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Identification of Domains of the HPV11 E1 Protein Required for DNA Replication *in Vitro*

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The HPV E1 and E2 proteins along with cellular factors, are required for replication of the viral genome. In this study we show that *in vitro* synthesized HPV11 E1 can support DNA replication in a cell-free system and is able to cooperate with E2 to recruit the host polymerase α primase to the HPV origin *in vitro.* Deletion analysis revealed that the N-terminal 166 amino acids of E1, which encompass a nuclear localization signal and a cyclin E-binding motif, are dispensable for E1-dependent DNA replication and for recruitment of pol α primase to the origin *in vitro.* A shorter E1 protein lacking the N-terminal 190 amino acids supported cell-free DNA replication at less than 25% the efficiency of wild-type E1 and was active in the pol α primase recruitment assay. An even shorter E1 protein lacking a functional DNA-binding domain due to a truncation of the N-terminal 352 amino acids was inactive in both assays despite the fact that it retains the ability to associate with E2 or pol α primase in the absence of ori DNA. We provide additional functional evidence that E1 interacts with pol α primase through the p70 subunit of the complex by showing that p70 can be recruited to the HPV origin by E1 and E2 *in vitro,* that the domain of E1 (amino acids 353–649) that binds to pol α primase *in vitro* is the same as that needed for interaction with p70 in the yeast two-hybrid system, and that exogenously added p70 competes with the interaction between E1 and pol α primase and inhibits E1-dependent cell-free DNA replication. On the basis of these results and the observation that pol α primase competes with the interaction between E1 and E2 in solution, we propose that these three proteins assemble at the origin in a stepwise process during which E1, following its interaction with E2, must bind to DNA prior to interacting with pol α primase.

INTRODUCTION

Papillomaviruses (PV) infect cutaneous and mucosal tissues resulting in the development of benign and malignant lesions. Replication of PV is coordinated with the differentiation of the keratinocytes from the basal layer of the stratified epithelium (Zur Hausen and de Villiers, 1994; Chow and Brocker, 1977). Mechanistic studies have revealed similarities in how the SV40 large T antigen (Bullock, 1997; Brush *et al.,* 1995; Hurwitz *et al.,* 1990) and bovine papillomavirus (BPV) E1 and E2 proteins (Stenlund, 1996; Ustav and Stenlund, 1991; Chiang *et al.,* 1992; Yang *et al.,* 1991) coordinate replication of their respective genomes with common host DNA replication proteins. Purified recombinant HPV11 E1 and E2 proteins can support replication of HPV11 ori-containing DNA in a human cell-free system with similar host factor requirements as the well-characterized BPV and SV40 *in vitro* DNA replication systems (Kuo *et al.,* 1994).

As an early step in the HPV DNA replication process the E2 protein binds to specific sequences at the viral origin and promotes the efficient recruitment of E1 to form a ternary complex (Kuo *et al.,* 1994; Frattini and Laimins, 1994; Chao *et al.,* 1999), a feature previously demonstrated for the BPV cognate proteins with the BPV ori (Lusky *et al.,* 1993, 1994; Seo *et al.,* 1993; Berg and Stenlund, 1997). For HPV E1, we and others have identified a region at the C-terminus of the protein, between amino acids 353 and 649, that is sufficient for interaction with the transactivation domain of E2 (Titolo *et al.,* 1999; Masterson *et al.,* 1998; Yasugi *et al.,* 1997; Sun *et al.,* 1998; Zou *et al.,* 1998). Following or concomitant with its recruitment to the origin, the E1 proteins of HPV11 and BPV1 probably assemble into hexameric structures that possess helicase activity (Lusky *et al.,* 1994; Liu *et al.,* 1998; Sedman and Stenlund, 1998; Fouts *et al.,* 1999). Based on the SV40 paradigm, it is likely that the helicase and ATPase activities of papillomavirus E1 are coupled with the activities of cellular replication factors to establish the replication forks (reviewed in Bullock, 1997; Brush *et al.,* 1995; Hurwitz *et al.,* 1990, and references therein).

The results of biochemical analyses of SV40 large T antigen (reviewed in Bullock, 1997; Brush *et al.,* 1995; Hurwitz *et al.,* 1990) and papillomavirus E1 (reviewed in Sverdrup and Myers, 1997), together with the observation of amino acid conservation among their carboxy-terminal regions (Clertant and Seif, 1984; Mansky *et al.,* 1997),

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suggest that these viral proteins encode common functions to facilitate replication of their respective genomes. Among these conserved functions are the ability to bind ori DNA, to self-oligomerize, to function as an ATPase/helicase, and to interact with host cell replication factors. Earlier studies have shown that the large T antigen of SV40 (Smale and Tjian, 1986; Dornreiter *et al.,* 1990, 1992, 1993) and polyomavirus (Bruckner *et al.,* 1995), as well as the E1 proteins of BPV E1 (Park *et al.,* 1994; Bonne-Andrea *et al.,* 1995), HPV11 E1 (Conger *et al.,* 1999), and HPV16 E1 (Masterson *et al.,* 1998), associate directly with the host polymerase α primase complex. This complex is composed of four subunits of 180 (p180), 70 (p70), 58 (p58), and 48 (p48) kDa, respectively (reviewed in Wang, 1991). P180 is the polymerase catalytic subunit, whereas p48 and p58 are the primase catalytic and auxiliary subunits, respectively. P70 has no known catalytic function. Pol α primase is essential for initiation of DNA synthesis and for lagging-strand DNA synthesis of the host genome (Wang, 1991). Studies have shown that the SV40 large T antigen (Dornreiter *et al.,* 1993), BPV E1 (Park *et al.,* 1994), and HPV11 E1 (Conger *et al.,* 1999) proteins interact with the p180 subunit of the pol α primase complex. One study further showed that a domain of p180 that binds to T antigen functions as a dominant-negative inhibitor of T-antigen-catalyzed cell-free DNA replication (Dornreiter *et al.,* 1993). In contrast, another study showed that T antigen does not associate with p180 but rather binds to the p70 subunit of pol α primase and that the T-antigen-binding domain in p70 also inhibits T-antigen-catalyzed cell-free DNA replication (Collins *et al.,* 1993). For the E1 protein of papillomavirus a similar controversy has recently emerged. In a study by Masterson *et al.* (1998), direct binding assays were used to demonstrate that the HPV16 E1 protein binds to the p70 subunit, but not the p180 subunit, of pol α primase. An independent study by Conger *et al.* (1999) demonstrated that the HPV11 E1 protein binds to both p70 and p180 and that free p180, but not free p70 could, inhibit E1-dependent cell-free DNA replication. Free p70 could, however, inhibit cell-free DNA replication that was promoted by both E1 and E2 (Conger *et al.,* 1999).

In this study, we report that *in vitro* synthesized HPV11 E1 is active in supporting cell-free DNA replication and have made use of this observation to identify a domain of E1 sufficient for this process. We adapted the McKay assay to detect the E1- and E2-dependent recruitment of pol α primase, or its p70 subunit, to the HPV origin. We show that the domain of E1 that is required for recruiting pol α primase to the origin is the same as that needed for cell-free DNA replication. In contrast, a shorter C-terminal domain of E1 is sufficient for binding to pol α primase and to p70 in the absence of ori DNA. In solution, binding of E1 to pol α primase and to E2 is mutually exclusive. Finally, we show that free p70 competes with the interaction between E1 and pol α primase and inhibits E1-catalyzed cell-free DNA replication. The implications of these findings for the mechanism of primosome assembly are discussed.

RESULTS

E1 synthesized by coupled transcription/translation *in vitro* is active in supporting HPV DNA replication in a cell-free system

We wished to establish an E1-dependent cell-free DNA replication system that would expedite our characterization of functional domains of E1 that are required for DNA replication. Toward this goal, we tested whether coupled *in vitro* transcription/translation of the E1 ORF in a rabbit reticulocyte lysate would provide a convenient source of active E1 for analytical experiments. *In vitro* synthesis of active E1 would offer the advantage that deletion derivatives or mutant forms of the protein could be prepared rapidly and assayed immediately in a cell-free DNA replication system.

An E1-containing reticulocyte lysate was assayed for its ability to catalyze replication of an HPV11 ori-containing plasmid in a human cell-free extract (CSH293). In these experiments, the reaction was staged to reduce background DNA repair synthesis, by preincubating the DNA in the cell-free extracts for 30 min in the absence of [^{33}P]dATP, before addition of the E1-containing lysate. Repair synthesis is generally limited to the first 30 min of incubation, and since the nucleotide triphosphate is not present at this time, the background DNA repair-mediated incorporation of [^{33}P]dAMP is dramatically minimized during the course of the replication reaction.

[^{33}P]dATP and aliquots of the E1-containing lysate were added to the prereplication reaction followed by incubation for 1.5 h at 37°C. E1-dependent synthesis from each reaction was assessed in two ways. Half of the reaction was used to quantify DNA synthesis by TCA precipitation of radiolabeled nucleic acids and the other half was analyzed by gel electrophoresis followed by autoradiography to identify ^{33}P-labeled DNA replication intermediates.

The addition of increasing amounts of E1-containing lysate resulted in a dose-dependent increase of [^{33}P]dAMP incorporation into acid precipitable DNA (Fig. 1A, lanes 5–7). Equivalent amounts of the mock lysate resulted in negligible DNA synthesis (lanes 2–4) similar to that observed in a reaction in which lysate was omitted (lane 1) and which is most likely attributable to residual background DNA repair synthesis. In the presence of 20 μl of E1-containing lysate, there was a ninefold increase in the amount of nucleotide incorporated compared to the amount obtained with mock lysate (compare lane 4 with lane 7). In these experiments, it is possible that factors present in the reticulocyte lysate contribute, together with E1, to the overall levels of cell-free DNA replication. Further experiments will be re-

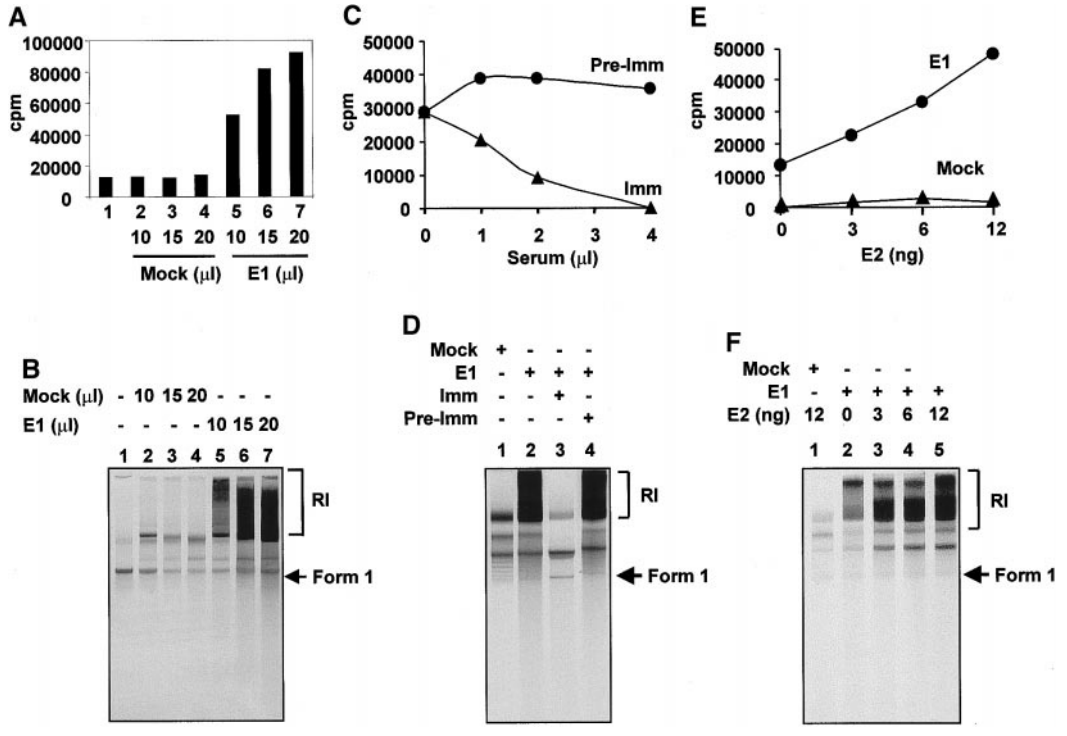

FIG. 1. *In vitro* synthesized HPV11 E1 catalyzes DNA replication in a cell-free system. (A) 10, 15, and 20 μl of E1-containing lysate (lanes 5–7) or mock lysate (lanes 2–4) was added to the DNA replication reaction. Lane 1 refers to a nonlysate control. The reactions were incubated at 37°C for 90 min and DNA synthesis was quantified by TCA precipitation of half of the reaction. (B) Analysis of DNA replication intermediates by gel electrophoresis. DNA was extracted from half of each replication reaction and subjected to electrophoresis on a 1% agarose gel. The gel was dried and exposed to film. The amounts of mock lysate and E1-containing lysate used in the reactions are indicated above the lanes (1–7). RI, DNA replication intermediates. The arrow indicates the position of form 1 DNA. (C and D) An E1 polyclonal antibody inhibits E1-catalyzed DNA replication. 20 μl of the E1-containing lysate was incubated at 4°C for 30 min with increasing amounts (1, 2, and 4 μl) of the E1 immune or preimmune serum as indicated, prior to addition to the prereplication reaction. DNA synthesis (C) and formation of replication intermediates (D) were analyzed as described above. (D) Lane 1, mock lysate; lane 4, E1-containing lysate; lanes 2 and 3, E1-containing lysate with 4 μl of preimmune serum and immune serum, respectively. (E) E2 stimulates the E1-dependent DNA replication reaction. Increasing amounts of pure E2 protein (0, 3, 6, and 12 ng) were added to 20 μl of the E1-containing lysate or mock lysate, followed by addition to the prereplication reaction. DNA synthesis (E) and formation of replication intermediates (F) were analyzed as described above. For the graph in E, the cpms plotted were subtracted from the cpm from the mock lysate (without E2).

quired to address this possibility. The gel analysis (Fig. 1B, lanes 5–7) indicated that slow mobility DNA replication intermediates (RI), which likely represent intact or nicked theta forms of replication intermediates, were produced only in reactions containing the E1 lysate and not in those containing the mock lysate (lanes 2–4). The faster migrating products, which are not unique to the E1-dependent replication reactions, are likely due to repair synthesis, since they are observed in the reactions with mock lysate (lanes 2–4) and the reaction lacking lysate (lane 1).

To confirm further that the replication reaction was dependent on E1, we made use of a polyclonal antibody directed against the N-terminal 249 amino acids of E1 (kindly provided by Dr. L. T. Chow, University of Alabama at Birmingham) that was previously shown to inhibit the HPV11 E1-dependent DNA replication reaction (Liu *et al.,* 1995). The experiment was carried out as described above, except that the respective lysates were preincubated with the immune and preimmune sera at 4°C for 30 min, before their addition to the reaction. Figure 1C shows that increasing concentrations of the immune serum resulted in a dose-dependent inhibition of the replication reaction, whereas the equivalent amounts of preimmune serum (from the same rabbit) had a slight stimulatory effect on the reaction. As shown in Fig. 1D, lane 3, 4 μl of the E1 antiserum resulted in a substantial decrease in the formation of replication intermediates (RI), but had little or no effect on the products derived from repair synthesis. The preimmune serum (lane 4) had no effect on the yield of RI, which was comparable to that of a reaction with E1 alone (lane 2). These results indicate that DNA synthesis, leading to the formation of replication intermediates, is dependent on E1.

As an additional piece of evidence that the replication reaction is dependent on functional E1 protein, we investigated whether E2 could stimulate replication of ori DNA in the presence of a limiting amount of E1. The experiment was carried out as described above using 5, 10, and 20 μl of E1-containing lysate with increasing

amounts of purified HPV11 E2. The results from using 20 μl of mock or E1-containing lysate are shown in Fig. 1E. This level of E1-containing lysate (no E2) could be stimulated in a dose-dependent manner by the addition of purified E2 to the reaction. The addition of 12 ng of E2 resulted in an approximately fourfold stimulation of DNA synthesis but had no effect on the reaction containing the mock lysate. An analysis of the replication products shown in Fig. 1F indicated that the stimulatory effect of E2 was the result of an increase in the amount of RI formed in the reaction containing E1 (compare lanes 2–5). No RI were produced in the reaction containing mock lysate and 12 ng of E2 (lane 1). Collectively, the results presented in Fig. 1 indicate that coupled *in vitro* transcription/translation is a rapid and convenient method for preparing E1 protein that is active in supporting cell-free DNA replication.

The N-terminal 165 amino acids of E1 are dispensable for E1-dependent replication *in vitro*

To analyze functional domains of the E1 protein required for *in vitro* replication, N-terminally truncated E1 proteins that encompass residues 72–649 (N72), 166–649 (N166), 191–649 (N191), 353–649 (N-353), and 435–649 (N435) of E1 were synthesized *in vitro.* SDS–PAGE analysis of the ^{35}S-labeled truncated proteins synthesized in the reticulocyte lysate showed that they migrated at positions, relative to prestained molecular weight standards, that are consistent with their predicted molecular weights (Fig. 2A, lanes 1–6, respectively). Smaller products were also observed, which are likely produced by translation initiation at internal ATG codons.

These truncated proteins were assessed for their ability to catalyze DNA replication of HPV11 ori DNA in the cell-free replication system. As shown from the acid precipitable counts given as pmoles of dAMP incorporated into DNA in Fig. 2B (bottom of the gel), the wild-type E1 and truncated derivatives N72 and N166 were almost equally active in catalyzing DNA synthesis (lanes 1–3). The truncated derivative N191 had reduced activity and supported replication at less than 25% the efficiency of wild-type E1 (lane 4). The two shorter derivatives, N353 and N435, were dramatically impaired in their ability to catalyze DNA synthesis. As expected, DNA RI were observed in reactions containing wild-type E1, N72, and N166 and to a lesser extent for N191, but were dramatically reduced in reactions containing N353 and N435. These results indicate that a C-terminal domain of E1 spanning amino acids 166–649 is sufficient for E1 function in DNA replication *in vitro.* This domain must therefore encode all functions necessary for DNA replication including DNA binding, oligomerization, helicase, ATPase, and binding to the host pol α primase complex.

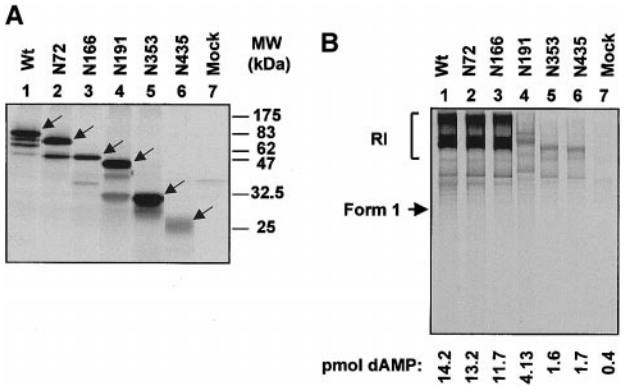

FIG. 2. Ability of truncated E1 proteins to support cell-free DNA replication. (A) E1 polypeptides synthesized in the lysate. The wild-type and truncated E1 polypeptides N72, N166, N191, N353, and N435 were synthesized in the lysate using ^{35}S-labeled methionine, and 2-μl samples of the respective lysates and of a mock lysate were analyzed on a 10% SDS–PAGE followed by autoradiography. The position of each truncated derivative is indicated by an arrow (lanes 1–7). Prestained molecular weight markers (Bio-Rad) were used as size standards. (B) Activity of N-terminal truncated E1 proteins in cell-free DNA replication. 20 μl of lysate containing either wild-type E1 or the indicated truncated polypeptide was assessed for their ability to catalyzed DNA replication as described in the legend to Fig. 1A. The pmol dAMP incorporation for the reaction are shown below the gel for each polypeptides (lanes 1–7). The reactions were processed as in Fig. 1B to assess the yield of RI.

E1- and E2-dependent association of pol α primase with the origin of HPV DNA replication

The E1 protein from papillomaviruses associates with the host polymerase α primase during both the initiation and the elongation phases of DNA replication. To determine whether the same domain of E1 that is needed for cell-free DNA replication, or a shorter domain, is required for the recruitment of pol α primase to the HPV origin during the initiation phase of DNA replication, we established the following assay. Previously, we had shown using the McKay assay that *in vitro* synthesized HPV11 E1 and E2 will bind cooperatively to a ^{33}P-labeled DNA fragment containing the HPV11 origin of replication to form a nucleoprotein complex that can be immunoprecipitated with E1-specific antibodies (Titolo *et al.,* 1999). We have now adapted this assay to assess whether E1 and E2 could promote the association of pol α primase with the HPV origin *in vitro.* For this assay, a fixed amount of the E1 and E2 proteins, made by the *in vitro* transcription/translation were mixed with increasing amounts of highly purified human pol α primase complex (Fig. 3A). These protein mixtures were then incubated with two ^{33}P-labeled DNA fragments containing and lacking, respectively, the HPV ori. The nucleoprotein complexes were immunoprecipitated with a monoclonal antibody specific for pol α primase. The coprecipitated DNA present in these complexes was then detected by gel electrophoresis and autoradiography and quantified by PhosphorImager analysis. As shown in Fig. 3B, increasing amounts of pol α primase resulted in a dose-

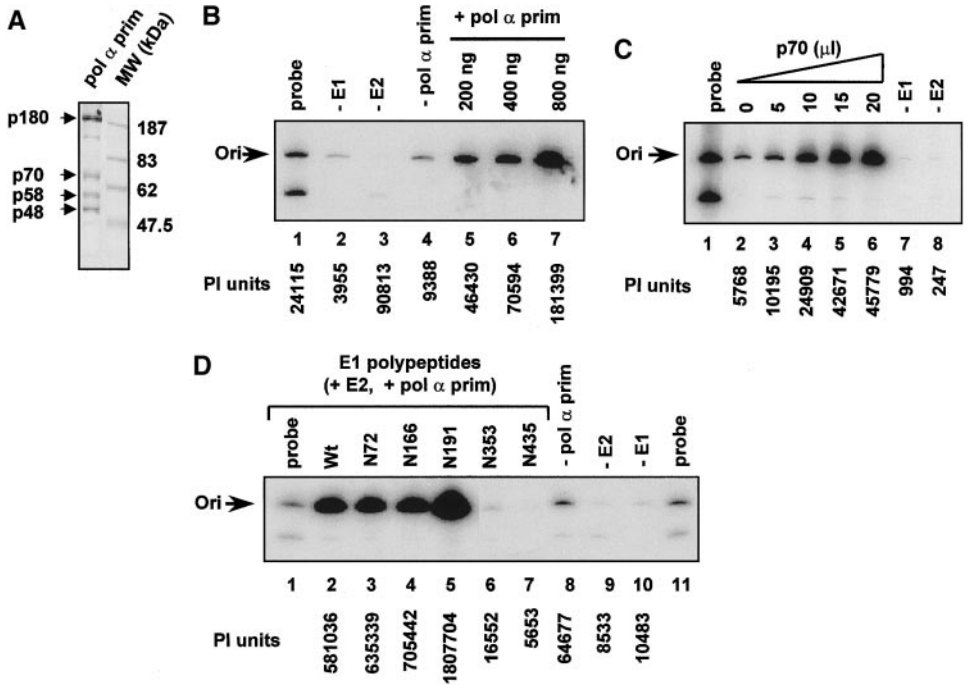

FIG. 3. E1- and E2-dependent association of pol α primase and p70 with the HPV origin and mapping of a domain of E1 required for this process. (A) Purified pol α primase complex. One microgram of purified complex was subjected to SDS–PAGE and stained with Commassie blue. The bands corresponding to the subunits and molecular weight standards are indicated on the left and on the right of the gel, respectively. (B) E1 and E2 promote association of the human polymerase α–primase complex with the HPV origin. A ^{32}P-labeled DNA fragment containing the HPV11 origin of replication as well as a ^{32}P-labeled nonspecific DNA fragment was incubated with 7.5 μl of E2-containing lysate, 25 μl of E1-containing lysate, and increasing amounts of purified pol α primase. The nucleoprotein complexes were immunoprecipitated with a pol α primase-specific monoclonal antibody, and the coprecipitated DNA was analyzed by gel electrophoresis and autoradiography. Lane 1 shows the position of ^{32}P-labeled probes. Lanes 2 and 4 are reactions without E1, E2, or the pol α primase complex, respectively. Lanes 5 to 7 are from complete reactions containing E1 and E2 in addition to 200, 400, and 800 ng of pol α primase complex. (C) E1 and E2 promote association of the p70 subunit of pol α primase with the HPV origin. The reactions were carried out as described in B for using increasing amounts of the FLAG-p70-containing lysate instead of pol α primase. Lanes 2–6 correspond to complete reactions containing 0, 5, 10, 15, and 20 μl of FLAG-p70, respectively. Lanes 7 and 8 correspond to reactions without E1 and E2, respectively. Lane 1 corresponds to the input DNA fragments. (D) Mapping of a domain of HPV11 E1 that is able to promote the association of polymerase α primase with the HPV origin, together with E2. The reactions were carried out as described in A using lysates containing respectively wild-type E1 or one of the truncated polypeptides N72, N166, N191, N435, and N353. Lanes 1 and 11, free DNA fragments; lanes 2 to 7, full-length E1 and truncated E1 proteins; lanes 8 to 10 are reactions in which pol α primase, E2, and E1, respectively, were omitted from the reactions.

dependent immunoprecipitation of the labeled ori fragment, but not of the nonspecific fragment included in the reactions as a control (lanes 5–7). The addition of 800 ng of pol α primase resulted in a 19-fold stimulation of ori DNA recovery compared to the reaction in which pol α primase was omitted (compare lane 7 with lane 4). No ori DNA was detected when E2 was omitted from the reaction (lane 3), but minor amounts of ori fragment were detected when either E1 (lane 2) or pol α primase (lane 4) was omitted. These results suggest that the pol α primase antibody may cross-react weakly with the E2 protein bound to the origin. With 800 ng of pol α primase, approximately four times more ori DNA could be immunoprecipitated using saturating amounts of an antibody directed against E1 than when using an anti-pol α primase antibody (data not shown). This result is consistent with the notion that approximately 25% of the E1-containing complexes formed in the binding reactions contain pol α primase. These results indicate that association of pol α primase with the HPV origin is stimulated by E1 and E2.

The p70 subunit associates in solution with the HPV16 (Masterson *et al.,* 1998) and HPV11 E1 proteins (Conger *et al.,* 1999; this study). To determine whether this subunit can associate with the HPV origin in the presence of E1 and E2, we performed the McKay assay described above under conditions in which the purified pol α primase complex was replaced by a FLAG epitope-tagged p70 subunit made by *in vitro* translation. For this experiment, the FLAG-p70, E1, and E2 proteins were synthesized separately in reticulocyte lysates. Increasing amounts of FLAG-p70 were then added to reactions containing fixed amounts of E1, E2, and the ^{33}P-labeled ori and control fragments. Following incubation, the complexes were immunoprecipitated with an excess of anti-FLAG monoclonal antibody and the coprecipitated DNA was analyzed as described above. The results in Fig. 3C, lanes 3–6, show a dose-dependent increase in the yield of the

ori fragment, indicating that p70 could associate with the HPV origin in the presence of E1 and E2. Twenty microliters of the p70-containing lysate resulted in a ninefold increase in the yield of ori fragment relative to the reaction in which mock lysate was used (compare lane 6 with lane 2). Recovery of the ori fragment was dependent on the addition of FLAG-p70, E1, and E2 since omission of any one of these proteins resulted in only low background levels of ori-fragment precipitation (lanes 2, 7, and 8, respectively). These data support the notion that the p70 subunit, like the pol α primase complex, can be recruited to the HPV11 origin of replication by the E1 and E2 proteins (see Discussion).

Next, the McKay assay described above was performed using ^{35}S-labeled truncated derivatives of E1 made in reticulocyte lysate to identify the domain of E1 that is required, together with E2, for association of pol α primase with the HPV origin. The results in Fig. 3D, lane 5, show that N191 was the smallest domain of E1 that allowed association of pol α primase with the origin. The increased recovery of the ori fragment in the reaction using N191 relative to the reaction performed with wild-type E1 (compare lane 5 to lane 2) was due to the addition of a larger amount of the N191 derivative, which was expressed at higher levels than wild-type E1 in the reticulocyle lysate (data not shown). The N353 and N435 E1 derivatives were inactive in this assay (lanes 6 and 7, respectively). Background levels of DNA were immunoprecipitated when pol α primase, E2, or E1 was omitted from the reaction (lanes 8–10, respectively). Thus, the same region of E1 that is needed to support DNA replication is required, together with E2, for association of pol α primase with the HPV origin.

E1-N353 interacts with the p70 subunit of pol α primase in the absence of ori DNA

The results from the McKay assay demonstrated that a region spanning amino acids 191–649 of E1 is required for E2-dependent recruitment of pol α primase to the HPV origin. Next, we wished to determine whether this entire domain, or a subdomain, is required for interaction with pol α primase in solution, in the absence of DNA. To this end, we established an ELISA assay (see Materials and Methods) to confirm the interaction of E1 with pol α primase and with its p70 subunit and used this assay to analyze the ability of the various truncated E1 proteins to bind to pol α primase. In these experiments, purified pol α primase and GST–p70 (Fig. 4A) were immobilized on plates, followed by incubation with increasing amounts of purified or *in vitro* synthesized E1 protein. Following several washes, the amount of bound E1 protein was detected using an anti-E1 antibody and a secondary antibody coupled to a colorimetric detection. Results presented in Fig. 4B illustrate that purified E1 interacted in a similar dose-dependent manner with GST–p70 and pol α primase, suggesting that the affinities of E1 for both proteins are comparable. No interaction was observed with GST alone as a control (data not shown; also see Fig. 5A).

Next, each of the truncated E1 proteins was tested for its ability to interact with immobilized pol α primase complex or with BSA as a control. The results shown in Fig. 4C indicate that the E1 derivatives N72 and N166 (lanes 3 and 4) bound to pol α primase as well as wild-type E1 (lane 2). The signal for interaction of the E1 derivative N72 (lane 3) was slightly higher than that observed for full-length E1, which was attributable to a higher level of N72 synthesized in the lysate relative to wild-type E1 (data not shown). For the shorter E1 derivatives N353 and N435, we reproducibly observed higher levels of nonspecific binding to BSA. Nevertheless, a signal higher than background could be detected for the binding of N353, but not of N435, to pol α primase (lanes 5 and 6). The mock lysate resulted in a low level of nonspecific signal (lane 7). These results indicate that a region spanning amino acids 353–649 of E1 is able to bind directly to pol α primase.

From the results described above, we predicted that amino acids 353–649 of E1 should be sufficient for interaction with the p70 subunit of pol α primase. To verify this prediction in the context of an *in vivo* interaction, we used the yeast two-hybrid system. In this assay, N-terminal deletion derivatives of E1 were expressed as fusions to the Gal4 activation domain (AD), whereas p70 was expressed as a fusion to the Gal4 DNA-binding domain (BD). Interaction between E1 derivatives and p70 was determined by measuring the levels of expression of β-galactosidase in yeast cells. Full-length E1 protein could not be tested in this system because it activates transcription of the LacZ reporter gene even in the absence of an interacting partner (Titolo *et al.,* 1999). As shown in Fig. 4D, N353 was the smallest E1 derivative that interacted efficiently with p70 (lane 3), giving rise to levels of β-galactosidase that were comparable to those measured for the interaction between N191 (delete-AD) or N330 (delete-AD) with p70 (lanes 1 and 2, respectively). In contrast, N435 interacted only weakly with p70 (lane 4). In a previous study (Titolo *et al.,* 1999), we showed that N435 is able to interact with E2 in the same two-hybrid system, therefore ruling out the possibility that this protein does not accumulate in yeast cells. These two-hybrid results on the interaction of E1 with p70 were confirmed in reciprocal experiments in which the truncated E1 proteins were fused to the Gal4 DNA-binding domain, and p70 was fused to the Gal4 activation domain (data not shown). Altogether, the results shown above demonstrate that pol α primase and its p70 subunit interact with a similar region of E1 (353–649).

Previously, we and others showed that amino acids 353–649 of E1 are sufficient for interaction with the transactivation domain of the E2 protein (Titolo *et al.,* 1999;

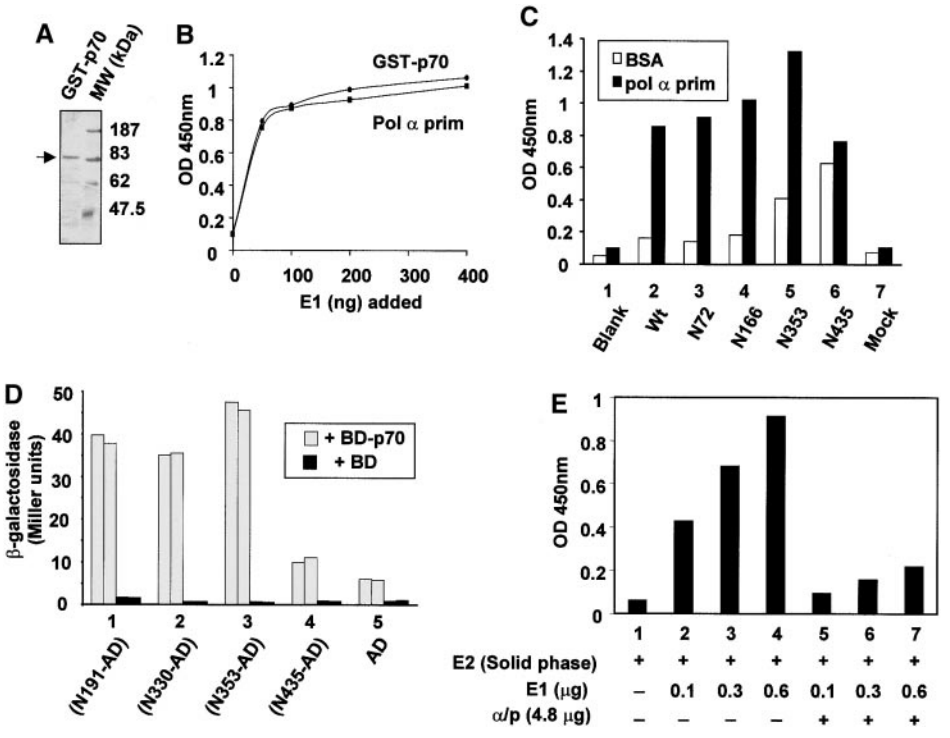

FIG. 4. Interaction of HPV11 E1 and truncated derivatives with polymerase α primase and p70 in ELISA and the yeast two-hybrid system. (A) Purified GST-p70. The protein was processed as described in the legend to Fig. 3A. The arrow on the left indicates the position of the GST–p70 fusion protein. (B) Interaction of the p70 subunit of pol α primase with E1 in ELISA. Purified GST–p70 or purified pol α primase was immobilized on the plate and incubated with increasing amounts of purified E1 (0, 50, 100, 200, and 400 ng). Detection of E1 bound to GST–p70 or pol α primase was carried out as described under Materials and Methods. (C) Mapping of a domain of E1 that interacts with pol α primase using ELISA. Pol α primase and BSA were immobilized on plates and challenged with 80 μl of lysate containing either wild type or one of the truncated derivatives N72, N166, N191, N353, and N435 (lanes 2–6). The background signal with mock lysate is shown in lane 7. (D) Mapping of a domain of E1 that interacts with p70 using the yeast two-hybrid system. The yeast two-hybrid analysis was carried out as described under Materials and Methods with the truncated E1 derivatives fused to the Gal4 activation domain (AD) in combination with p70 fused to the Gal4 BD or in combination with the Gal4 BD as a control. Duplicate β-galactosidase readings are reported for two independent transformants for each combinations of plasmids. Lane 1–4, N191-AD, N330-AD, N353-AD, and N435-AD, respectively. Lane 5, BD (control). (E) The binding of pol α primase complex and E1 to E2 is mutually exclusive. Purified E2 protein was immobilized on plates and challenged with increasing amounts of purified E1 in the presence or in the absence of 4.8 μg (molar excess) of pol α primase complex as indicated. The amount of E1 bound to E2 following incubation was quantified as described under Materials and Methods. Lane 1, no E1 or pol α primase. Lanes 2–4, 0.1, 0.3, and 0.6 μg of E1, respectively. Lanes 5–7, 0.1, 0.3, and 0.6 μg of E1, respectively, in the presence of 4.8 μg of pol α primase.

Masterson *et al.,* 1998; Yasugi *et al.,* 1997; Sun *et al.,* 1998; Zou *et al.,* 1998). Furthermore, it was shown recently that E2 and free p70 compete for binding to E1 in solution (Masterson *et al.,* 1998; Conger *et al.,* 1999). From these results we anticipated that pol α primase should also compete with E2 for binding to E1, if binding occurs through the p70 subunit. This was tested by determining whether pol α primase could compete for the interaction between E1 and E2 in ELISA. Increasing amounts of E1 were tested for binding to immobilized E2 in the presence of a molar excess of pol α primase. As shown in Fig. 4E, lanes 2–4, increasing amounts of purified E1 resulted in a dose-dependent increase in the yield of the E1:E2 complex. The presence of a fixed molar excess of pol α primase resulted in a lower yield of the E1:E2 complexes (lanes 5–7). An approximately twofold molar excess of pol α primase over E1 resulted in an 80% reduction of the E1:E2 complexes (lane 7). These results indicate that pol α primase, like p70, competes with E2 for binding to E1, and provide additional evidence that E1 binds to pol α primase via its p70 subunit.

The p70 subunit of pol α primase inhibits E1-dependent cell-free DNA replication

From the results presented above we predicted that the free p70 subunit of pol α primase should compete with the interaction between E1 and the holoenzyme complex. This was tested in an ELISA competition experiment wherein increasing concentrations of purified GST–p70 or GST were mixed with 0.2 μg of purified E1 prior to incubation with immobilized pol α primase. Because both E1 and pol α primase are DNA-binding proteins whose purified preparations could be contaminated with small amounts of DNA, these experiments were performed in the presence of 20 μg/ml ethidium

bromide to disrupt any protein:DNA interactions that may artificially mediate their association. The amount of E1 that remained bound to the pol α primase complex is shown in Fig. 5A. As anticipated, GST–p70 effectively inhibited in a dose-dependent manner the interaction between E1 and immobilized pol α primase (lanes 2–4), whereas increasing amounts of GST had no measurable inhibitory effect on the interaction (lanes 6–8). These results indicate that the binding of p70 and pol α primase to E1 is mutually exclusive and reinforces the notion that E1 interacts with pol α primase through the p70 subunit.

A logical prediction from the results described above is that an excess of free p70 should prevent the interaction between E1 and pol α primase in the cell-free replication system and result in inhibition of E1-catalyzed DNA replication. Because an earlier study (Conger *et al.,* 1999) detected inhibition of HPV11 E1-catalyzed DNA replication *in vitro* only in the presence of both E1 and E2, we decided to reinvestigate the effect of exogenous p70 on DNA synthesis catalyzed by E1 alone. In these experiments, the E1 produced in the reticulocyte lysate was preincubated with increasing amounts of GST–p70, and GST protein as a control, before addition to the cell-free replication system. The results in Fig. 5B show that increasing amounts of GST–p70 resulted in a dose-dependent inhibition of DNA synthesis, whereas GST did not. We estimated that the lysate contained about 20 ng/μl of E1 and that about 33% of the GST–p70 material is intact (i.e., not proteolysed) after purification. Based on this, a calculated molar ratio of p70/E1 of 2 resulted in a substantial 80% reduction in DNA synthesis, whereas a similar GST/E1 ratio had no measurable inhibitory effect. As expected, the inhibitory effect manifested with increasing GST–p70/E1 ratio, correlated with a dose-dependent loss of replication intermediates, whereas a high GST/E1 ratio had no effect (Fig. 5C, compare lane 1 with lanes 3–6). These data provide further evidence that the interaction of E1 with the p70 subunit of the pol α primase complex is functionally relevant.

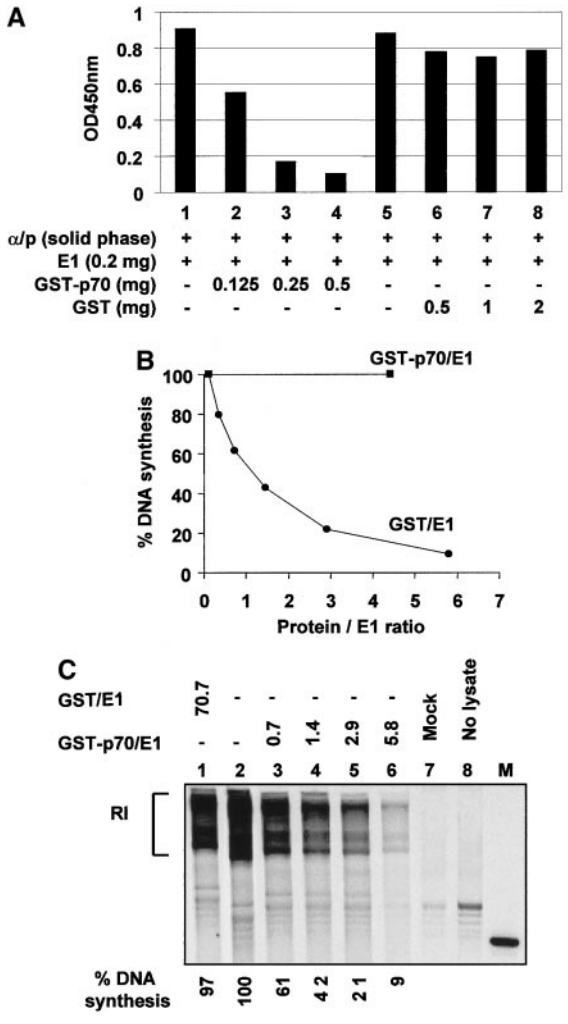

FIG. 5. Inhibition of pol α primase–E1 interaction and E1-catalyzed DNA replication by exogenous p70. (A) The p70 subunit competes for the interaction between E1 and the pol α primase complex in ELISA. Purified pol α primase was immobilized on the plate and incubated with 0.2 μg of pure E1 (lanes 1–8) and either increasing concentrations of GST–p70 (lanes 2–4) or GST (lanes 6–8) as indicated. Detection of E1 bound to pol α primase was carried out as described under Materials and Methods. The values of OD_{450} plotted were corrected by subtracting the background nonspecific signals obtained with the incubation of E1 on BSA. (B) Exogenous free p70 subunit inhibits E1-catalyzed DNA replication *in vitro.* Reactions were assembled with 20 μl of E1-containing lysate and increasing amounts of GST–p70 or GST, followed by addition to the prereplication reaction mix. The samples were processed as described in the legend to Fig. 1A to quantify the level of DNA synthesis. The level of E1-dependent synthesis in the absence of GST–p70 or GST was set at 100%. The graph indicates the percentage DNA synthesis obtained at various GST/E1 and GST–p70/E1 ratios. (C). Exogenous free p70 inhibits formation of DNA replication intermediates. The samples were processed as in Fig. 1B. The molar ratios of GST/E1 (lane 1) and GST–p70/E1 (lanes 3–6) are indicated above the lanes. Lane 2, E1-containing lysate without GST or GST-E1; lane 7, mock lysate; lane 8, no lysate.

DISCUSSION

Domains of E1 required for DNA replication

Our observation that *in vitro* synthesized HPV11 E1 protein can support DNA replication in a human cell-free extract greatly facilitated our characterization of domains of this protein essential for its DNA replication function *in vitro.* We have identified a region encompassing amino acids 166–649 as sufficient for replication of HPV ori DNA *in vitro.* A shorter E1 polypeptide spanning amino acids 191–649 (N191) functioned at less than 25% the efficiency of the wild-type E1 polypeptide, whereas one composed of residues 353–649 (N353) was inactive. We have adapted the McKay assay to demonstrate that *in vitro* synthesized E1, together with E2, can recruit the pol α primase complex to the HPV11 origin. In this assay E1-N191 but not E1-353-649 was able to recruit pol α primase to the HPV ori. We further delineated a region spanning amino acids 353–649 of E1 as being essential for direct interaction with the pol α primase complex in

the absence of DNA. Collectively our results suggest that a region spanning amino acids 191–353 of E1, which encode part of the DNA-binding domain (Sun *et al.,* 1998; our unpublished observation), is required together with the pol α primase interaction domain (residues 353–649) for E1 to support cell-free DNA replication and recruitment of pol α primase to the origin. The C-terminal region of E1 (amino acids 353–649) also contains an ATPase domain that is characteristic of the superfamily 3 of helicases (Gorbalenya *et al.,* 1990) that is undoubtedly required for helicase activity during DNA synthesis. The high degree of conservation of this C-terminal domain among the initiator proteins from the small DNA tumor viruses (*Papovaviridae*), which extends beyond sequences required for ATP and Mg binding, suggests that analogous domains in these proteins may also interact with the human pol α primase complex.

The N-terminal 166 amino acids of E1, which are dispensable for E1 replication function *in vitro,* contain a nuclear localization sequence (Lentz *et al.,* 1993; Leng and Wilson, 1994) and a functional cyclin-binding motif (Ma *et al.,* 1999). Recently, it was shown that cyclin E–Cdk2 kinase can bind to and phosphorylate the E1 proteins of HPV11 (Ma *et al.,* 1999) and BPV (Cueille *et al.,* 1998). Furthermore, it was shown that an HPV11 E1 mutant protein that is defective in binding cyclin E, because of amino acid substitutions in the cyclin-binding motif, functioned at about 15% the efficiency of wild-type E1 in a transient cell-based DNA replication assay (Ma *et al.,* 1999). These results pointed to a possible functional association between E1 and cyclin E in HPV ori DNA replication in cells. Our results indicate that the cyclin-binding motif is not essential for E1-catalyzed DNA synthesis *in vitro,* which is consistent with the findings of another study on BPV E1 that showed that the addition of cyclin E–Cdk2 to an *in vitro* DNA replication reaction resulted in a small (2.5-fold) stimulatory effect (Cueille *et al.,* 1998). Thus, it appears that the cyclin-binding motif in E1, which is highly conserved in the E1 proteins of other papillomaviruses, may be required primarily *in vivo* rather than *in vitro,* perhaps as part of a mechanism to synchronize the onset of viral and host DNA synthesis.

Because of the requirement for the cyclin-binding motif and the NLS of E1 *in vivo,* it is difficult to compare the activities of N-terminally truncated E1 proteins in catalyzing DNA replication *in vitro* to their activities *in vivo.* However, we note that results from earlier transient DNA replication studies are not inconsistent with our *in vitro* findings. Two truncated E1 polypeptides encoding amino acids 186–649 of HPV11 E1 (Sun *et al.,* 1998) and amino acids 132–605 of BPV-1 (Ferran and McBride, 1998) supported transient viral DNA replication, with about 6 and 10%, respectively, the activity of wild-type E1. Based on our *in vitro* results, these truncated E1 polypeptides should be able to interact with E2 and pol α primase and support DNA replication, although with reduced efficiency in the case of the HPV11 protein, which is only 6 amino acids longer than our partially defective N191 E1 polypeptide. The decrease in replication activity *in vivo* of the HPV11 (186–649) polypeptide is likely exacerbated by the combined loss of the cyclin-binding motif and of the NLS. For the BPV E1 mutant protein, loss of replication function is probably due in part to the loss of the cyclin-binding motif rather than to a nuclear targeting defect since this polypeptide contained an engineered heterologous NLS (Ferran and McBride, 1998). It is also possible that the N-terminus of E1 encodes an as yet undiscovered function that is essential for DNA replication *in vivo,* but which, according to our results, would not be essential *in vitro.*

Interaction of E1 with pol α primase

In this study we have provided evidence that confirm and extend the observations by others that the E1 protein associates with the pol α primase complex via the p70 subunit (Masterson *et al.,* 1998; Conger *et al.,* 1999). Specifically we have shown that E1 interacts efficiently with the p70 subunit of the pol α primase complex in an ELISA and in the yeast two-hybrid system and that p70 competes for the interaction between E1 and pol α primase *in vitro.* Similarly to Masterson *et al.* (1998), who found that p70 binds to the C-terminal domain of HPV16 E1 *in vitro,* we determined that amino acids 353–649 of HPV11 E1 are sufficient for interaction with p70 in the yeast two-hybrid system. In addition, we determined that the minimal domain of E1 (amino acids 353–649) that interacts with p70 in yeast is similar to that required for interaction with pol α primase in ELISA. These results strengthen the notion that the C-terminal domain of E1 interacts with the pol α primase complex via its p70 subunit. We have now provided additional evidence that the interaction of E1 with p70 is functionally significant by demonstrating with a modified McKay assay that E1 and E2 can efficiently recruit p70 to the HPV origin, similar to what we observed for the entire pol α primase complex. These results suggest that p70, like pol α primase, can interact with DNA-bound E1. On the basis of these findings and the observation that GST–p70 competes with the interaction between E1 and pol α primase in ELISA, we anticipated that exogenously added GST–p70 would inhibit E1-dependent cell-free DNA replication. Our finding that GST–p70, but not GST, inhibited E1-dependent DNA replication supports the notion that the interaction of E1 with pol α primase, mediated by p70, is essential during DNA synthesis. Although a reasonable interpretation of these results is that GST–p70 inhibits E1-catalyzed cell-free DNA replication by competing with the interaction between E1 and pol α primase, we cannot rule out the possibility that GST–p70 inhibits by another mechanism, such as by binding to pol α primase and inhibiting its activity. However, we find this last possibility

to be unlikely given that all four subunits of pol α primase are purified from cell-free extracts as a stable holoenzyme complex with no indication that p70 can reversibly associate (Gronostajski *et al.,* 1984). Our finding that GST–p70 inhibits E1-catalyzed cell-free DNA replication is in apparent contradiction with the results from another group (Conger *et al.,* 1999), which found that exogenously added p70 did not inhibit, but rather stimulated weakly E1-catalyzed DNA replication in a cell-free system. At this time we do not know the reason for this discrepancy, but we note that there are differences in the source of E1 (*in vitro* translated vs purified recombinant protein), in the type of p70 added to the replication reaction (GST fusion versus polyhistidine tagged), and in the methods used for preparation of cell-free extracts for the replication assays. Our results on the interaction of E1 with p70 are reminiscent of those reported by Collins *et al.* (1993), who demonstrated a functional association of the p70 subunit with SV40 large T antigen, by showing that the T-antigen-binding domain of p70 inhibited T-antigen-catalyzed replication of ori DNA *in vitro.* Thus our results together with these findings suggest that binding to p70 may be a conserved mechanism by which helicases of the small DNA tumor viruses interact with the pol α primase complex.

Finally, it is worth pointing out that similarly to Masterson *et al.* (1998), but in contrast to Conger *et al.* (1999), we were unable to detect an interaction between E1 and the p180 subunit of pol α primase by ELISA (data not shown). Likewise, we found that under our ELISA conditions, enzymatically active p180 was unable to compete with the interaction between E1 and pol α primase (data not shown). From these results we would not anticipate that p180 can inhibit E1-catalyzed cell-free DNA replication by competing with the interaction of E1 with pol α primase.

Recruitment of pol α primase to the origin by E1 and E2

In this study we have shown that the C-terminal domain of E1 (amino acids 353–649) is sufficient for interaction with the pol α primase complex, or with its p70 subunit, in solution. We and others had shown previously that this same domain of E1 is also sufficient for binding to E2 (Titolo *et al.,* 1999; Masterson *et al.,* 1998; Yasugi *et al.,* 1997; Sun *et al.,* 1998; Zou *et al.,* 1998). Our ELISA competition results clearly indicated that the binding of E2 and pol α primase to E1 are mutually exclusive, a result consistent with the recent observation by others that free p70 and E2 compete for binding to E1 (Masterson *et al.,* 1998; Conger *et al.,* 1999). In contrast we obtained the seemingly contradictory result that E1 and E2 can recruit pol α primase or its p70 subunit to the HPV origin, in the modified McKay assay. We reconcile these observations by suggesting a temporally staged reaction in which E2 first promotes the efficient binding and assembly of E1 at the origin, followed by the release of E2 with the recruitment of pol α primase by E1 bound to the origin. The evidence in support of such a model is discussed below. We have found that residues 191–353 of E1, in addition to the C-terminal domain (amino acids 353–649), were required together with E2 for recruitment of pol α primase to the origin. Amino acids 191–352 of E1 are required together with the C-terminal domain (353–649) to bind stably to DNA (Sun *et al.,* 1998; our unpublished observation) but are dispensable for interaction with E2 or with pol α primase in solution (Titolo *et al.,* 1999; this study). Thus, our results suggest that the DNA-binding function of E1 is required for recruitment of pol α primase to the origin by E1 and E2. We have previously argued that under the conditions of our McKay assay, E1 probably oligomerizes upon binding DNA, following its interaction with E2 (Titolo *et al.,* 1999). In support of this hypothesis, we have found recently that mutant E1 proteins that are unable to oligomerize do not bind stably to the HPV origin (in preparation). These results are consistent with the notion that binding and oligomerization of E1 onto DNA, following its interaction with E2, may be a prerequisite for recruitment of pol α primase to the origin. Binding and oligomerization of E1 onto DNA may serve as part of a mechanism to break the interaction between E1 and E2 and allow for interaction with the p70 subunit of the complex. Studies with purified BPV E1 protein have indeed indicated that ATP-stimulated oligomerization of E1 was accompanied by dissociation of E2 from the origin (Lusky *et al.,* 1994; Sanders and Stenlund, 1998). Furthermore, it has been reported recently that pol α primase can interact with multimerized HPV11 E1 in solution (Conger *et al.,* 1999). Although our results have pointed to a role for DNA binding and oligomerization of E1 in recruitment of pol α primase to the origin, they have not addressed whether E2 is retained within the complex nor have they addressed the stochiometry of E1, E2, and pol α primase on the origin. Further experiments with purified proteins will be required to elucidate the temporal order of binding of E2, E1, and pol α primase to the HPV origin and the spatial arrangements of the proteins during this process.

MATERIALS AND METHODS

Expression plasmids

Plasmid pTM1, which contains an IRES from EMCV (Moss *et al.,* 1990), was used to express the various truncated E1 proteins by coupled transcription and translation in a rabbit reticulocyte lysate (Promega TNT kit). Plasmids pTM1-E1 and pCR3-E2, which contain the HPV11 E1 and E2 ORFs downstream of the bacteriophage T7 promoter, respectively, were described previously (Titolo *et al.,* 1999). The various truncated E1 ORFs were amplified by PCR using appropriate primers containing an *Nco*I (forward

primer) or a *Bam*HI restriction site (reverse primer) and cloned as *Nco*I–*Bam*HI cut fragments into pTM1. Sequence of the various pairs of primers will be provided upon request. These deletion derivatives of E1 encode C-terminal polypeptides spanning amino acids 72–649, 166–649, 191–649, 353–649, and 435–649 and are referred to as N72, N166, N191, N353, and N435, respectively, throughout this text (Titolo *et al.,* 1999). To construct the plasmid used for expression of the FLAG-tagged p70 subunit of the human pol α primase complex, the p70 ORF was amplified with the following pair of primers: 5′-CCCAGATCTCCATGGACTACAAGGACGACGATGACAAGTCCGCATCCGCCAGCAGC-3′ (encoding the FLAG epitope) and 5′-CCCGGATCCTCAGATCCTGACGACCTGCAC-3′. The resulting PCR fragment was then digested with *Nco*I and *Bam*HI and inserted between the *Nco*I and *Bam*HI sites of pTM1. ^{35}S-labeled proteins were synthesized in the rabbit reticulocyte lysate according to the manufacturer's instructions (Promega).

Yeast two-hybrid analysis

The yeast two-hybrid analysis to map the region of E1 that interacts with p70 was carried out as described (Titolo *et al.,* 1999). The plasmid vectors used in the assay contain the Gal4-DNA-binding domain (BD) and the Gal4-activation domain (AD), respectively (Durfee *et al.,* 1993). For this assay, the full-length E1 ORF and N-terminal deletion derivatives thereof were cloned as *Nco*I–*Bam*HI fragments into the plasmid containing the Gal4 AD as described (Titolo *et al.,* 1999). The plasmids encoding the Gal4 AD fused to E1 derivatives lacking the N-terminal 191, 330, 353, and 435 amino acids are referred to as 191-AD, 330-AD, 353-AD, and 435-AD, respectively. The p70 ORF was also cloned as a *Nco*I–*Bam*HI PCR fragment into plasmid pAS1 (Durfee *et al.,* 1993) encoding the Gal4 BD to produce plasmid p70-BD. The respective pairs of plasmids were used to cotransform yeast strain Y153 to tryptophan and leucine prototrophy as described (Titolo *et al.,* 1999). Interaction between p70 and E1 proteins in yeast was detected by measuring β-galactosidase activity using a liquid culture assay with chlorophenol red-β-D-galactopyranoside as the colorimetric substrate, as described previously (Titolo *et al.,* 1999). The assay was done for two independent isolates of transformants in duplicate.

E1- and E2-dependent recruitment of pol α primase to ori

The McKay assay (McKay, 1981) that we used previously to study the E2-stimulated binding of the E1 protein onto the HPV11 origin of DNA replication (Titolo *et al.,* 1999) was adapted to study the E1- and E2-dependent recruitment of pol α primase or of the FLAG-epitope-tagged p70 subunit to the HPV11 origin. For this assay, a ^{32}P-labeled DNA fragment containing the HPV11 origin of replication as well as a ^{32}P-labeled nonspecific DNA fragment were prepared as described (Titolo *et al.,* 1999) and incubated with 7.5 μl of E2-containing lysate, 25 μl of E1-containing lysate, and increasing amounts of either the purified pol α primase or *in vitro* translated FLAG-p70. The nucleoprotein complexes were immunoprecipitated with the pol α primase-specific monoclonal antibody, SJK 237, or the FLAG M2 monoclonal antibody (Kodak) coupled to protein G beads (Pharmacia). The coprecipitated DNA was recovered by phenol:chloroform extraction and ethanol precipitation. The DNA samples were then subjected to agarose gel chromatography followed by autoradiography. The recovered ^{32}P-labeled DNA fragments were quantified on a PhosphorImager (Molecular Dynamics).

Purification of pol α primase, p180, GST–p70, and HPV11 E1

The reconstituted pol α primase holoenzyme was purified from insect cells to homogeneity by immunoaffinity purification as described previously, with modifications (Copeland and Wang, 1991; Stadlbauer *et al.,* 1994). The four recombinant baculoviruses were used to coinfect SF21 cells at an m.o.i. of 5 and the cells were grown for 48 h. Cells were harvested, pelleted, and washed with 1 vol of PBS, and the pellet stored frozen at −80°C until needed. A 10 μg/ml concentration of each of the protease inhibitors leupeptin, antipain, aprotinin, and antipain (Sigma) and 1 mM pefabloc (Pentafarm AG) were included in all buffers. Cells were thawed and resuspended into 2 vol of lysis buffer (50 mM Tris, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$) and dounced with a homogenizer 20 times, and NaCl was slowly added to the lysate to a final concentration of 0.35 M. The lysate was placed at 4°C with stirring for 20 min after which it was spun at 35,000 rpm for 45 min. The supernatant was adsorbed overnight to an immunoaffinity column containing the SJK237 mAb against the p180 subunit (Copeland *et al.,* 1991). The column was washed with 20 vol of Buffer A (25 mM Tris, pH 7.5, 1 mM EDTA, 10% glycerol) containing 0.35 M NaCl and 0.01% NP-40 (Sigma), and the enzyme was eluted with Buffer A containing 0.5 M NaCl, 50% ethylene glycol, and 0.01% NP-40. Fractions were assayed for polymerase and primase activities as described previously (Stadlbauer *et al.,* 1994), and fractions enriched for both activities were pooled and dialyzed in 20 mM Tris, pH 8.5, 0.1 M NaCl, 50% glycerol, 0.25 mM EDTA, 0.01% NP-40, and 1 mM DTT for 5 h. The enzyme was concentrated on a Millipore Ultrafree-15 Centrifugal Filter Device as suggested by the manufacturer, and the enzyme was aliquoted, frozen, and stored at −80°C. The yield of the preparation from 0.5 liter of cells was 1.9 mg of enzyme/2500 units of polymerase and 3000 units of primase. The free p180 subunit was purified and assayed as described above, following expression of the recom-

binant p180 baculovirus in insect cells. The specific activity of polymerase was 4218 units/mg.

A construct that has the p70 gene in the expression vector pGEX-2T (Pharmacia) was used for the production and purification of GST–p70 from bacterial strain BL21 (provided by Dr. H. P. Nasheuer, Institut fur Molekulare Biotechnologie, Jena, Germany). The cells were diluted 1/100 from an overnight culture into Circlegrow medium (Bio 101), supplemented with 0.2% glucose, and 100 μg/ml ampicillin and grown at 37°C for 1.5 h to an OD_{600nm} of 0.4. Then 1 mM IPTG was added, and cells were cooled to 22°C and grown at this temperature for 5 h. The cells were pelleted and stored at −80°C until needed. The cells were thawed and resuspended into 4 vol of lysis buffer, and then lysozyme was added (1 mg/g cells). The cells were incubated on ice for 15 min, followed by the addition of Brij 58 detergent to 0.06%. The lysate was sonicated and cleared by centrifugation at 35,000 rpm for 1 h, and the supernatant was coupled to glutathione–Sepharose beads (Pharmacia) for 1 h. The beads were washed with 20 vol of Buffer A containing 0.8 M NaCl, 0.01% NP-40, and 1 mM DTT followed by 2 vol of Buffer A containing 0.1 M NaCl, 0.01% NP-40, and 1 mM DTT. The protein was eluted in the latter buffer adjusted to 100 mM Tris, pH 8, and containing 20 mM reduced glutathione. Fractions containing the protein were pooled and dialyzed overnight against Buffer A containing 50 mM NaCl, 0.01% NP-40, and 1 mM DTT. The protein was concentrated on a Millipore filter, aliquoted, and stored frozen at −80°C. The HPV11 E2 was purified from a baculoviral expression system as described (Kuo *et al.,* 1994).

HPV11 E1 was expressed as an N-terminal six-histidine fusion protein in insect cells using the baculovirus system and purified to near homogeneity by nickel chelate affinity chromatography. Details of the expression and purification procedures will be published elsewhere.

In vitro replication assay

The E1 and E1/E2 cell-free DNA replication was carried out essentially as described for other viral cell-free systems with minor modifications (Kuo *et al.,* 1994). The CSH 293 cell-free extracts were prepared as described (Amin and Hurwitz, 1992). A 60-μl prereplication mix reaction contained 40 mM creatine phosphate, pH 7.5, 7 mM $MgCl_2$, a 200 μM concentration each of rUTP, rGTP, and rCTP, 4 mM ATP, a 100 μM concentration each of dGTP, dCTP, and dTTP, 20 μM dATP, 100 μg cell extract, 0.3 μg of HPV11 ori-containing plasmid, pN9 (Lu *et al.,* 1993), and 100 μg/ml creatine kinase. The E1 used for *in vitro* replication was derived from coupled *in vitro* transcription/translation of plasmid pTM1-E1 or derivatives thereof. We routinely used 10 to 20 μl of the transcription/translation reaction for each replication reaction. We estimated previously (Titolo *et al.,* 1999) by Western blotting of different amounts of E1-containing lysate and of purified E1 as standards that approximately 5 to 20 ng of E1 were synthesized per microliter of *in vitro* transcription/translation reaction. Replication reactions were preincubated without E1 at 37°C for 30 min followed by the addition of [^{33}P]dATP (specific activity of dATP ~7000 cpm/pmol) and the E1-containing lysate. Following this, the reactions were then incubated for 90 min at 37°C. Reactions were terminated with 0.1% SDS, 40 mM EDTA, and 0.5 mg/ml Proteinase K (Boehringer Mannheim) at 37°C for 30 min, and 20 μl was used for TCA precipitation as described previously (Amin and Hurwitz, 1992). For analysis of DNA replication products, 1 vol of TE was added to the remaining reaction followed by two extractions with phenol:chloroform (1:1). The DNA was coprecipitated with 5 μg sonicated salmon sperm DNA, 0.5 vol of 7.5 M ammonium acetate, and 2.5 vol of 100% ethanol. The DNA was rinsed with 70% ethanol, dried, resuspended into 20 μl TE, and subjected to electrophoresis on a 1.2% agarose gel in TBE buffer. The gel was dried and exposed to X-OMAT AR film (Kodak).

ELISA

To detect an interaction between E1 and the pol α primase complex, 300 ng of the purified complex was plated on Nunc Immunoplates overnight at 4°C. The next day, the solution was discarded and the wells blocked with 200 μl of PBS:3% BSA, for 1 h. The solution was discarded, and the E1 protein was added, followed by incubation for 2 h at room temperature. The solution was discarded, and the wells washed four times with 200 μl PBS:0.05% Tween 20 (wash buffer). One hundred microliters of PBS:0.5% BSA:0.05% Tween 20 (antibody buffer) containing 0.125 μl of a polyclonal antibody directed against the C-terminal 14 amino acids of HPV11 E1 (K71) was added, followed by incubation for 1 h. The wells were washed as described above, and 100 μl of antibody buffer containing 1/5000 dilution of goat anti-rabbit conjugated horseradish peroxidase (GAR-HRP) (Sigma) was added to wells, followed by incubation at room temperature for 1 h. The wells were washed and developed with *o*-phenylenediamine substrate as described by Sigma. Colorimetric readings were taken at $OD_{450\,nm}$ using a Titertek plate reader. To detect an interaction between p70 and E1, 500 ng of GST-p70 was plated and challenged with the E1 protein as described above.

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