JOURNAL OF VIROLOGY, Apr. 1995, p. 2341–2350 Vol. 69, No. 4
0022-538X/95/$04.00+0

Bovine Papillomavirus E1 Protein Binds Specifically DNA Polymerase α but Not Replication Protein A

CATHERINE BONNE-ANDREA,* SABINE SANTUCCI, PHILIPPE CLERTANT, AND FRANÇOISE TILLIER

Centre de Biochimie, Unité 273 de l'Institut National de la Santé et de la Recherche Médicale (INSERM), Nice, France

Received 28 September 1994/Accepted 13 January 1995

Extracts prepared from either mouse cells or monkey cells were examined for the ability to support in vitro bovine papillomavirus type 1 (BPV1) DNA replication, and they were used in parallel as a source of host replication proteins for affinity chromatography. DNA synthesis exhibited an absolute requirement for BPV1 E1 protein. In contrast to previous observations, we found that low levels of E1 were highly efficient in initiating DNA replication in the absence of the BPV1 transcription factor E2. Surprisingly, COS-1 cell extract allowed a high rate of BPV1 DNA replication, supporting an efficient production of mature circular DNA molecules, whereas in mouse cell extracts, the replication products mostly consisted of replicative intermediates. Submitting the extracts to affinity chromatography allowed specific binding of DNA polymerase α-primase to E1 protein, up to a total depletion of the extract, regardless of the origin of the cell extract. Furthermore, replication protein A was not retained on E1 affinity columns, even when E2 was complexed with E1. These data confirm that the interactions between E1 and DNA polymerase α-primase do not exhibit cell-type specificity, as had already been suggested by data from in vivo and in vitro replication assays, but they imply that other cellular proteins may affect the level of E1-dependent replication.

The small circular chromosome of simian virus 40 (SV40) has provided a simple model to study the mechanisms of DNA replication in mammalian cells. The virus parasitizes the host cell for virtually all of the replication proteins required for viral replication, with one exception being the SV40 T antigen, the initiator of DNA replication which allows the virus to avoid the regulatory mechanisms of the host cell. Thus, each viral genome replicates multiple times within one cell cycle, ultimately killing the host cell (for reviews, see references 2, 7, and 41). The SV40 genome contains a single well-defined origin of replication, from which replication occurs bidirectionally. Since an in vitro SV40 DNA replication system was developed (19), cellular replication factors required for the bidirectional replication of SV40 have been identified and the initiation and elongation stages have been reconstituted with purified proteins (11, 15, 29, 47). Only four factors—the viral T antigen, the three-subunit single-stranded DNA-binding protein, the replication protein A (RPA), and the topoisomerase I and DNA polymerase α-primase complex—are sufficient to initiate viral DNA replication (11, 15). Numerous studies have indicated that the initiation stage consists of successive events that could be directed by specific protein-protein interactions between the participating factors (reviewed in reference 7). The first step is the ATP-dependent assembly of the T antigen into a double hexamer at the origin of replication, causing structural distortion of the DNA (6, 31). Subsequent unwinding of the template is then mediated by the DNA helicase function of the SV40 T antigen (40). At this step, topoisomerase I relieves torsional stress generated by unwinding while RPA both binds to the single-stranded DNA and interacts with the T antigen (9, 23). The T antigen-RPA complex then binds polymerase α-primase, and a nascent RNA-DNA is synthesized at the origin (4, 10, 29).

Initial in vitro studies of SV40 DNA replication performed with soluble cell extract showed that the capacity of the extract prepared from cells of different species reflected the ability of those cells to replicate SV40 DNA in vivo (20, 42, 49). Subsequent studies suggested that the host cell specificity of SV40 replication was determined by the interaction of the T antigen and origin DNA with the host cell DNA polymerase α-primase complex (30).

In contrast to SV40 or polyomavirus, the papillomaviruses achieve DNA replication in different mammalian cell lines with an apparent lack of host cell specificity. In fact, replication of a bovine papillomavirus type 1 (BPV1) origin DNA was possible in normal nonpermissive cells only when the viral proteins required for BPV1 replication were provided from heterologous expression vectors (3, 46). Otherwise, in cultured cells, only rodent and bovine fibroblasts have been shown to support replication of BPV1 DNA, concomitant with oncogenic transformation (see reference 17). These results suggested that the in vivo replication specificities of BPV1 were likely due to restrictions at the level of gene expression and probably not to species-specific interactions with the host replication machinery.

BPV1 presents other features distinguishing it from SV40 and polyomavirus, making this virus an interesting model to provide new insights into the mechanism of DNA replication control in mammalian cells. An important aspect is that BPV1 has the potential to replicate as a stable chromosomal plasmid in the nucleus of mammalian cells (18). In bovine or rodent fibroblasts transformed in vitro by this virus, BPV1 replication does not continue after reaching a certain number of copies per cell, allowing the virus to be maintained for many generations (22). Recently, progress has been made in understanding the mechanism of initiation of BPV1 replication. In vivo and in vitro studies identified a single origin of replication (44, 46, 51). Only two viral proteins, E1 and E2, are required for

* Corresponding author. Mailing address: Centre de Biochimie, Unité 273 de l'INSERM, Parc Valrose, 06108 Nice Cedex 2, France. Phone: (33) 93529945. Fax: (33) 93529917.

BPV1 DNA replication in vivo (45). As for SV40, BPV1 replication relies entirely on the cellular DNA replication machinery. It is therefore likely that the differences between SV40 and BPV1 lie with the proteins involved in the initiation of replication. The E1 protein, which shares a number of biochemical properties with the T antigen, has been clearly established as the initiator of BPV1 replication (33, 35, 36, 43, 52). The role of the E2 transactivator protein in BPV1 replication is less clear. In a previously reported cell-free replication system, E2 was absolutely required at low levels of E1 and was shown to markedly stimulate the DNA synthesis reaction in the presence of higher E1 concentrations (51–53). It was suggested that as E2 interacts with E1, E2 may be required to facilitate the binding of E1 to the BPV1 origin of replication.

In view of the species-specific association among SV40 T antigen, polymerase α-primase, and RPA at the SV40 origin and of the apparent lack of host cell specificity for BPV1 replication in cultured cells, it was of interest to determine whether these cellular replication proteins also interact with E1 or with the E1-E2 complex. In the in vitro system reported by Yang et al. (51), the cellular extract supporting replication was obtained from a mouse cell line (FM3A). In this study, we compared extracts derived from mouse and simian cells for their ability to support BPV1 replication in vitro in the presence of E1. The E1 protein, isolated in a way to optimize recovery of its activity, was highly efficient at low concentrations in initiating DNA replication in the absence of E2. Surprisingly, the activities of extracts derived from a simian cell line (COS) were at least fourfold higher than those of mouse cell extracts. By using specific protein affinity columns, we studied interactions between E1 or E1-E2 and cellular components present in active cell extracts. Our in vitro results confirm the lack of BPV replication specificity and show that affinity columns containing either E1 or E1-E2 complex specifically and quantitatively retain the DNA polymerase α but not RPA, irrespective of the source of the replication extract.

MATERIALS AND METHODS

Expression and purification of GST fusion proteins. To generate plasmids encoding pGEX-E1, the BPV *Nru*I-*Stu*I restriction fragment (nucleotides 838 to 3351), which is ligated to *Bam*HI linkers, was inserted into the unique *Bam*HI site of pGEX 3X (Pharmacia) and transformed into *Escherichia coli* HB101 cells. Expression of the glutathione *S*-transferase (GST) fusion protein in bacteria and purification were essentially as described by Smith and Johnson (38), with some modifications. Cells were collected 1 h after the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to decrease the level of proteolytic degradation products. Bacterial cell pellets were frozen in liquid nitrogen, thawed, and resuspended in 1/10 volume of buffer A (20 mM Tris-HCl [pH 8.0], 300 mM NaCl, 50 mM $MgSO_4$, 5 mM dithiothreitol [DTT], 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, leupeptin at 10 μg/ml, and aprotinin at 10 μg/ml). The resuspended material was sonicated on ice five times for 10 s at a setting of 50 on a Vibracell 72434. After centrifugation, the fusion protein was bound to glutathione-Sepharose beads batchwise at 4°C for 1 h. The GST protein was prepared as the GST-E1 protein, although the induction time was increased and the cells were harvested after 3 to 4 h. Approximately 1 μg of GST-E1 was recovered in 1 ml of culture.

The GST-E1 protein was also expressed from a recombinant baculovirus. To construct the AcG-3X-E1 recombinant, the BPV *Nru*I-*Stu*I restriction fragment (nucleotides 838 to 3351), which is ligated to *Bam*HI linkers, was inserted into the unique *Bam*HI site of the pAcG-3X transfer plasmid, kindly provided by A. Davies and D. H. L. Bishop (NREC Institute, Oxford, United Kingdom). The GST-E1-E2 protein complex was recovered from Sf9 cell extract coinfected with AcG-3X-E1 and vFE2, prepared as described previously for the E2 protein (34). The GST-E1 fusion protein, as the complex GST-E1-E2 from the soluble nuclear extract of insect cells, was purified as described above for the GST-E1 protein recovered from bacteria.

Purification of E1 protein. In order to produce recombinant E1 from AcNPV-E1 (33), cells were harvested 48 h after infection. Infected cell pellets were frozen in liquid nitrogen and stored at −70°C. All procedures were carried out at 4°C. A total of 10^9 cells were lysed by thawing them in 10 ml of buffer B (20 mM Tris acetate [pH 8.2], 5 mM potassium acetate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, leupeptin at 10 μg/ml, and aprotinin at 10 μg/ml). After 30 min on ice, the suspension was centrifuged at 2,000 rpm for 5 min in an HB4 rotor. The pellet, containing the nuclei, was first suspended in 10 ml of buffer B plus 150 mM potassium acetate and incubated on ice for 30 min. After centrifugation, the nuclei were then resuspended in 10 ml of buffer B containing 50 mM $MgSO_4$ and 300 mM potassium acetate and left on ice for 45 min with occasional agitation. The nuclei were sedimented at 35,000 rpm in an SW50.1 rotor for 30 min, and the resulting supernatant, adjusted with 5% glycerol, was passed through a fast protein liquid chromatography (FPLC) column filter (Pharmacia) before being directly applied to an FPLC Mono Q column (Pharmacia) equilibrated in the same buffer. After it was washed, the column was eluted stepwise with the same buffer that first contained 0.5 M potassium acetate and then contained 1 M potassium acetate. E1 (identified by immunoblotting) was found in the fractions eluted with 1 M potassium acetate. These fractions were pooled and dialyzed against a buffer containing 20 mM Tris acetate (pH 8.0), 200 mM potassium acetate, 10 mM $MgSO_4$, 5 mM DTT, and 10% glycerol. The final dialysate was then aliquoted and stored at −70°C. Under these conditions, the E1 protein appears to be stable, since no noticeable loss of activity has been observed over the course of 12 months.

Nuclear extracts of Sf9 cells infected with recombinant baculovirus that expressed the GST-E1 protein were prepared as described above for the E1 protein, with some modifications. The first pellet that contained the nuclei was resuspended in a buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 50 mM $MgSO_4$, 5 mM DTT, and 0.5% Nonidet P-40 plus the protease inhibitors. After 30 min, the suspension was centrifuged at 10,000 rpm in a Beckman JA20 rotor for 30 min. The supernatant was mixed with glutathione-Sepharose beads (Pharmacia) previously equilibrated in the same buffer and rocked for 1 h at 4°C. For quantitative fusion protein recovery, the amount of GST-E1 present in the extract must not exceed 200 μg per ml of 50% glutathione–Sepharose beads. After absorption, the beads were collected by brief centrifugation and washed with at least 50 bead volumes of the same buffer and then washed in buffer with 1 M NaCL. The beads were then equilibrated in a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgSO_4$, 100 mM NaCl, 10% glycerol, and 5 mM DTT (phenylmethylsulfonyl fluoride is no longer added because it inhibits factor Xa). GST-E1 was eluted with 1 bead volume of the same buffer containing 10 mM reduced glutathione (Sigma). Specific proteolysis to release E1 protein was at 4°C in elution buffer containing 1 mM $CaCl_2$ and factor Xa (Boehringer), at an enzyme-to-GST-E1 ratio of 1/50. As efficient cleavage occurred within 3 to 4 h, the isolation of E1 can be accomplished in less than 10 h, which will keep E1 activity as high as possible. Freeze-thawed samples have to be used immediately, because as judged by the in vitro DNA replication assay, the E1 activity drops quickly within a few minutes at 4°C.

Affinity chromatography. A maximum of 8 mg of GST-E1 purified from either bacteria or insect cells was bound per ml of glutathione-Sepharose beads, and at least 12 mg was bound in the case of GST. GST-E1 and GST columns were equilibrated with buffer C (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.5], 5 mM KCl, 8 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, leupeptin at 10 μg/ml, and aprotinin at 10 μg/ml) containing 0.03 to 0.2 M NaCl. Equal aliquots of soluble extracts adjusted to the various NaCl concentrations were applied at 4°C at a flow rate of 1 ml/h to two columns containing GST-E1 or GST. Each column was washed extensively and eluted with buffer C containing 1 M NaCl. A subsequent 2 M NaCl wash did not remove detectable proteins from the columns. However, analysis of the beads after this step revealed at least one protein which remained specifically bound to the GST-E1 column. When smaller amounts of extract and resin were used, the extract was mixed with the desired amount of packed beads in a small column and incubated for 1 h with frequent stirring before extensive washing. The bound proteins were then released from the beads by boiling them in a gel sample buffer containing sodium dodecyl sulfate (SDS).

In vitro DNA replication assays. Cytoplasmic extracts from COS-1 cells were prepared as described by Li and Kelly (19). The isolation of cytoplasmic extracts from FM3A and Ts85 mouse cell lines (24) grown in suspension at 32°C was as described previously (42). Soluble extracts that had been centrifuged for 1 h at 100,000 × *g* were either used directly or frozen in liquid nitrogen and stored at −70°C. Supercoiled pBPV+ plasmid DNA carrying the *Hin*dIII-*Eco*RI fragment of the BPVI genome (positions 6958 to 2113) in pJLO (20) was used as the template. The pBPV origin minus (pBPV−) was generated by insertion of an *Xho*I linker into the unique *Hpa*I site of pBPV. The *Xho*I linker separates the half-sites of the palindromic motif for E1 binding (14). The standard reactions (100 μl) were as described by Stillman and Gluzman (42), except that the mixtures contained 0.5 μg of DNA template, 0.03 to 0.48 μg of E1 protein, and 400 to 500 μg of cell cytoplasmic extract and 5 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham). After being prepared on ice, the reaction mixtures were incubated at 37°C for the times indicated in the figure legends. DNA synthesis was monitored on aliquots by measuring the radioactivity incorporated into acid-insoluble material, while samples to be analyzed by agarose gel electrophoresis were brought to 20 mM EDTA and 0.2% SDS. Proteinase K (200 μg/ml) was added, and after further incubation for 30 min at 37°C, the mixtures were twice extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitated. *Dpn*I digestion was performed with 0.2 M NaCl. DNA samples incubated with or without *Dpn*I were fractionated by electrophoresis through 1.2% agarose gels in Tris-borate-EDTA buffer. Agarose gel electrophoresis was performed in the presence of ethidium bromide (1 μg/ml) so that relaxed,

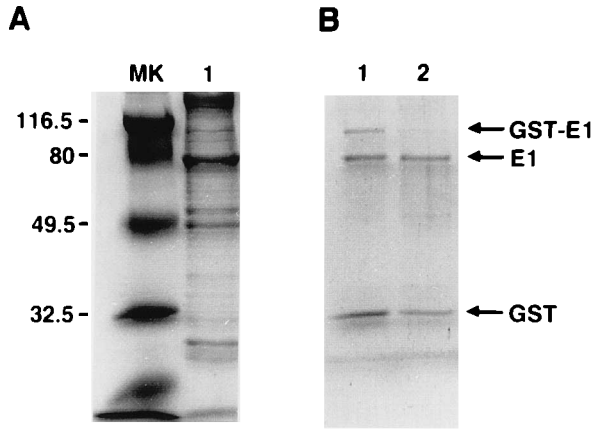

FIG. 1. SDS gel electrophoresis of E1 preparations. (A) E1 fraction eluted with 1 M potassium acetate from an FPLC Mono Q column. The fraction (30 μl) was separated on an SDS–10% polyacrylamide gel which was stained with Coomassie blue (lane 1). The positions of the size markers (MK) (in kilodaltons) are indicated at the left. (B) Protease cleavage of purified baculovirus GST fusion protein. Purified GST-E1 was digested with factor Xa at an enzyme-to-substrate molar ratio of 1:50 at 4°C and analyzed on an SDS–10% polyacrylamide gel which was stained with Coomassie blue. Lane 1, 2 h after the addition of factor Xa; lane 2, 4 h after addition of factor Xa.

covalently closed DNA circles (Form Io) migrating at the position of the supercoiled DNA could be observed.

ECL Western immunoblotting. The ECL Western blotting detection system (Amersham) was used to detect either DNA polymerase α or the p70 subunit of RPA. The protocol provided by Amersham was followed, with minor modifications. To eliminate a high background obtained with the anti-DNA polymerase α-primase antiserum, kindly provided by H. P. Nasheuer, the rabbit antiserum raised against calf thymus DNA polymerase α-primase was used at a 1:5,000 dilution, and after exposure, the membranes were extensively washed and blocked again with 3% milk before being incubated with peroxidase-conjugated anti-rabbit immunoglobulin G. Under these conditions, the p48 primase subunit and p70 DNA polymerase α subunit were barely detectable, and with this additional blocking step, the p180 subunit was the only subunit that was readily detected. RPA (p70) was probed with polyclonal antibodies raised against two peptides of the p70 subunit (amino acids 112 to 126 and 127 to 141) coupled to keyhole limpet hemocyanin (a gift from J. Imbert).

RESULTS

Purification of E1 protein. The isolation of the BPV-encoded E1 protein from extracts of baculovirus-infected Sf9 cells is summarized in Materials and Methods. The development of a purification procedure was made difficult by the extreme lability of the E1 protein activity subsequent to its chromatographic elution. To obtain a more active E1 preparation, the time required for purification must be kept to a minimum. E1 activity was indeed always lost when the protein was subjected to more than one step of chromatography. The best purification result was obtained after a simple chromatographic step in which the protein is adsorbed and eluted from an FPLC Mono Q column (Fig. 1A). In most cases, the partially purified E1 fraction was free of endodeoxyribonuclease activity. In order to further purify this protein, we developed another rapid procedure allowing us to obtain active preparations of the E1 protein. We attempted to produce E1 as a GST fusion protein in a recombinant vector in Sf9 insect cells to permit its rapid purification in a single step via glutathione affinity chromatography followed by removal of the GST by factor Xa cleavage (38). As can be seen from Fig. 1B, E1 produced by this system appears free of detectable contaminants other than the GST protein. However, the activity of the purified protein appears much more unstable than in the partially purified fraction, and variability in the amount of activity was observed, even when the same frozen cells were used.

Mouse cell extracts are less active than primate cell extracts for BPV DNA synthesis in vitro. We have first determined whether cell extracts from normal nonpermissive cells (COS-1) were capable of supporting BPV DNA replication as efficiently as cell extracts from permissive cells (Ts85). As can be seen from Fig. 2A, the amount of DNA synthesis obtained with equal amounts of similarly prepared extracts supplemented with E1 was approximately fourfold less with extracts from Ts85 cells than those from COS cells. Such a difference was observed with extracts derived from two other primate cell lines, monkey CVI cells and human 293 cells, which were as active as COS cell extracts, and with several other mouse cell lines, C127 cells, FM3A cells, and ID14 cells (a BPV-transformed C127 cell line). Extracts from mouse Ts85 cells (a temperature-sensitive mutant isolated from FM3A) were slightly more efficient and were chosen for in vitro replication assays. Products formed during the time course of the DNA synthesis presented in Fig. 2A were analyzed by agarose gel electrophoresis with or without treatment with restriction endonuclease *Dpn*I in order to rule out the possibility that the in vitro DNA synthesis represented repair synthesis. This assay relies on the fact that the *Dpn*I restriction enzyme cleaves DNA at specific sites that are methylated at adenine residues on both strands (32). The semiconservative replication of such templates prepared in *E. coli dam*$^+$ cells results in the production of hemimethylated DNA molecules which are completely resistant to *Dpn*I digestion. As can be seen from Fig. 2B, a comparison of the replication products synthesized with monkey and mouse cell extracts supplemented with E1 protein shows major differences. The electrophoretic pattern of *Dpn*I-digested products synthesized in Ts85 extracts showed no detectable fully replicated molecules (*Dpn*I-resistant Form Io), demonstrating that the band at the position of Form Io DNA in the nondigested sample was mainly due to repair synthesis and not to de novo DNA replication. In these extracts, replicative intermediates accumulated and failed for the most part to mature into completely replicated molecules. In contrast, in COS extracts, efficient production of completely replicated molecules which were completely resistant to *Dpn*I digestion was observed. Additional properties of the in vitro replication reaction in COS cell extract will be described elsewhere (1).

Requirement for a functional origin in Ts85 and COS cell extracts. Purified E1 protein was added to reaction mixtures containing either Ts85 extract or COS extract and either BPV *ori*$^+$ or BPV *ori* mutant plasmid DNA as a template. Neither extract catalyzed significant DNA synthesis in the absence of E1. However, addition of increasing amounts of the viral protein stimulated DNA synthesis in the different assays, albeit at a much lower level in mouse extracts (Fig. 3A). In both extracts, the specific requirement for an intact BPV1 origin of replication decreased with increasing E1 concentration, but surprisingly, the extent of DNA synthesis observed with extracts from COS cells and pBPV *ori* mutant DNA was threefold more than that observed with pBPV *ori*$^+$ DNA and extracts from mouse cells. Moreover, with 450 ng of E1 and COS extracts, the efficiency of DNA synthesis with pBPV *ori* mutant DNA reached almost 75% of that observed with pBPV *ori*$^+$ DNA. As can be seen from Fig. 3A, DNA synthesis with 50 ng of E1 was detected only for pBPV *ori*$^+$ DNA incubated with COS extracts. Incorporation of radioactive precursors was detected at approximately the same E1 concentration (200 ng) for pBPV *ori*$^+$ with mouse extracts and mutant pBPV *ori* with COS extracts, while *ori*-independent DNA synthesis became detectable at much higher E1 concentrations (450 ng) with

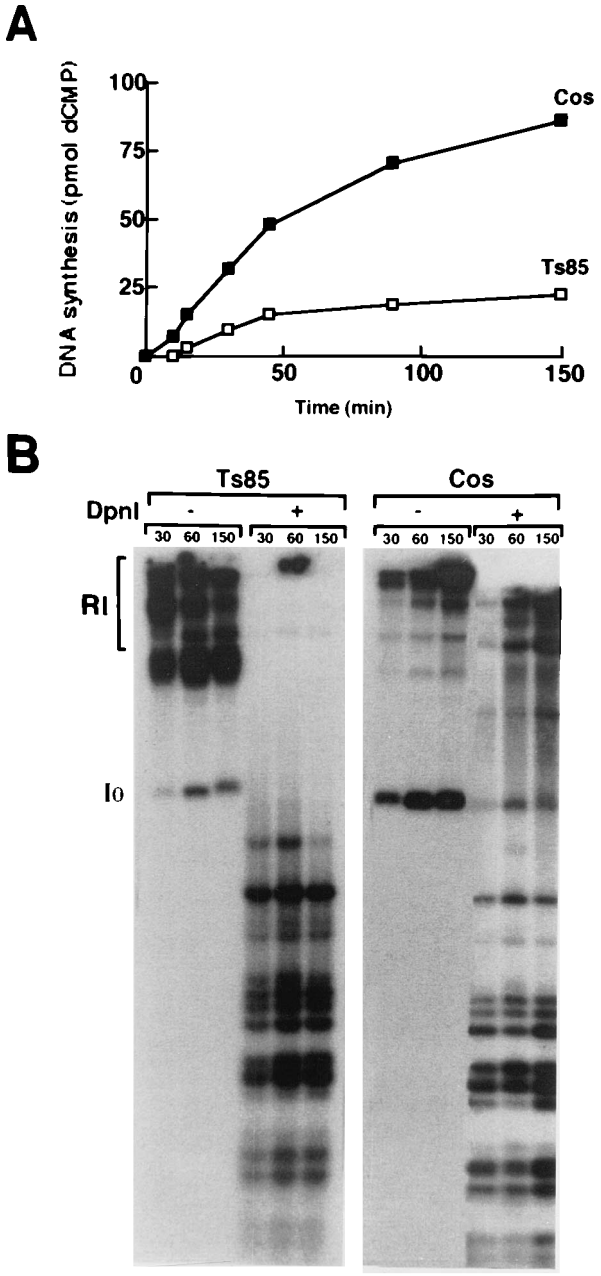

FIG. 2. BPV DNA replication in Ts85 and COS cell extracts. DNA was synthesized in replication reaction mixtures containing partially purified baculovirus-expressed E1 protein (240 ng) and 500 μg of either Ts85 or COS cell extract and 0.45 μg of pBPV *ori*$^+$ DNA which contains a wild-type BPV origin. (A) Time-dependent DNA synthesis supported by extracts from Ts85 and COS cells. The reaction mixtures were incubated at 37°C for the indicated times, and the acid-insoluble radioactivity was determined. (B) Analysis of DNA replication products with restriction endonuclease *Dpn*I. Samples were withdrawn at 30, 60, and 150 min, subjected to product analysis, and then fractionated by electrophoresis in 1.2% agarose in the presence of ethidium bromide. The time of incubation and subsequent incubation with (+) or without (−) *Dpn*I are indicated above each lane. Equal volumes of the reaction mixtures were added to each lane. RI and Io designate the migration positions of replicative intermediate DNA and pBPV Form Io (relaxed, covalently closed DNA circles migrating in the presence of ethidium bromide at the position of the supercoiled monomer DNA), respectively. The position of Form Io DNA was based on that of the supercoiled pBPV DNA electrophoresed in parallel.

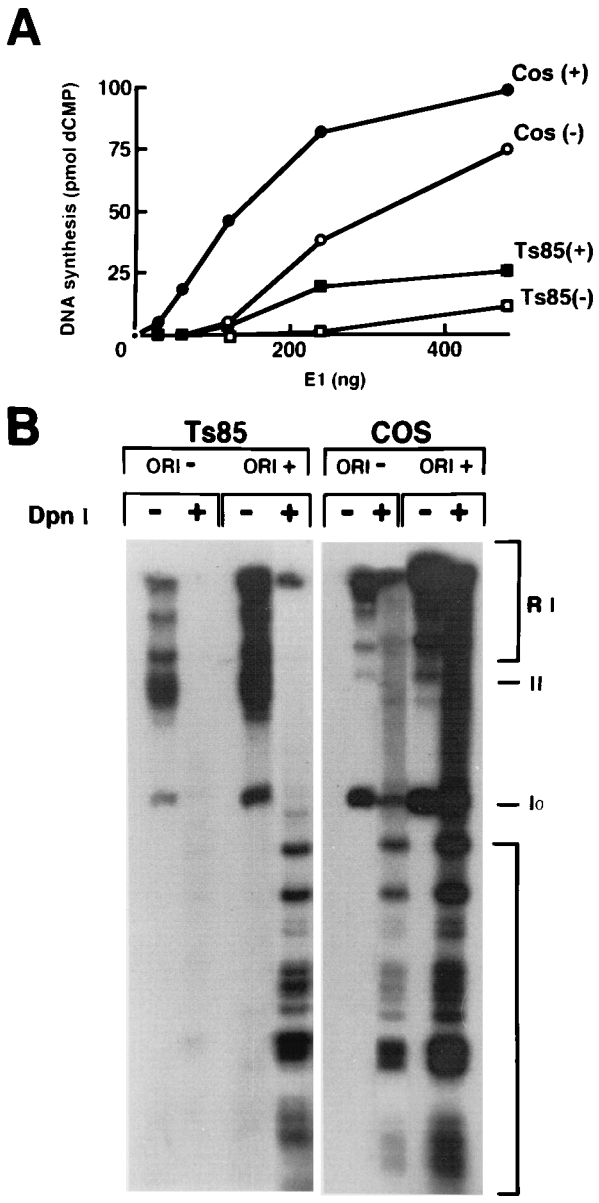

FIG. 3. Stimulation of DNA synthesis by increasing amounts of E1 protein. Reaction mixtures (100 μl) containing 450 μg of either Ts85 or COS cell extract, 0.45 μg of either pBPV+ or pBPV−, and purified E1 protein produced from the baculovirus GST-E1 fusion protein were incubated for 2 h at 37°C. (A) Samples were withdrawn and subjected to acid precipitation. + and −, wild-type and mutant *ori* DNA templates, respectively. The absolute amount of [α-^{32}P]dCMP incorporation was measured by scintillation counting and converted to picomoles of synthesis. (B) *Dpn*I assay of replication products. The radioactive products were incubated with (+) or without (−) *Dpn*I before agarose gel electrophoresis was performed as described in the legend to Fig. 2. Equal volumes of the reaction mixtures were added to each lane. RI, Io, and II designate the migration positions of the replicative intermediate, Form Io (supercoiled monomer circle), and Form II (nicked monomer circle, respectively), and the unlabeled bracket at the right indicates the positions of the *Dpn*I-generated DNA fragments of pBPV.

mouse extracts. Fig. 3B shows gels of the replication products comparing *ori*$^+$ and mutant *ori* templates in the presence of equal amounts of E1 protein (250 ng) in both extracts. In the mouse extract, DNA products made with the mutant *ori* template were totally *Dpn*I sensitive, while in those made with the *ori*$^+$ template, *Dpn*I-resistant fragments almost reached the size of monomer molecules. By contrast, in the COS cell ex-

tract, mutant pBPV *ori* supported significant DNA replication as determined by the *Dpn*I resistance of the reaction products, which is consistent with the semiconservative replication of full-length DNA strands. The products of this reaction were almost the same as those obtained with pBPV *ori*$^+$ (Fig. 3B). Thus, the high efficiency in COS cell extracts was due to the efficient production of completely replicated molecules. Furthermore, *Dpn*I-resistant replicating intermediates were shown to issue from replicated molecules used for new rounds of DNA synthesis (1). From these results, it was clear that in vitro BPV DNA synthesis can be efficiently supported with extracts from nonpermissive COS cells. Moreover, these extracts were considerably more active in cooperating with E1 to replicate BPV DNA molecules than were extracts prepared from permissive cells. In total, these results suggested that the requirements for the formation of an initiator complex in the BPV system are not as stringent as those for the SV40 system and led us to further investigate the interactions of E1 with cellular proteins known to be involved in the SV40 initiation process (e.g., polymerase α-primase and RPA).

DNA polymerase α and several other proteins from mouse cell extract bind specifically to GST-E1 protein columns. To detect cellular proteins that interact with E1, active cell extracts used for in vitro replication assays were applied to affinity columns containing E1. The E1 protein was expressed in bacteria as a fusion protein with GST (GST-E1), and the fusion protein was purified on a glutathione-Sepharose column in order to be used for affinity chromatography. The control columns contained only the GST protein. In the first set of experiments, active extracts were prepared from FM3A cells and chromatographed under ionic strength conditions used for in vitro DNA replication. Bound proteins (0.3 and 0.7% of the input) were eluted with 1 M NaCl from the GST and GST-E1 columns, respectively. SDS-polyacrylamide gel electrophoresis and Coomassie blue staining allowed the visualization of several proteins with apparent molecular masses of 82, 70, 60, 47, 44, 34, 33, and 23.5 kDa specifically retained onto the GST-E1 column (Fig. 4).

The single-stranded DNA-binding protein, RPA, consists of three tightly complexed subunits with molecular masses of 70, 34, and 11 kDa (12, 50). Two polypeptides of the expected size were reproducibly retained on the GST-E1 columns. To determine if RPA was indeed present in the GST-E1 eluate fractions, Western blot experiments with polyclonal antibodies against the 70-kDa subunit of RPA were performed to monitor the RPA polypeptide in the flow-through and the salt-eluted fractions from the GST-E1 and GST columns. As can be seen in Fig. 5, the 70-kDa polypeptide was present in both flow-through fractions (lanes 2 and 4) but not in eluates from columns containing GST-E1 and GST, indicating that RPA was not retained on the columns. The same type of experiment was performed with an anti-polymerase α-primase antiserum (Fig. 6). The mammalian DNA polymerase α-primase complex, which is highly conserved in structure, contains polypeptides with sizes of 180 (the catalytic subunit), 70, and 58 and 48 kDa (two subunits) that comprise the DNA primase subunits (48). As can be seen in Fig. 6A, one polypeptide with a size of 180 kDa that was recognized by the antiserum was present in the eluates from the GST-E1 column (lane 6) but not in those from the GST column (lane 3). Furthermore, a concomitant decrease in the apparent level of p180 polypeptide in the GST-E1 flow-through fractions was observed (lane 5). A sample of the first GST-E1 flow-through was passed over a second GST-E1 column. This second flow-through was almost totally depleted of the p180 subunit (lane 7). Although the three other subunits of the DNA polymerase α-primase cannot be monitored in the input extract and in the flow-through fractions, the p58 subunit of the DNA primase was detected in the fractions eluted from the GST-E1 column (lane 6). To eliminate the possibility that DNA serves to mediate the interactions between E1 and DNA polymerase α, cellular extracts were preincubated with DNase I and micrococcal nuclease, and it was noted that the presence of both nucleases throughout the chro-

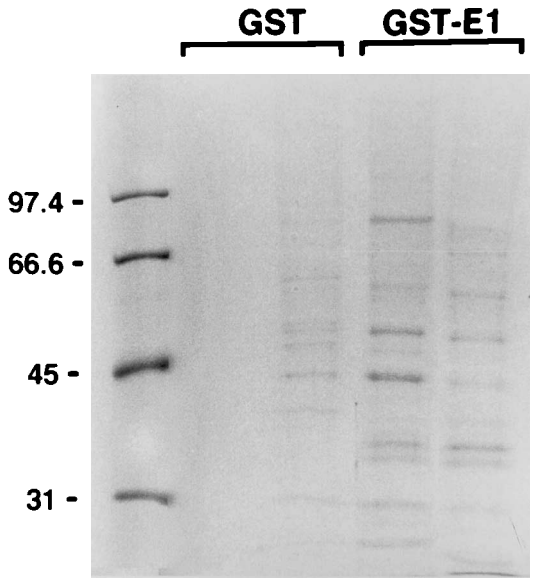

FIG. 4. Binding of mouse proteins to the BPV E1 protein. A soluble extract obtained from FM3A cells (3 ml at 7 mg/ml) was adjusted to 30 mM NaCl– 8 mM $MgCl_2$ and applied to a 0.5-ml column containing *E. coli* GST-E1 protein and to a 0.5-ml column containing GST. Bound proteins eluted in two successive fractions by 1 M NaCl were resolved by electrophoresis in an SDS–10% polyacrylamide gel and visualized by staining with Coomassie blue. Protein molecular markers in kilodaltons are labeled at the left.

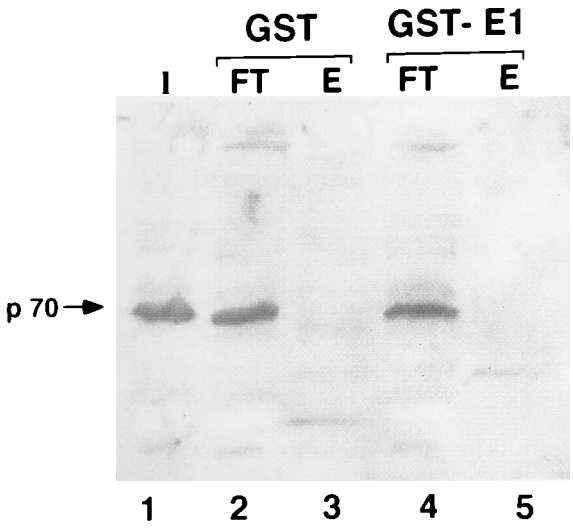

FIG. 5. RPA is not retained on the GST-E1 column. Aliquots of the flow-through (FT) and of the pooled 1 M salt-eluted (E) fractions from each column discussed in the legend to Fig. 3 were resolved on an SDS–10% polyacrylamide gel, transferred onto a nitrocellulose membrane, blotted with a polyclonal antibody against the p70 subunit of RPA, and revealed by ECL. Lane 1, input (I) FM3A extract (20 μl); lanes 2 and 3, flow-through (FT) (20 μl) and 1 M eluate (E) (40 μl) from the GST column, respectively; lanes 4 and 5, flow-through (20 μl) and 1 M eluate (E) (40 μl) from the GST-E1 column, respectively.

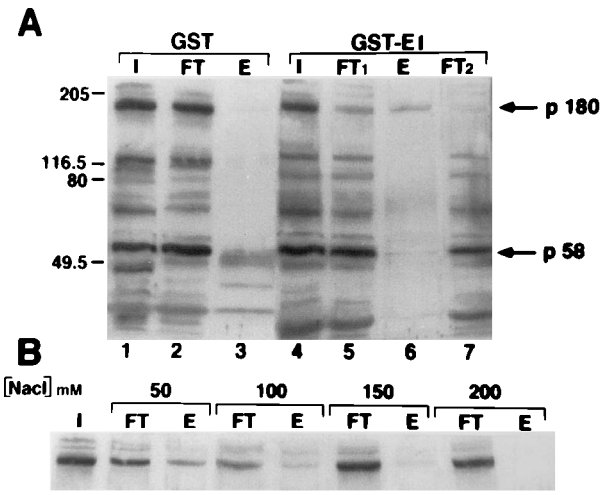

FIG. 6. Mouse DNA polymerase α-primase binds specifically to the GST-E1 column. (A) Western blot with anti-DNA polymerase α-primase antiserum of the input (I) (20 μl), flow-through (FT) (20 μl), and 1 M salt eluate (E) (40 μl) from each column discussed in the legend to Fig. 3. FT2 was obtained by passing an aliquot of the pooled GST-E1 flow-through fractions (500 μl) directly on a second GST-E1 column (50 μl). The positions of size markers are shown at the left (in kilodaltons). (B) Binding of DNA polymerase α to GST-E1 in the presence of increasing salt concentrations. GST-E1 columns (25 μl) were used to assess the binding of DNA polymerase α in the presence of salt. Samples of FM3A extract (200 μl at 7 mg/ml) were adjusted to the NaCl concentrations indicated and processed as described previously. The bound proteins were released from the beads by boiling them in SDS sample buffer. I, FT, and E designate the input FM3A extract, the flow-through, and the eluate fractions, respectively.

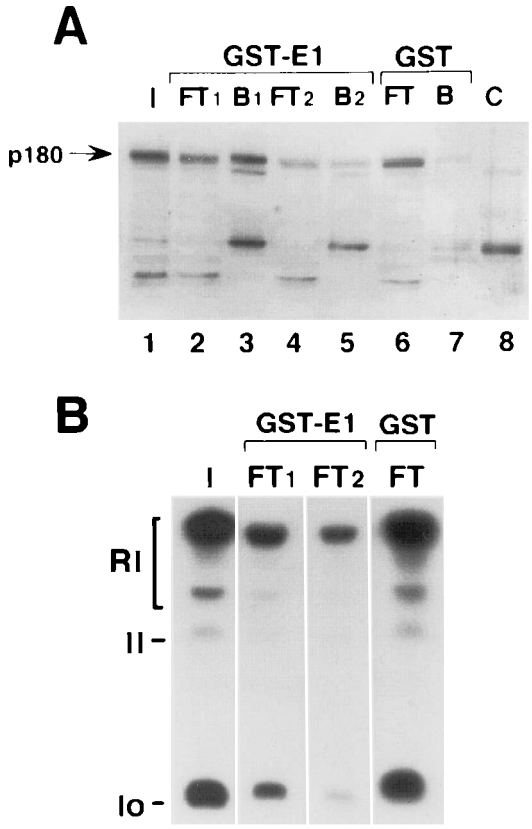

FIG. 7. GST-E1 column retains the DNA polymerase α from monkey cell extract. (A) Samples of extract obtained from COS cells (300 μl at 7.5 mg/ml) were adjusted to 30 mM NaCl–8 mM $MgCl_2$ and applied to a 50-μl column containing the *E. coli* GST-E1 protein and to a 50-μl column containing GST. The GST-E1 flow-through (FT1) was then loaded onto a second GST-E1 column (25 μl). After the beads were washed, they were collected, the bound proteins were released by boiling them in SDS sample buffer, and they were resolved by electrophoresis in an SDS–7% polyacrylamide gel, transferred onto a nitrocellulose membrane, and blotted with anti-polymerase α-primase antisera. I, FT, and B designate the input COS extract (20 μl), flow-through (20 μl), and eluted fractions (total eluate), respectively, of the first (FT1 and B1) and second (FT2 and B2) GST-E1 columns. C (lane 8) corresponds to GST-E1 beads (50 μl) not incubated with cellular extract. (B) In vitro BPV replication supported by COS cell extracts passed through GST-E1 columns. Aliquots of the input COS cell extract (I), the GST-E1 flow-through (FT1 and FT2), and the GST flow-through (FT) were tested for replication in the presence of 100 ng of E1 and 0.4 μg of pBPV as a DNA template. The reaction mixtures were incubated for 90 min at 37°C. Under these conditions, 16, 5.7, 0.5, and 14.8 pmol of dCMP were incorporated with I, FT1, FT2, and FT, respectively. DNA products were purified and electrophoresed in a 1.2% agarose gel in the presence of ethidium bromide. The positions of supercoiled (Io) and nicked DNA (II) were determined with DNA standards run in parallel. RI designates the migration positions of replicative intermediates.

matography did not affect the binding of DNA polymerase α to GST-E1 (data not shown). Binding of the DNA polymerase α-primase to GST-E1 in the presence of increasing salt concentrations is shown in Fig. 6B. Polymerase α was still able to bind to E1 at 100 mM NaCl, but a further increase in the salt concentration resulted in a large reduction in the binding. It should be noted that the initiation of replication in the in vitro system presents a similar sensitivity with regard to ionic strength. These experiments showed that among the mouse cellular proteins specifically retained on a GST-E1 column, the DNA polymerase α-primase was present and the RPA was not.

GST-E1 columns specifically bind monkey DNA polymerase α-primase. We used a similar approach to determine whether a GST-E1 column might similarly retain DNA polymerase α-primase present in active COS cell extracts. The COS extract was applied to a GST-E1 column and to a GST column as a control. A sample of the first GST-E1 flow-through was reapplied to a second GST-E1 column, and bound proteins were released from the beads by boiling them in an SDS-containing gel sample buffer. As can be seen in Fig. 7A, the catalytic subunit (p180) of the monkey DNA polymerase α was the only subunit recognized by the polyclonal anti-DNA polymerase α-primase antiserum in the input extract (lane 1). The DNA polymerase α polypeptide was present in the flow-through (lane 6) but again not in the proteins eluted from the GST column. As for the mouse DNA polymerase α, the monkey DNA polymerase α was specifically and quantitatively depleted from the COS extract, as shown by the recovery of the p180 subunit in the proteins eluted from the GST-E1 columns (lanes 3 and 5) and the concomitant decrease in the amount of p180 remaining in the flow-through (lanes 2 and 4). The GST-E1 flow-throughs were assayed for their capacities to support BPV in vitro replication. The amount of BPV DNA synthesis permitted by the first GST-E1 flow-through was 35% of that observed with the input extract, while that of the GST flow-through was 87%. Passing this first GST-E1 flow-through over a second GST-E1 column resulted in the near total inactivation of the COS extract for BPV in vitro replication. As can be seen from Fig. 7B, the products of these reactions were almost the same as those obtained with the input extract, but the number of replicated DNA molecules was markedly decreased in the reactions carried out with GST-E1 flow-throughs. Since the DNA polymerase α-primase is the only enzyme in mammalian cells capable of starting DNA chains de novo, it is likely that this effect can be correlated with the selective retention of the DNA polymerase α-primase on the successive GST-E1 col-

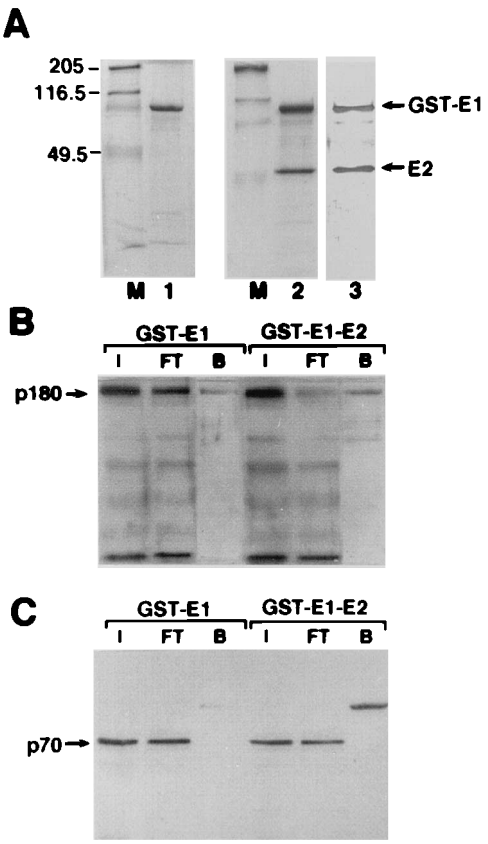

FIG. 8. A GST-E1-E2 column retains DNA polymerase α but not RPA. (A) GST-E1 protein was obtained from a recombinant baculovirus in Sf9 cells as described previously. Lane 1, GST-E1 purified on glutathione-Sepharose beads. Lane 2, GST-E1-E2 complex obtained by coinfection of Sf9 cells with GST-E1 and E2 recombinant baculoviruses and purified on glutathione-Sepharose beads. The proteins bound to the glutathione-Sepharose beads were subjected to electrophoresis on an SDS-polyacrylamide gel and visualized by Coomassie blue staining. Lane 3, Western blot with anti-E1 and anti-E2 antisera of the same sample shown in lane 2. The lanes labeled M show molecular markers. The numbers at the left are kilodaltons. (B) Samples of extract obtained from COS cells (150 μl at 6.9 mg/ml) were adjusted with 50 mM NaCl and 8 mM $MgCl_2$ and applied to a 25-μl column containing GST-E1 protein and to a 25-μl column containing GST-E1-E2. After the beads were washed, they were collected, the bound proteins were released by boiling them in SDS sample buffer, and the proteins were resolved by electrophoresis in an SDS–7% polyacrylamide gel, transferred onto a nitrocellulose membrane, and blotted with anti-polymerase α-primase antiserum. I, FT, and B designate the input COS extract (20 μl), flow-through (20 μl), and eluted fractions (half of the eluate), respectively. (C) Western blot of the equivalent amounts of the same samples shown in panel B probed with polyclonal antibodies against p70 RPA. Abbreviations are the same as those for panel B.

umns. However, it cannot be excluded that some of the proteins selectively retained on GST-E1 columns (Fig. 4) could also affect the level of DNA synthesis. This point can be directly tested with more purified protein fractions.

RPA is not depleted by a GST-E1-E2 column. Since RPA was not retained either on a GST-E1 column (our results) or on a GST-E2 column (21), we decided to determine if the E1-E2 complex was more susceptible to binding by the single-stranded DNA-binding protein than E1 or E2 alone. GST-E1 and GST-E1-E2 complexes were isolated from insect cells singly or doubly infected with the GST-E1- and E2-encoding baculoviruses and purified on glutathione-Sepharose beads (Fig. 8A). Samples of COS cell extracts were applied on columns containing GST-E1 and GST-E1-E2 recovered from insect cells. As can be seen in Fig. 8B, the catalytic subunit of the DNA polymerase α was present in the eluted proteins of both columns, demonstrating that the source of the GST-E1 as well as the presence of E2 does not impede the binding of the DNA polymerase α to E1. However, RPA was again not retained on the GST-E1 and GST-E1-E2 columns (Fig. 8C).

DISCUSSION

In this study, we have shown that BPV DNA synthesis can be carried out in extracts from both primate cells and mouse cells. DNA synthesis was totally dependent on the virus-encoded E1 protein. The replication activity of our E1 preparations was significantly higher than the activities reported in previous studies (26, 36, 51). This discrepancy might be explained by the different procedures used to isolate the protein. Indeed, E1 behaves in our purification schemes as an unstable protein, and recovery of a high activity was obtained only when the time required for E1 purification was kept to a minimum. Interestingly, the systems exhibit a strict requirement for the BPV origin of replication only at very low E1 concentrations. DNA synthesis was observed with a template containing an origin mutation known to drastically abolish BPV DNA replication in vivo (39, 46). Moreover, plasmids that do not contain any BPV sequences can be replicated in COS cell extract supplemented with E1 (data not shown). These results extend the initial observation reported by Yang et al. (52) and Müller et al. (26) by showing that origin-independent replication can, in fact, be very efficient even at low E1 levels. One may imagine that this capacity of E1 to initiate replication outside the origin site plays a significant role in in vivo BPV replication, especially during the vegetative phase of viral amplification.

Our in vitro studies have confirmed the lack of species specificity for BPV DNA replication observed in vivo by Ustav et al. (46) in their transient replication assays. In these experiments, BPV E1 and E2 proteins expressed from heterologous expression vectors were capable of replicating BPV *ori* plasmids in human 293 cells and human HeLa cells. The study by Chiang et al. (3) extended this finding by showing that E1 and E2 proteins from BPV and human papillomavirus 11 were also able to function in CHO cells as well as in simian COS cells on different papillomavirus *ori*-containing plasmids. Quite unexpectedly, in in vitro BPV DNA synthesis assays, primate cell extracts were found to be much more active than normal permissive mouse cells. During the preparation of this manuscript, data concerning in vitro BPV DNA replication and showing that extracts and purified replication proteins provided from HeLa cells supported in vitro BPV DNA synthesis were reported by Müller et al. (26). However, the amount of BPV DNA synthesized by HeLa cell extracts was only 70% of that observed with FM3A extracts, and no difference in the pattern of replication products was observed. By contrast, our results show that mouse and primate cell extracts gave rise to a totally different pattern of replication products (Fig. 2 and 3). The high efficiency of the reaction performed with the COS extract was due to the efficient production of completely replicated molecules, whereas in the different mouse cell lines tested, the major products were replicating intermediates, which accumulated and failed to mature for the most part into completely replicated molecules. Endogenous SV40 T antigen is present in cytoplasmic COS extracts. Although E1 was able to drive an extensive DNA synthesis in CV1 and 293 cell extracts which do not contain any T antigen, we nevertheless verified that the addition of purified T antigen to mouse extracts did not enhance the production of mature DNA molecules. However, the activities of mouse extracts complemented with T antigen were

approximately the same as those in its absence, and replicating intermediates were still the major DNA products in the reactions (data not shown). The pattern of replication products that we observed in mouse cell extracts is quite similar to those previously observed with high E1 levels and both E1 and E2 in several studies using the in vitro BPV replication system (21, 51–53). However, as these authors did not use the *Dpn*I assay to accurately identify the real amount of completely replicated molecules, it is difficult to compare the results. Nevertheless, in their studies, DNA synthesis was totally dependent on the addition of E2 when E1 was present at low concentrations. Our results show that E1 alone at low concentrations is highly efficient in initiating DNA replication independently of E2. This observation is in accord with those of the recent study by Müller et al. (26) and demonstrates that the only viral-encoded protein absolutely required for in vitro BPV DNA synthesis is E1. The discrepancies observed in vitro may likely be explained by differences in the E1 activity level, since E1 is a rather unstable protein. We do not yet know what the effect of E2 will be in our system, but in the report of Müller et al. (26), the stimulation of DNA replication by E2, even at low levels of E1, was minimal. It is possible that the role of E2 in the in vivo BPV replication process is different from that initially suggested.

The accumulation of replicating intermediates in mouse extracts could indicate that these extracts are devoid of factors required for efficient elongation of DNA synthesis. However, mouse cell extracts supplemented with polyomavirus T antigen support extensive DNA synthesis of the polyomavirus DNA template (28), suggesting that, as is the case for primate extracts, they should contain all the cellular proteins needed for replication. These results could therefore suggest that permissive cells contain factors that inhibit or exert some kind of control on BPV replication. On the other hand, it is also possible that primate extracts contain cellular factors which stimulate DNA synthesis either directly or indirectly. These factors could be limiting in mouse cell extracts. In all cases, different observations suggest that this effect would not be mediated through specific BPV sequences. First, accumulation of replicating intermediates within mouse extracts was observed with pBPV which contains the complete BPV upstream regulatory region (this study) and with plasmids containing only the minimal origin sequence in the previously reported in vitro system of Yang et al. (51, 52). Second, origin-independent replication appears to be repressed at a level similar to that for origin-dependent replication, as can be seen in Fig. 3. Taken together, these observations suggest that a cellular factor(s) could interact directly with the proteins involved in the initiation of replication. Thus, the development of specific protein affinity chromatography provides a tool to purify and identify such cellular factors. We are currently attempting to determine whether mouse factors retained on GST-E1 columns and GST-E1-E2 columns can repress BPV synthesis in COS cell extracts and, conversely, whether monkey factors can stimulate BPV replication in mouse extracts.

The first results obtained with GST-E1 affinity columns, as presented in this study, show that several proteins are specifically retained on the GST-E1 column. Among these proteins, we have identified the DNA polymerase α-primase by using a polyclonal anti-DNA polymerase α-primase antiserum. This result shows that E1 has a DNA polymerase-binding activity. It is likely that this activity is essential for E1-dependent initiation of replication. Cellular extracts derived from either mouse or monkey cells can be totally depleted in DNA polymerase α by GST-E1 affinity chromatography. These results are significantly different from those obtained with a T-antigen affinity column. The initial study by Smale and Tjian (37) showed that only a small fraction of the DNA polymerase α activity was selectively retained on an SV40 T-antigen affinity column when crude extracts were used. More recently, Moses and Prives (25) have obtained similar results with a polyomavirus T-antigen affinity column, showing that only a specific subfraction of the total murine polymerase α-primase activity present in crude extracts was selectively retained on a T-antigen column. In addition, the polyomavirus T-antigen affinity column selectively retained the DNA polymerase α-primase from permissive mouse cells and not from nonpermissive human cells. Such a discrimination is clearly not observed with the GST-E1 column, indicating that the interactions between E1 and cellular polymerase α-primase are not species specific. If these results adequately reflect the lack of replication specificities exhibited by BPV in vitro and in cultured cells, they do not account for the difference in the replication efficiencies of the different extracts used in the DNA replication assays, reinforcing the idea that other cellular factors must interact with E1 to modulate its activity.

We have shown that the baculovirus-encoded GST-E1 binds the DNA polymerase α-primase as well as the *E. coli* GST-E1 protein. Therefore, posttranslational modifications, such as phosphorylation, do not appear to be critical for DNA polymerase-binding activity. In addition, the presence of E2 did not prevent the association of E1 with DNA polymerase α-primase. Further studies are now required to define the molecular specificity of these interactions. In the case of the T antigen, a direct physical interaction with the catalytic subunit of DNA polymerase α was detected by an immunoblotting assay (8–10). Although it was not detected by this assay, specific binding of the T antigen to the 70-kDa subunit of DNA polymerase α was recently demonstrated with a bacterial recombinant fusion protein, GST-p70, coupled to glutathione beads (5).

In addition to its ability to bind polymerase α-primase, the T antigen also binds the single-stranded DNA-binding protein, RPA. An interaction between the T antigen and RPA was also detected by immunoblotting techniques (9, 23), and this interaction is supported by functional assays (9, 11, 23, 27). From these studies, it was suggested that the T antigen mediates the loading of DNA polymerase α-primase onto a DNA template via interactions with RPA bound to single-stranded DNA, a function analogous to that of several prokaryotic single-stranded DNA-binding proteins (16). Binding of RPA was not detected with E1 affinity columns, regardless of the source of the E1 protein and the cellular extract. It is therefore possible that interactions between E1 and RPA are too weak to be detected, even by affinity chromatography. E1 may also interact with RPA only upon binding to DNA. On the other hand, it is also possible that E1 does not need to interact with RPA in order to tether the polymerase α-primase to the DNA template, since RPA forms specific complexes with DNA polymerase α-primase and associates efficiently with the isolated primase (9). An obvious possibility is that RPA binds only to a specific initiation complex formed between E1 and E2. A recent report (13) has shown that the transactivation domains of transcriptional factors such as VP16, p53, and Gal4 can increase the efficiency of DNA replication by binding to RPA. Li and Botchan (21) demonstrated that cellular extracts depleted of RPA by passing them through a GST-VP16 affinity column were incapable of supporting in vitro BPV replication. However, a GST-E2 affinity column was not able to retain RPA from cellular extracts, and it was by a Far-Western assay that an interaction between the p70 subunit of RPA and GST-E2 was detected. Our results show that neither the E1-E2 complex nor the separate E1 and E2 proteins were able to bind RPA. It

is possible that a direct association of RPA with the viral proteins is not essential to direct the formation of an initiator complex in the BPV system.

The results of this study further demonstrate the similarities between the T antigen and E1, in that both initiators bind DNA polymerase α-primase. However, the results also extend the differences between the two systems. Besides the fact that no interactions between E1 or E1-E2 and RPA were detected, our results confirm that the initiation of BPV replication does not require species-specific interactions between the participating components and principally that E1-dependent initiation of replication does not absolutely require specific origin sequences. This last observation is certainly the most striking difference between E1 and the T antigen and highlights the use of BPV as a model to provide new insights into fundamental aspects of replication initiation in eukaryotic cells.

ACKNOWLEDGMENTS

We thank H. P. Nasheuer for providing a sample of anti-DNA polymerase α-primase antiserum and Jean Imbert for providing the polyclonal antibodies directed against the 70-kDa subunit of RPA. We are also grateful to François Cuzin and Fergus McKenzie for careful reading of the manuscript and Yan Fantéi and Corinne Cibré for help in preparing photographs.

This work was supported by grant 6112 from the Association pour la Recherche contre le Cancer.

REFERENCES

1. **Bonne-Andrea, C., S. Santucci, and P. Clertant.** Unpublished data.
2. **Chalberg, M., and T. J. Kelly.** 1989. Animal virus DNA replication. Annu. Rev. Biochem. **58:**671–717.
3. **Chiang, C. M., M. Ustav, A. Stenlund, T. F. Ho, T. R. Broker, and L. T. Chow.** 1992. Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral replication origins. Proc. Natl. Acad. Sci. USA **89:**5799–5803.
4. **Collins, K. L., and T. J. Kelly.** 1991. The effects of T antigen and replication protein A on the initiation of DNA synthesis by DNA polymerase α-primase. Mol. Cell. Biol. **11:**2108–2115.
5. **Collins, K. L., A. A. R. Russo, B. Y. Tseng, and T. J. Kelly.** 1993. The role of the 70kDa subunit of human polymerase α in DNA replication. EMBO J. **12:**4555–4566.
6. **Dean, F. B., J. A. Borowiec, T. Eki, and J. Hurwitz.** 1992. The simian virus 40 T antigen double hexamer assembles around the DNA at the replication origin. J. Biol. Chem. **267:**14129–14137.
7. **Diffley, J. F. X.** 1992. Early events in eukaryotic DNA replication. Trends Cell Biol. **2:**298–303.
8. **Dornreiter, I., W. C. Copeland, and T. S.-F. Wang.** 1993. Initiation of simian virus 40 DNA replication requires the interaction of a specific domain of human DNA polymerase α with large T antigen. Mol. Cell. Biol. **13:**809–820.
9. **Dornreiter, I., L. F. Erdile, I. U. Gilbert, D. Von Winkler, T. J. Kelly, and E. Fanning.** 1992. Interaction of DNA polymerase α-primase with cellular replication protein A and SV40 T-antigen. EMBO J. **11:**769–776.
10. **Dornreiter, I., A. Höss, A. K. Arthur, and E. Fanning.** 1990. SV40 T antigen binds directly to the catalytic subunit of DNA polymerase alpha. EMBO J. **9:**3329–3336.
11. **Erdile, L. F., K. L. Collins, A. Russo, P. Simancek, D. Small, D. Umbricht, D. Virshup, L. Cheng, S. Randall, D. Weinberg, L. Moarefi, E. Fanning, and T. J. Kelly.** 1991. Initiation of SV40 DNA replication; mechanism and control. Cold Spring Harbor Symp. Quant. Biol. **56:**303–313.
12. **Fairman, M. P., and B. Stillman.** 1988. Cellular factors required for multiple stages of SV40 replication in vitro. EMBO J. **7:**1211–1218.
13. **He, Z., B. T. Brinton, J. Greenblatt, J. A. Hassell, and J. C. Ingles.** 1994. The transactivator proteins VP16 and Gal4 bind replication factor A. Cell **73:** 1223–1232.
14. **Holt, S. E., G. Schuller, and V. G. Wilson.** 1994. DNA binding specificity of the bovine papillomavirus E1 protein is determined by sequences contained within an 18-base-pair inverted repeat element at the origin of replication. J. Virol. **68:**1094–1102.
15. **Hurwitz, J., F. B. Dean, A. D. Kwong, and S.-H. Lee.** 1990. The in vitro replication of DNA containing the SV40 origin. J. Biol. Chem. **265:**18043–18046.
16. **Kornberg, A., and T. A. Baker.** 1991. DNA replication, p. 286–292, 328–338. W. H. Freeman and Co., New York.
17. **Lambert, P. F., C. Baker, and P. M. Howley.** 1988. The genetics of bovine papillomavirus type 1. Annu. Rev. Genet. **22:**235–258.
18. **Law, M. F., D. R. Lowy, I. Dvoretzky, and P. M. Howley.** 1981. Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. Proc. Natl. Acad. Sci. USA **78:**2727–2731.
19. **Li, J. J., and T. J. Kelly.** 1984. Simian virus 40 DNA replication in vitro. Proc. Natl. Acad. Sci. USA **81:**6973–6977.
20. **Li, J. J., and T. J. Kelly.** 1985. Simian virus 40 DNA replication in vitro: specificity of initiation and evidence for bidirectional replication. Mol. Cell. Biol. **5:**1238–1246.
21. **Li, R., and M. R. Botchan.** 1993. The acidic transcriptional activation domains of VP16 and P53 bind the cellular replication protein A and stimulate in vitro BPV1 DNA replication. Cell **73:**1207–1221.
22. **Mecsas, J., and B. Sugden.** 1987. Replication of plasmids derived from bovine papillomavirus type 1 and Epstein-Barr virus in cells in culture. Annu. Rev. Cell Biol. **3:**87–108.
23. **Melendy, T., and B. Stillman.** 1993. An interaction between replication protein A and SV40 T antigen appears essential for primosome assembly during SV40 DNA replication. J. Biol. Chem. **268:**3389–3395.
24. **Mita, S., H. Yasuda, T. Marunouchi, S. Ishiko, and M. Yamada.** 1980. A temperature-sensitive mutant of cultured mouse cells defective in chromosome condensation. Exp. Cell Res. **126:**407–416.
25. **Moses, K., and C. Prives.** 1994. A unique subpopulation of murine DNA polymerase α/primase specifically interacts with polyomavirus T antigen and stimulates DNA replication. Mol. Cell. Biol. **14:**2767–2776.
26. **Müller, F., Y. S. Seo, and J. Hurwitz.** 1994. Replication of bovine papillomavirus type 1 origin-containing DNA in crude extracts and with purified proteins. J. Biol. Chem. **269:**17086–17094.
27. **Murakami, Y., T. Eki, and J. Hurwitz.** 1992. Studies on the initiation of simian virus 40 replication *in vitro*; RNA primer synthesis and its elongation. Proc. Natl. Acad. Sci. USA **89:**952–956.
28. **Murakami, Y., T. Eki, M. Yamada, C. Prives, and J. Hurwitz.** 1986. Species-specific *in vitro* synthesis of DNA containing the polyomavirus origin of replication. Proc. Natl. Acad. Sci. USA **83:**6347–6351.
29. **Murakami, Y., and J. Hurwitz.** 1993. Functional interactions between SV40 T antigen and other replication proteins at the replication fork. J. Biol. Chem. **268:**11008–11017.
30. **Murakami, Y., C. R. Wobbe, L. Weissbach, F. B. Dean, and J. Hurwitz.** 1986. Role of DNA polymerase α and DNA primase in simian virus 40 DNA replication *in vitro*. Proc. Natl. Acad. Sci. USA **83:**2869–2873.
31. **Parsons, R. E., J. E. Stenger, S. Ray, R. Welker, M. E. Anderson, and P. Tegtmeyer.** 1991. Cooperative assembly of simian virus 40 T-antigen hexamers on functional halves of the replication origin. J. Virol. **65:**2798–2806.
32. **Peden, K. W. C., J. M. Pipas, S. Pearson-White, and D. Nathans.** 1980. Isolation of mutants of an animal virus in bacteria. Science **209:**1392–1396.
33. **Santucci, S., E. J. Androphy, C. Bonne-Andréa, and P. Clertant.** 1990. Proteins encoded by the bovine papillomavirus E1 open reading frame: expression in heterologous systems and in virally transformed cells. J. Virol. **64:**6027–6039.
34. **Santucci, S., C. Bonne-Andrea, and P. Clertant.** 1992. Bovine papillomavirus (BPV1) replication protein E1: associated ATPase and binding to the viral E2 transactivator, p. 399–416. *In* M. Kohiyama, J. Campbell, and E. Fanning (ed.), DNA replication: the regulatory mechanisms. Springer-Verlag, Berlin.
35. **Seo, Y.-S., F. Müller, M. Lusky, E. Gibbs, H.-Y. Kim, B. Phillips, and J. Hurwitz.** 1993. Bovine papilloma virus (BPV)-encoded E2 protein enhances binding of E1 protein to the BPV replication origin. Proc. Natl. Acad. Sci. USA **90:**2865–2869.
36. **Seo, Y. S., F. Müller, M. Lusky, and J. Hurwitz.** 1993. Bovine papillomavirus (BPV)-encoded E1 protein contains multiple activities required for BPV DNA replication. Proc. Natl. Acad. Sci. USA **90:**702–706.
37. **Smale, S. T., and R. Tjian.** 1986. T-antigen-DNA polymerase α complex implicated in simian virus 40 DNA replication. Mol. Cell. Biol. **6:**4077–4087.
38. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67:**31–40.
39. **Spalholz, B. A., A. A. McBride, T. Sarafi, and J. Quintero.** 1993. Binding of bovine papillomavirus E1 to the origin is not sufficient for DNA replication. Virology **193:**201–212.
40. **Stahl, H., P. Dröge, and R. Knippers.** 1986. DNA helicase activity of SV40 large tumor antigen. EMBO J. **5:**1939–1944.
41. **Stillman, B.** 1989. Initiation of eukaryotic DNA replication *in vitro*. Annu. Rev. Cell Biol. **5:**197–245.
42. **Stillman, B. W., and Y. Gluzman.** 1985. Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. Mol. Cell. Biol. **5:**2051–2060.
43. **Sun, S., L. Thorner, M. Lentz, P. MacPherson, and M. Botchan.** 1990. Identification of a 68-kilodalton nuclear ATP-binding phosphoprotein encoded by bovine papillomavirus type 1. J. Virol. **64:**5093–5105.
44. **Ustav, E., M. Ustav, P. Szymanski, and A. Stenlund.** 1993. The bovine papillomavirus origin of replication requires a binding site for the E2 transcriptional activator. Proc. Natl. Acad. Sci. USA **90:**898–902.
45. **Ustav, M., and A. Stenlund.** 1991. Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. EMBO J. **10:**449–457.
46. **Ustav, M., E. Ustav, P. Szymanski, and A. Stenlund.** 1991. Identification of

the origin of replication of bovine papillomavirus and characterization of the viral origin recognition factor E1. EMBO J. **10:**4321–4329.
47. **Waga, S., and B. Stillman.** 1994. Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication *in vitro*. Nature (London) **369:**207–212.
48. **Wang, T. S.-F.** 1991. Eukaryotic DNA polymerases. Annu. Rev. Biochem. **60:**513–552.
49. **Wobbe, C. R., F. Dean, L. Weissbach, and J. Hurwitz.** 1985. *In vitro* replication of duplex circular DNA containing the simian virus 40 DNA origin site. Proc. Natl. Acad. Sci. USA **82:**5710–5714.
50. **Wold, M. S., and T. Kelly.** 1988. Purification and characterization of replication protein A, a cellular protein required for *in vitro* replication of simian virus 40 DNA. Proc. Natl. Acad. Sci. USA **85:**2523–2527.
51. **Yang, L., R. Li, I. L. Mohr, R. Clark, and M. Botchan.** 1991. Activation of BPV-1 replication *in vitro* by the transcription factor E2. Nature (London) **353:**628–632.
52. **Yang, L., I. Mohr, E. Fouts, D. A. Lim, M. Nohaile, and M. Botchan.** 1993. The E1 protein of bovine papilloma virus 1 is an ATP-dependent DNA helicase. Proc. Natl. Acad. Sci. USA **90:**5086–5090.
53. **Yang, L., I. Mohr, R. Li, T. Nottoli, S. Sun, and M. Botchan.** 1991. Transcription factor E2 regulates BPV-1 DNA replication *in vitro* by direct protein-protein interaction. Cold Spring Harbor Symp. Quant. Biol. **56:**335–346.