) through to pool G (Fig. 8). The protein(s) that gives rise to the slower migrating complex 5 (Fig. 8) became evident as three distinct bands in pool G and was only detectable at concentrations of 1 μg of protein. A faster migrating complex 4 (Fig. 8) is greatly enriched in pool G and can be detected with amounts of protein as low as 50 ng. The same complex 4 can also be detected in pool E with 500 ng of protein. This faster migrating complex could not be reproducibly observed in replicate purification attempts and may represent a degradation product.

The enrichment of the OBA through the different purification steps was quantitated by densitometry scanning of the different complexes that

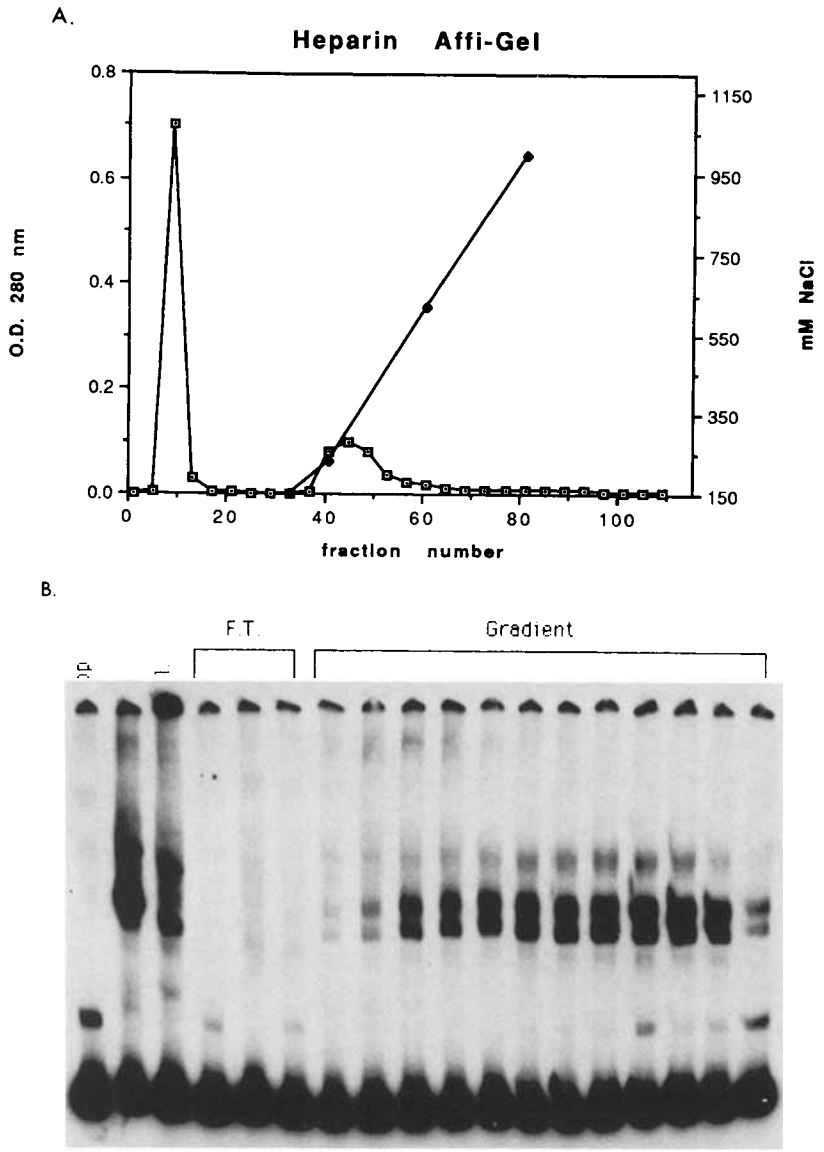

Fig. 6. Heparin Affi-Gel column. Pool B from the DEAE-Sephadex A-50 column was loaded onto a Heparin Affi-Gel column. **A:** Protein profile of the Heparin fractionation. The OBA was fractionated on a Heparin column as described in Materials and Methods. The protein absorbance at 280 nm (dotted squares) and the NaCl molarity (solid squares) are shown. **B:** Three fractions from the flowthrough (FT) and the protein peak eluted at 300 mM NaCl (pool E) were assessed with the 186 bp fragment in band-shift assay. The DNA probe alone and in the presence of pool B dialyzed against the DEAE Sephadex (B) and Heparin (B dial) buffers are shown.

were observed in the total extracts (NC) and the three pools (B, E, and G) from Figure 8. In pool G, at least a fortyfold enrichment of OBA was attained (Fig. 8 graph).

The degree of OBA purification at each step was also measured in terms of amounts of protein recovered (data not shown). Since the degree of protein purification in pool G was approximately 1,000-fold, it is likely that some origin binding activity was lost due to denaturation during various purification steps.

Proteins Recovered by Band-Shift Elution

The range of proteins in total cell extract (NC) and pools B, E, and G was analyzed by SDS-PAGE (8%) followed by silver-staining (Fig. 9A). At least five bands can be detected as enriched in

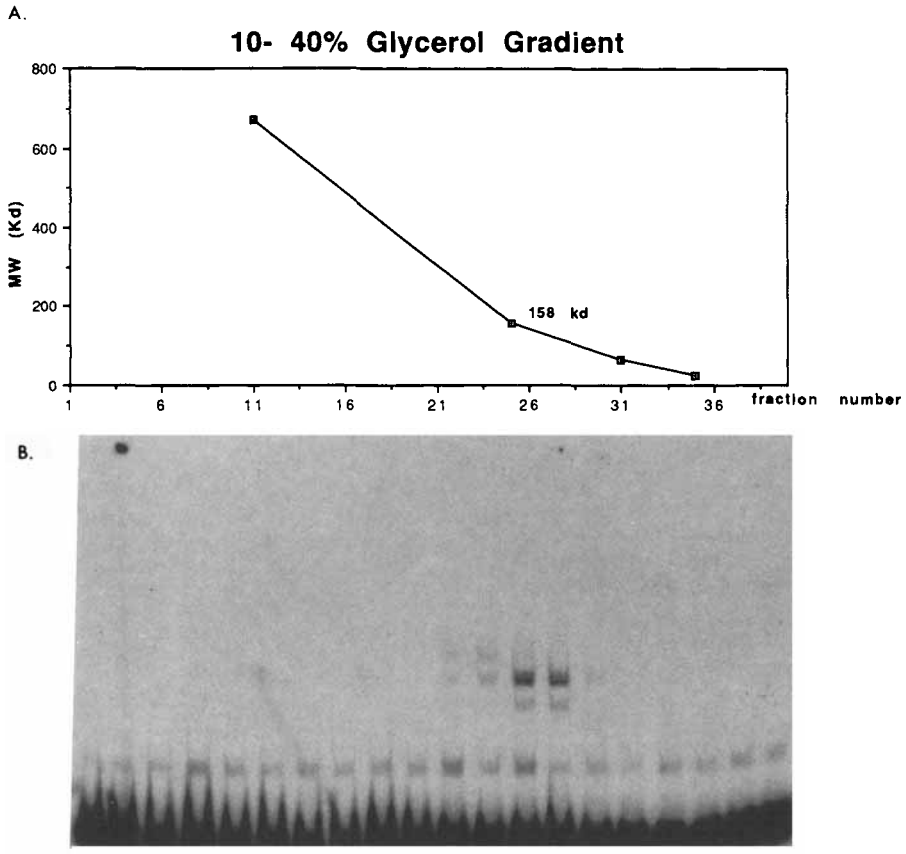

Fig. 7. Glycerol gradient sedimentation. Pool E from Heparin Affi-Gel column was loaded onto a 10–40% glycerol gradient. **A:** Sedimentation profile of protein markers used in the calibration of the gradient (thyroglobulin: 669,000; aldolase: 158,000; hemoglobin: 64,500; and chymotrypsinogen A: 25,000). **B:** Every second fraction from the gradient was tested for the binding activity to the 186 bp fragment. Fractions (21–27) with positive band-shift activity were pooled, concentrated, and dialyzed (pool G).

pool G, ranging in molecular weights from approximately 50,000 to 160,000.

In order to determine the molecular weight of the protein(s) in pool G that interacted specifically with the 186 bp fragment of *ors* 8, band-shift reactions were performed, in which the various protein-DNA complexes (complexes 2, 4, and 5) and the unbound DNA (see Fig. 8) were excised from the gel and the proteins and the DNA were eluted by isotachophoresis and analyzed by SDS-PAGE (8%) (Fig. 9B). Control reactions performed in the absence of DNA (protein control lane), as well as the unbound DNA alone (186 bp), were run in parallel. The proteins again were visualized by silver-staining of the gel (Fig. 9B), while the DNA was visualized by exposing the gel overnight for autoradiography, to localize the position of the radioactively labeled DNA probe (arrow). Two prominent bands were detectable from such an analysis of band-shift complex 2, corresponding to molecular weights of approximately 146,000 and 154,000 (Fig. 9B, arrowhead). These molecular weights are consistent with the estimated molecular weight (approximately 150 kDa) of the OBA from the glycerol gradient. Two similar protein bands, which were enriched in pools E and G (Fig. 9A), were also detectable by SDS-PAGE analysis throughout the purification. Some other faint bands of lower molecular weight could also be detected. Since no bands were present in the protein control lane, they may represent proteins that were also bound to the DNA. The dark staining at the edges of the lane containing the proteins eluted from the excised complex 2 is believed to be due to micelle formation caused by the presence of SDS and/or ϵ-amino-N-caproic acid (used in isotachophoresis) and their reaction with reagents used in silver-staining. Such aberrations in staining have been observed in other similar band-shift and protein complex elution assays [Gaillard and

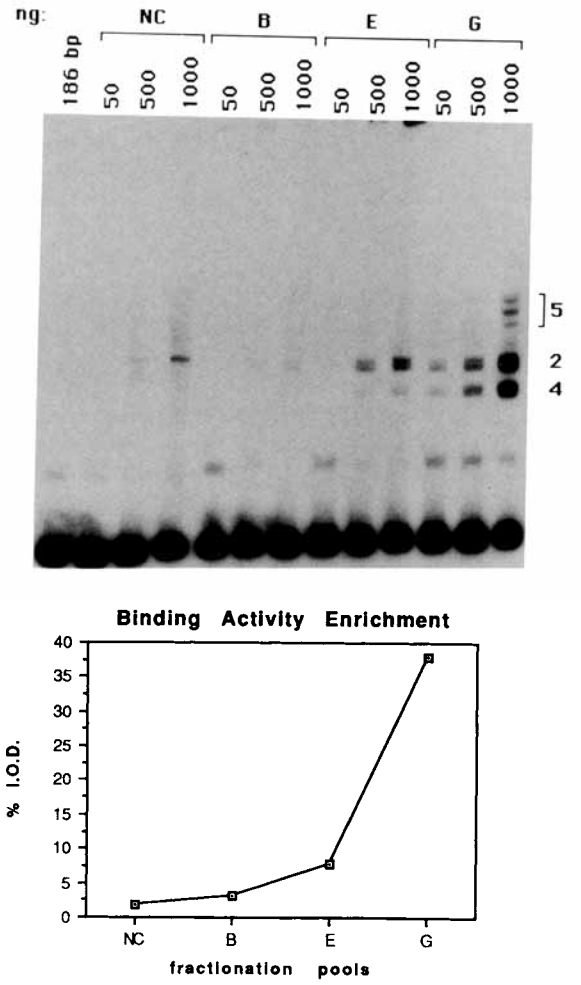

Fig. 8. Enrichment of the OBA through the purification. Increasing amounts (ng) of protein from total cell extracts (NC) and from the different pools used in the fractionation (B, E, and G) were tested in a gel retardation assay with 0.2 ng of radioactively labeled 186 bp fragment. The Bio-Imager quantitation is presented below, in percent of Integral Optical Density (%I.O.D) of the film.

Strauss, 1990]. Finally, no visible protein bands were recovered from complexes 4 and 5 (not shown).

Polymerases α and δ, Topoisomerase II, and RP-A Are Present in Pool G

To analyze which known replication proteins cofractionated with OBA, Western blot analyses of total cell extracts and pools A–G were performed using monoclonal antibodies against polymerases α and δ, RP-A, RP-C, and PCNA, as well as polyclonal antibodies against topoisomerases I and II (Table I). The predominant proteins cofractionating with OBA in pool G were polymerases α and δ and RP-A (Fig. 10), as well as topoisomerase II (not shown).

Pool G Is Able to Support In Vitro Replication of *Ors* 8

Finally, we tested the ability of pool G to support the in vitro replication of *ors* 8 plasmid. The products of the in vitro reaction were digested with *DpnI* as described previously [Pearson et al., 1991; Nielsen et al., 1994]. Typically, *DpnI*-resistant bands, corresponding to DNA forms II and III, were produced (Fig. 11), suggesting that pool G contains the minimal proteins necessary for sustaining the in vitro replication of *ors* 8, including PCNA and RP-C, albeit in levels below those that can be detected by Western blot (Fig. 10; Table I).

DISCUSSION

Molecular studies of DNA replication in well-defined systems have resulted in a general model for the initiation of bidirectional DNA synthesis [Bramhill and Kornberg, 1988]. This model suggests that initiation is mediated by successive assembly of nucleoprotein complexes at the origin of replication, involving binding of an initiator protein to origin DNA and subsequent duplex opening of adjacent sequences. The formation of each intermediate is accompanied by structural changes in the origin that culminate in strand separation, DNA unwinding, and initiation. The Ori core is the minimal essential cis-acting sequence required to initiate DNA replication, normally consisting of an origin recognition element (ORE), a DNA-unwinding element (DUE), and an A-T–rich region [for review see DePamphilis, 1993a,b].

Based on our observation that there exist specific cellular recognition proteins in HeLa cell extracts for the 186 bp fragment of *ors* 8, we have used it as a probe to monitor the purification of the DNA binding activity. In this study, we describe the partial purification of a protein fraction from HeLa cell extracts, which interacts specifically with the 186 bp fragment and is able to participate in supporting in vitro replication of *ors* 8 plasmid.

Multiple protein-DNA complexes are detected in total cell extracts (Fig. 2) and throughout the purification (Fig. 8). Many of the protein-DNA interactions are specific, since they can be competed only by the 186 bp *ors* 8 fragment and not by the nonspecific competitor pBRfg.

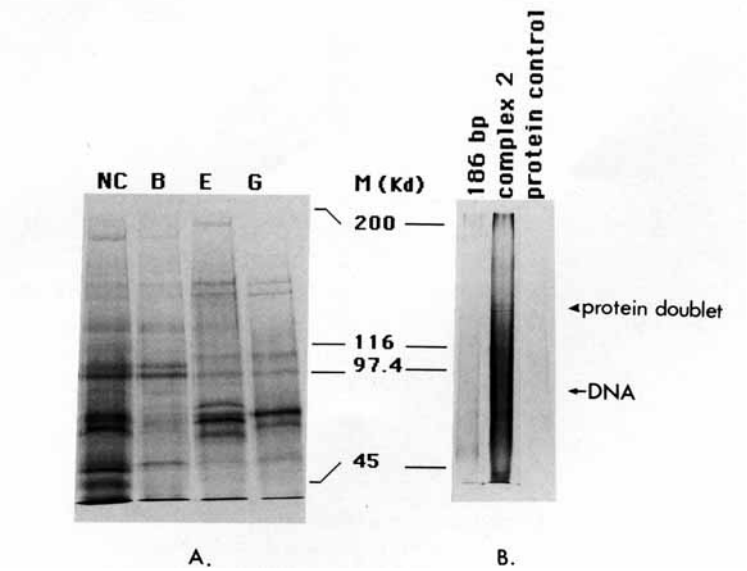

Fig. 9. SDS-PAGE analysis of the various pools through the purification protocol and of complex 2. **A:** Eight percent SDS-PAGE of the different fractionation pools (NC, B, E, G). **B:** Band-shift elution analysis of complex 2. Complex 2 (A, pool G) was purified by preparative gel retardation, eluted by isotachophoresis, and analyzed by 8% SDS-PAGE followed by silver-staining (Materials and Methods). A control reaction containing all the components except the specific DNA was loaded in parallel on the retardation gel; the area corresponding to complex 2 was excised from the gel, eluted, and analyzed as above (protein control). The location of the radioactive DNA (arrow), the protein doublet detected in complex 2 (arrowhead), and the position of the protein molecular weight markers (M, kDa) are indicated.

TABLE I. Summary of the Localization of Some Replication Proteins as Detected by Western Blot Experiments

Protein	Molecular weight[a] (kDa)	NC	A	B	C	D	E	G
PCNA[b]	36	+			+++			
Topo I[c]	100	+	++					
Topo II[c]	172	+	+	+			++	+++
Polymerase δ[d]	125	+	+	++			+++	+++
	50							
Polymerase α[d]	180		++	+			+++	+++
	70							
RP-C[e]	100–140						+	
	37–41							
RP-A[d]	70	+		++	+		+++	+++
	34							
	11							

[a]Molecular weight as characterized by Western blot analysis and previously reported [Hurwitz et al., 1990; Stillman, 1989].
[b]The Western blot was performed on 10% SDS-PAGE using 10 μg of protein. The transfer buffer contained 20% methanol.
[c]The Western blot was performed on 8% SDS-PAGE using 20 μg of protein. The transfer buffer contained 5% methanol + 0.05% SDS. No secondary antibody was used.
[d]The conditions are described in Figure 10.
[e]The Western blot was performed on 8% SDS-PAGE using 20 μg of protein. The transfer buffer contained 5% methanol + 0.05% SDS.

In band-shift elution experiments of the specific complex 2, which is highly enriched through the purification, we detected, by silver-staining, two major protein bands with molecular weights of 146,000 and 154,000, respectively (Fig. 9B, arrowhead). Two protein bands of similar molecular weight were enriched in pools E and G (Fig. 9A). SDS-PAGE performed under reducing conditions showed the same pattern of protein bands (data not shown). The identity of these

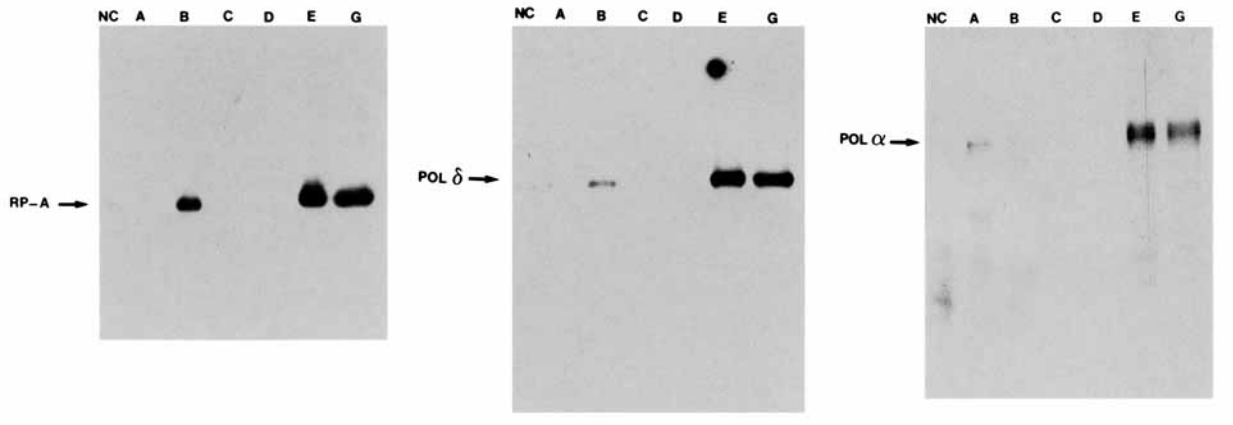

Fig. 10. Western blot analysis. Protein (20 μg) from total cell extracts (**NC**) and from pools (**A, B, C, D, E, and G**) was loaded on 10% PAGE for RP-A and on 8% PAGE for polymerases α and δ. The proteins were transferred to an Immobilon-P membrane for 2 h at 40 v in transfer buffer containing 10% methanol for RP-A and 5% methanol + 0.05% SDS for polymerases α and δ. The blots were incubated with the specific primary antibodies and developed with a secondary rabbit antimouse antibody, followed by the incubation of the membrane with protein A-I^{125}. For DNA polymerase α the Western blot performed with the SJK 237 monoclonal antibody is shown.

proteins is unknown; they may either represent distinct proteins or be due to microheterogeneity caused by posttranslational modification differences or to proteolytic digestion during extraction and purification. A precedent for such differences in replication proteins has been observed for polymerase α, in which the apparent molecular weight for the catalytic polypeptide ranges from 125,000–180,000 [So and Downey, 1992]. No protein bands could be detected from band-shift elution of the other complexes; it is conceivable that the amount of protein participating in those interactions may be below the threshold of detection in the conditions that were used.

The molecular weights (146,000 and 154,000) of the two major protein bands recovered after the band-shift elution of complex 2 are in agreement with the estimation of size (approximately 150,000) based on sedimentation in a glycerol gradient. From these results we conclude that at least two proteins of molecular weight of 146,000 and 154,000, respectively, are involved in the recognition of the minimal origin sequence fragment (186 bp) of *ors* 8.

Western blot experiments detected the presence in pool G of polymerases α and δ, topoisomerase II, and the replication factor A (RP-A). These four proteins have cofractionated throughout the purification procedure. Dornreiter et al. [1990, 1992] reported the specific physical interaction between RP-A and DNA polymerase α, both of which interact with the SV40 large T-antigen. They proposed that these proteins assemble at the SV40 origin of replication in the presence of ATP to form a specific initiation complex. By analogy, it is conceivable that RP-A and DNA polymerase α interact with a putative and yet unidentified cellular initiator protein(s) and assemble at the Ori core element. Significantly, pool G, which contains these two proteins, interacts specifically with the 186 bp fragment of *ors* 8, which has been found to act as the minimal sequence required for the autonomous replication function of *ors* 8, in vivo and in vitro [Todd et al., in press].

PCNA, an accessory protein of polymerase δ, and RP-C are necessary for elongation of SV40 DNA replication in vitro but are not required for its initiation [Stillman, 1989]. Both these proteins are thought to play a role in the control of the replication fork movement [Stillman, 1994]. Since we have previously shown that the in vitro system used in this study can specifically initiate and complete one round of semiconservative replication of *ors* 8 plasmid [Pearson et al., 1991, 1994; Todd et al., in press], we surmise that both PCNA and RP-C must be present in pool G, albeit in levels lower than can be detected by Western blot analysis.

The molecular weight of PCNA, a homodimer of 37 kDa [Hurwitz et al., 1990; So and Downey, 1992], is within the range of the molecular weights represented in pool G (Fig. 9A), as is that of RP-C, which consists of a complex of polypeptides that migrate in two clusters on SDS-PAGE (37–41 kDa and 100–140 kDa) [Tsurimoto and Stillman, 1989].

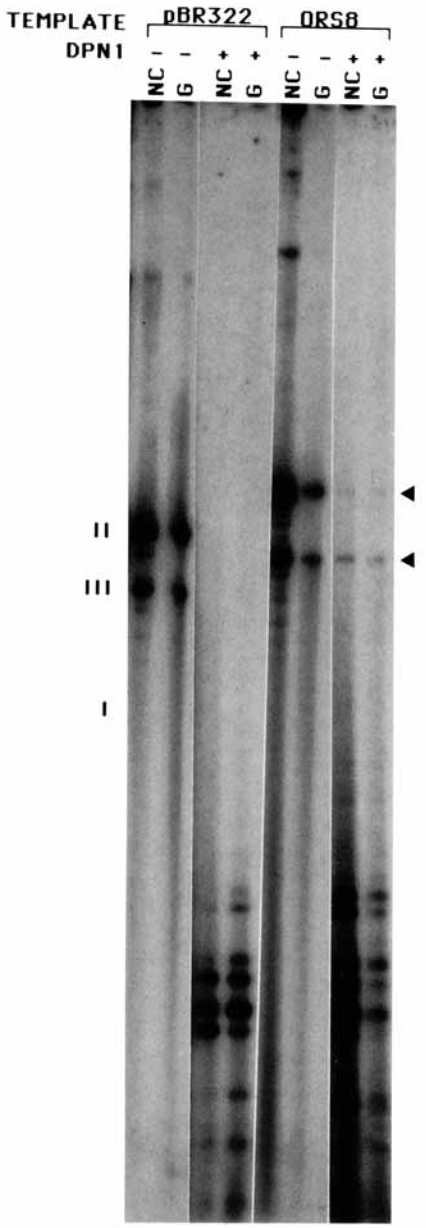

Fig. 11. Pool G is able to support in vitro replication of *ors* 8 plasmid. Protein (4 μg) from the total extracts (NC) and pool G was tested for in vitro replication activity in the presence of 50 ng of pBR322 plasmid or pBR/*ors* 8 plasmid as template. The products were divided into two equal parts and analyzed by electrophoresis on 1% agarose gel either undigested (−) or after digestion by *DpnI* (1 U) for 6 h at 37°C (+). The migration position of forms I, II, and III is shown. The *DpnI*-resistant bands are indicated by arrowheads.

In our study, pool G gave rise to multiple protein-DNA complexes (Fig. 8, complex 5), suggesting either the involvement of different proteins in the interaction with the 186 bp fragment of *ors* 8 or protein-protein interactions. The possibility of multimeric binding or the formation of a multiprotein complex that includes polymerases α and δ, topoisomerase II, RP-A, and/or the OBA at the *ors* cannot be excluded. Experiments are under way to further characterize the protein(s) which represents the OBA in pool G.

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