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# Human Hsp70 and Hsp40 Chaperone Proteins Facilitate Human Papillomavirus-11 E1 Protein Binding to the Origin and Stimulate Cell-free DNA Replication*

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**Human papillomavirus replication initiator, the E1 helicase, binds weakly to the origin of DNA replication. Purified human chaperone proteins Hsp70 and Hsp40 (HDJ-1 and HDJ-2) independently and additively enhanced E1 binding to the origin. The interaction between E1 and Hsp70 was transient and required ATP hydrolysis, whereas Hsp40 bound to E1 directly and remained in the complex. A peptide of 20 residues spanning the HPD loop and helix II of the J domain of YDJ-1 also stimulated E1 binding to the origin, alone or in combination with Hsp70 or Hsp40. A mutated peptide (H34Q) had a reduced activity, while an adjacent or an overlapping peptide had no effect. Neither Hsp70 nor the J peptide altered the E1/DNA ratio in the complex. Electron microscopy showed that E1 mainly bound to DNA as a hexamer. In the presence of Hsp40, E1 primarily bound to DNA as a dihexamer. Preincubation of chaperones with viral E1 and template shortened the lag time and increased replication in a cell-free system. Since two helicases are essential for bidirectional replication of human papillomavirus DNA, these results demonstrate that, as in prokaryotes, chaperones play an important role in the assembly of preinitiation complexes on the origin.**

Molecular chaperones regulate many cellular processes such as protein folding and translocation and the assembly and disassembly of multiprotein complexes (reviewed in Ref. 1). Two major *Escherichia coli* chaperones DnaK and DnaJ were originally identified as genes required for the initiation of bacterial or bacteriophage DNA replication. Mutations in *DnaK* and *DnaJ* lead to defects in DNA and RNA synthesis, cell division, and proteolysis (for reviews, see Refs. 2–4). DnaK is a weak ATPase with the ability to bind unfolded polypeptides (5, 6). DnaJ functions as a dimer (7, 8) and is considered a co-chaperone, since it dramatically stimulates the ATPase activity of DnaK in the presence of GrpE (6, 9). Together, these proteins facilitate the binding of the replication initiator protein to the origin (*ori*) and the initiation of DNA replication (Ref. 7; for reviews, see Refs. 10–14).

The families of eukaryotic heat shock protein 70 (Hsp70/Hsc70) and heat shock protein 40 (Hsp40) have a high degree of homology to DnaK and DnaJ, respectively. Hsp70 and Hsp40 proteins are co-localized to the cytosol and also function in the nucleus (15, 16). As in prokaryotes, the Hsp40 proteins function as co-chaperones of Hsp70, but they also have weak, independent activity (5, 7, 17–20). All of the DnaJ homologues, such as HDJ-1, HDJ-2, and YDJ-1, members of the human and yeast Hsp40 family, have a conserved J domain at the amino terminus. Truncated *E. coli* or YDJ-1 containing only the J domain is sufficient to modulate the ATPase activity of DnaK/Hsp70 (20, 21). The corresponding J domain of the human Hsp40 proteins is also thought to mediate interactions with Hsp70 and regulate its ATPase activity (for a review, see Ref. 8). Within the J domain, there is a highly conserved HPD tripeptide loop flanked by two $\alpha$-helices, designated helix II and helix III (22–25). Mutations in the HPD motif or in the external faces of the $\alpha$-helices significantly reduce its activity (21, 26–28). Recent evidence suggests that helix II interacts with Hsp70 and that peptides encompassing helix II and the HPD loop inhibit the co-chaperone function of DnaJ/Hsp40, presumably because they compete for binding to Hsp70 (28, 29).

A connection between chaperone proteins and eukaryotic DNA replication was found recently. SV40 and polyomavirus DNA replication require the viral large T antigen (Tag),[1] which serves as the initiator (for a review, see Ref. 30). In addition, the SV40 Tag is also the viral oncoprotein capable of transforming cells. The amino-terminal domain shared by the large, small, and tiny Tag of the SV40/polyomaviruses exhibits a sequence and functional homology with the J domain of the Hsp40 family (31–36). The J domain, especially the HPD tripeptide loop, of the SV40 Tag plays a critical role in interacting with the Hsc70 (31, 33), stimulating its ATPase activity (35, 36). This interaction is proposed to promote efficient viral DNA replication (33) and also correlates with its transforming ability (35, 37), suggestive of a role of chaperone function in eukaryotic DNA replication. But the mechanisms of the Tag J domain function are not understood.

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[1] The abbreviations used are: Tag, T-antigen; HPV, human papillomavirus; E1BS and E2BS, E1 and E2 protein binding site, respectively; EMSA, electrophoretic mobility shift assay; bp, base pair(s); EE, glutamate-rich epitope; AMP-PNP, 5′-adenylyl-$\beta,\gamma$-imidodiphosphate; ATP$\gamma$S, adenosine 5′-*O*-(thiotriphosphate); EM, electron microscopy.

As do SV40 and polyomaviruses, the papillomaviruses have served as model systems for eukaryotic DNA replication. Human papillomaviruses (HPVs) are prevalent and medically significant human pathogens (for a review, see Ref. 38). To support HPV *ori* replication, the virus supplies two proteins, E1 and E2, while the host cell provides all other known replication proteins, including DNA polymerases $\alpha$ and $\delta$, replication protein-A, replication factor-C, proliferating cell nuclear antigen, and topoisomerases (for reviews, see Refs. 39 and 40). The partition of the HPV replication activities into two proteins simplifies their functional dissections relative to the Tag of SV40/polyomavirus. To this end, we and others have established cell-free replication systems for HPV or bovine papillomavirus type 1 (BPV-1) *ori* plasmids by using human 293 cell or mouse cell extracts supplemented with E1 and E2 proteins purified from insect cells infected with recombinant baculoviruses (41, 42) or have used bacterially expressed proteins (43–45).[2]

Both the E1 and E2 proteins are necessary for initiation of replication, but only E1 is additionally required for elongation (46). The E1 protein is analogous to the SV40/polyomavirus Tag in its role during replication. It has specific as well as nonspecific DNA binding activities, and it is an ATPase and helicase (46–51). The E1 protein also interacts with the 180-kDa catalytic subunit and the 70-kDa subunit of the DNA polymerase $\alpha$ (52, 53),[3] thereby recruiting the host replication machinery to the *ori*. The E2 protein binds to its cognate binding sites (E2BS) in the *ori*, which consists of one E1BS and several copies of E2BS, and is located within the viral enhancer and promoter region. The bound E2 protein helps recruit E1 to the origin (39, 40). In addition, E2 protein, in its role as a transcription factor, excludes nucleosome formation over the *ori*, facilitating the assembly of the initiation complex (54). However, neither the E1 nor the E2 protein has a J domain.

In this report, we tested the hypothesis that host chaperone proteins are involved in the initiation of HPV DNA replication, as shown for bacteriophages. Our results demonstrate that these chaperones function independently and additively to facilitate the association between the E1 protein and the *ori* as demonstrated by electrophoretic mobility shift assays (EMSAs). Hsp70 interacts with E1 transiently and requires ATP hydrolysis. In contrast, Hsp40 or a 20- amino acid-long J peptide remains associated with E1 independent of ATP, and this is the first example of the J domain of Hsp40 specifically associating with a protein with ATPase activity other than Hsp70/Hsc70. Electron microscopic examination of the E1-*ori* DNA complexes demonstrates that, in the presence or absence of Hsp70 or the J peptide, the majority of the E1 bound to *ori* DNA has a size corresponding to a hexameric complex. Strikingly, Hsp40 promotes an efficient formation of a bilobed multimeric E1 complex on the DNA. Each lobe had the size of a hexamer, as required for bidirectional replication known to occur for papillomaviruses. Preincubation of chaperone proteins with E1 and *ori* shortens the lag time in cell-free replication. These results strongly suggest that the human chaperone proteins facilitate the assembly of the preinitiation complex on the HPV *ori*.

## MATERIALS AND METHODS

*Plasmids*—HPV *ori* plasmids pUC7730–99, pUC7874–99, and pUC7874–20 were named by inclusive nucleotides of the HPV-11 DNA fragment cloned from the double-stranded circular genome of 7933 bp (46). For example, pUC7730–99 spans nucleotide 7730–7933/1–99 and contains three copies of E2BS flanking a single E1BS. pUC7874–99 also contains the same *ori* sequences but is shorter at the 5′ sequence. pUC7874–20 contains only one copy each for E2BS and E1BS. Each was chosen for a different assay as described. Hsp70 and HDJ-1 expression vectors, pET-11a Hsp70 and pET-21d HDJ-1, were obtained from Dr. Richard Morimoto (55, 56). The cDNA of HDJ-2 was obtained from Dr. T. Mohanakumar (57). A polymerase chain reaction amplification was used to add an *Nco*I site at the 5′-end and a *Bam*HI site at the 3′-end of the cDNA, which was then inserted into the multiple cloning site of pET9d to generate the HDJ-2 expression vector, pET-9d HDJ-2.

*Protein Expression and Purification*—HPV-11 E1 protein, which was tagged at the amino terminus with a glutamate-rich epitope (EE), was purified from recombinant baculovirus-infected Sf9 cells (42, 46). Human chaperone proteins Hsp70, HDJ-1, and HDJ-2 were expressed and purified from *E. coli* BL21(DE3)pLysS as follows. Protein induction was achieved by the addition of 0.5 mM isopropylthiogalactoside to cells at midlog phase for 3 h at 37 °C. Cells were disrupted by sonication in lysis buffer (20 mM HEPES-$K^+$, pH 7.5, 1.5 mM $MgCl_2$, 400 mM NaCl, 1 mM dithiothreitol). The purification of Hsp70 was modified from a protocol described previously (55). Briefly, the soluble fraction was diluted with buffer Q (20 mM Tris-HCl, pH 7.0, 10 mM 2-mercaptoethanol) to a final NaCl concentration of 50 mM, applied to a 10-ml Q-Sepharose column (Bio-Rad), and eluted with a 0–500 mM NaCl gradient in buffer Q. The fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and those containing Hsp70 were pooled and applied to an ATP-agarose column (Sigma). Hsp70 proteins were eluted with 1 mM ATP or ADP in buffer Q, recaptured in a 1-ml Q-Sepharose column (Econo-Pac, Bio-Rad) and then eluted with 300 mM NaCl in Buffer Q, yielding a purity of about 95% at 2 mg/ml. Identical procedures were used to purify HDJ-1 and HDJ-2. Hsp40 eluted from the Q-Sepharose column with buffer Q containing 250 mM NaCl was then diluted with buffer Q to a final concentration of 50 mM NaCl and applied to an S-Sepharose column (Econo-Pac, Bio-Rad). Proteins were eluted at about 150–200 mM NaCl in a 50–300 mM NaCl gradient. The final purity of Hsp40 was over 95% at 0.3–0.5 mg/ml.

*Cell-free Replication Assay*—The HPV-11 origin-containing plasmid pUC7874–99 with one E1BS and three E2BS was used as an *ori* template. The conditions for cell-free replication have been described (42, 46) with the following modifications. The amount of viral E1 protein used was reduced due to a more active viral protein preparation, and the 293 cell extracts were minimized to reduce the endogenous chaperone proteins. In the preincubation experiments, 12 ng of HPV-11 E1 protein (in 0.1 mg/ml bovine serum albumin), 40 ng of template, 4 mM ATP were preincubated at room temperature for 15 min in the presence or absence of 100 ng of Hsp70 and 100 ng of HDJ-1 proteins. The balance of the replication reaction (including 8 ng of E2) and 2.5 $\mu$Ci of [$\alpha$-$^{32}$P]dCTP (Amersham Pharmacia Biotech) were then added, followed by an additional incubation at 37 °C for various times. The reaction was terminated by a stop solution containing SDS and proteinase K. Replication products were analyzed on a 0.8% agarose gel and exposed to Hyperfilm (Amersham Pharmacia Biotech) or a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

*Peptides and Antiserum*—Peptides containing sequences from the J domain of YDJ-1 protein, p21–40, p41–60, and p21–40 H34Q were as described (28). Peptide p33–52 was synthesized by Dr. David Klapper of the University of North Carolina at Chapel Hill. Mouse monoclonal antibody KA2A5.6 (Hsp40/HDJ-2) and SPA-815 (Hsc70/Hsp70) were purchased from Neomarkers and StressGen. Hybridoma cells producing monoclonal antibody against the EE epitope were obtained from Dr. Gernot Walter (58), and the antibodies were harvested from the culture medium and enriched by Q-Sepharose column chromatography.

*EMSA*—The 130-bp fragment that contained the minimal HPV-11 *ori* of one E1BS and one E2BS was liberated from pUC7874–20 by digestion with *Eco*RI and *Hin*dIII and then 5′-end labeled with T4 polynucleotide kinase (Life Technologies, Inc.) and [$\gamma$-$^{32}$P]ATP (Amersham Pharmacia Biotech). In a 10-$\mu$l reaction mixture, 80 ng of HPV–11 EE-E1 protein were mixed with 100 ng of poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech) and 20 fmol (about 6000 cpm) of labeled DNA fragments in the binding buffer (25 mM HEPES-$K^+$, pH 7.5, 4 mM ATP, 7 mM $MgCl_2$, and 1 mM dithiothreitol) with or without purified chaperone proteins or peptides as indicated in each figure. The binding reactions were conducted at room temperature for 20 min followed by the addition of glutaraldehyde to a final concentration of 0.2% and incubation for another 10 min at room temperature. For supershift experiments, antibodies were added at 10 min into the initial incubation. The protein-DNA complexes were then analyzed in a 1.5% agarose

[2] J.-S. Liu, S.-R. Kuo, T. R. Broker, and L. T. Chow, unpublished results.

[3] K. Conger, J.-S. Liu, S.-R. Kuo, L. T. Chow, and T. S.-F. Wang, unpublished results.

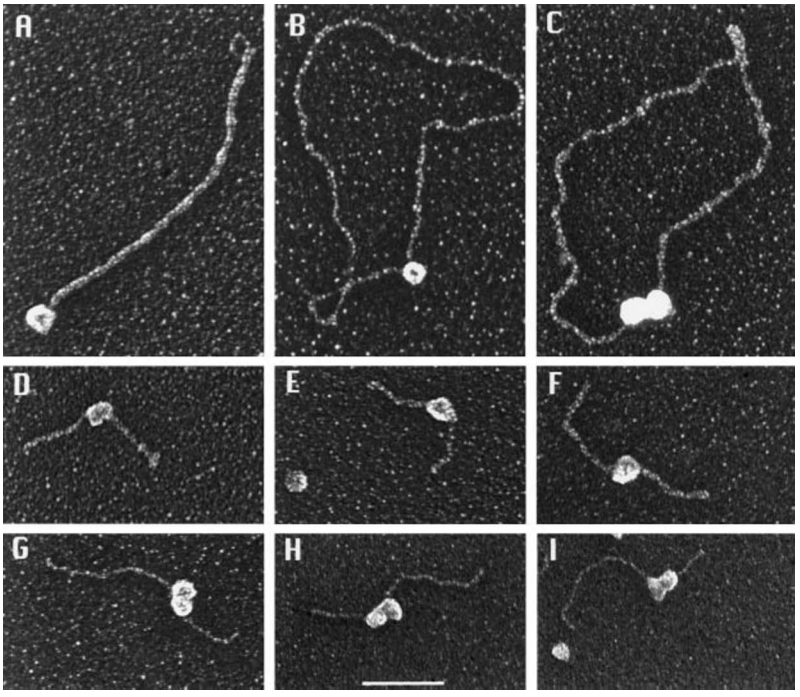


FIG. 1. **Visualization of E1 protein bound to HPV-11 origin-containing DNA.** HPV E1 protein alone (*A–F*) or together with HDJ-2 protein (*G–I*) was incubated with the 2.77-kilobase pair plasmid pUC7874–20 containing the HPV origin of replication (*A–C*) or a 600-bp restriction fragment thereof in which the E1BS is located near the center (*D–I*) as described under "Materials and Methods." Following incubation, the samples were fixed with glutaraldehyde, adsorbed to thin carbon foils, dehydrated, air-dried, and rotary shadow-cast with tungsten in a high vacuum. The plasmid DNA in *A* is a supercoil, while those in *B* and *C* are relaxed circles. In the absence of HDJ-2, the majority of the bound E1 complexes are of a size equivalent to one hexamer, although dihexamers (*C*) can be seen. In the presence of HDJ-2 (*G–I*), 86% of the complexes are a bilobed dihexamer. The somewhat different sizes of the E1 complexes on the DNA molecules reflect differences in the amount of metal deposited during different shadow castings. Images are shown in reverse contrast. *Bar*, 500 bp.

gel with 1× Tris acetate/EDTA buffer at 4 °C. Gels were dried and exposed to Hyperfilm for 18 h or to a PhosphorImager.

*Co-immunoprecipitation Assays*—100 ng of purified HPV-11 EE-E1 protein, 500 ng of purified HDJ-2 protein, and 2 $\mu$g of bovine serum albumin were incubated in the binding buffer (20 mM HEPES-$K^+$, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol) at room temperature for 20 min before 100 ng of KA2A5.6 anti-Hsp40 antibody and 10 $\mu$l of protein-A Sepharose 4B beads (Amersham Pharmacia Biotech) were added. After washing with binding buffer containing 0.1% Nonidet P-40, the precipitates were resuspended in sample buffer (20 mM Tris-HCl, pH 7.0, 2% SDS, 100 mM 2-mercaptoethanol) and boiled for 5 min. Proteins were analyzed by 8% SDS-polyacrylamide gel electrophoresis, transferred to a Hybond-C membrane (Amersham Pharmacia Biotech), and probed with anti-EE antibody and then with peroxidase-conjugated goat anti-mouse IgG antibody (Sigma). Signals were detected by enhanced chemiluminescent reactions (ECL) (Amersham Pharmacia Biotech) and exposed to Hyperfilm.

*Electron Microscopy*—100 ng of a 600-bp *Pvu*II DNA fragment from the plasmid pUC7730–99 or the supercoiled plasmid was used for electron microscopy. This plasmid was chosen because the E1BS was approximately located at the center of the restriction fragment. The DNA was incubated with 600 ng of E1 with or without 1.5 $\mu$g of chaperone protein (Hsp70 or HDJ-2) or 2.8 $\mu$g of peptide p21–40 in a buffer containing 20 mM HEPES-$K^+$, pH 7.5, 7 mM $MgCl_2$, 4 mM ATP, and 10% glycerol at room temperature for 20 min. The protein-DNA complexes were then cross-linked with 0.3% glutaraldehyde at room temperature for 10 min and separated from free proteins by filtration through 2-ml columns of Bio-Gel A-5m (Bio-Rad) equilibrated with 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA. The filtrate was mixed with a buffer containing 2 mM spermidine and 0.15 M NaCl, adsorbed to glow-charged thin carbon foils, dehydrated through a graded water-ethanol series, and rotary shadow-cast with tungsten as described (59). Samples were visualized in a Philips CM12 electron microscope. Micrographs were scanned from negatives using a Nikon multiformat film scanner, and the contrast was optimized and panels were arranged using Adobe Photoshop. Morphometry measurements were conducted by using NIH Image Software and a Summagraphics digitizer coupled to a Macintosh computer programmed with software developed by J. D. G.

## RESULTS

*HPV-11 E1 Protein Binds to DNA as a Hexamer*—The physical state of HPV-11 E1 protein was examined by electrophoresis through a native 5–12% gradient polyacrylamide gel. In the presence or in the absence of ATP, the majority of the E1 protein existed as monomers, with the minority as homo-oligomers as revealed by Western blot (data not shown). When native E1 protein was adsorbed to thin carbon supports and examined by transmission electron microscopy (EM) after negative staining, fields of particles of different sizes representing monomers to oligomers were observed. At a high concentration (50 $\mu$g/ml) and in the presence or absence of 4 mM ATP or AMP-PNP, the majority of E1 was oligomeric. Large particles of a similar size, possibly hexamers (see below), were common, and occasionally ring-like oligomers were observed (data not shown). For size determination, glutaraldehyde-fixed E1 protein was rotary shadow-cast on separate supports but side-by-side with apoferritin (443 kDa). The projected areas of the large uniform E1 particles ($n$ = 69) were measured and compared with those of the apoferritin particles ($n$ = 23). The results showed that E1 particles were 1.0 times the average volume of the apoferritin (60), a value consistent with a hexamer of a 73-kDa protein.

As demonstrated previously by EMSA (46), HPV-11 E1 binding to the *ori* is ATP-dependent. To visualize the binding of E1 to HPV *ori* DNA, superhelical forms of the 2.77-kilobase pair plasmid pUC7874–20 were incubated with E1 protein in the presence of ATP, and the complexes were fixed with glutaraldehyde, filtered through Bio-Gel A-5m to remove the fixative, and prepared for EM. A small fraction of the supercoiled DNA became nicked upon contact with the glow-charged grids. Supercoiled (Fig. 1*A*) or open circular DNAs (Fig. 1*B*), each associated with a large protein complex, were observed. The majority of the complexes (73%; $n$ = 38) each contained a single spherical particle of a size close to that of the free E1 hexamers in the background. The remaining complexes (27%; $n$ = 14) contained two side-by-side spherical particles on the DNA, here termed bilobed (Fig. 1*C*). When a 600-bp fragment of pUC7730–99 containing the E1BS close to the center was incubated with E1 protein and ATP (Fig. 1, *D–F*), E1 particles were observed on 20–30% of the DNA fragments. In 80–90% of these complexes, the E1 particle was close to the center, while 10–20% showed E1 close to an end, possibly due to nonspecific binding, since no nonspecific competitor was included as was in EMSA (see below). Analyses of the projected size of individual E1 protein particles on the DNA showed that they were 1.2–1.3 times the average volume of the apoferritin particles, probably

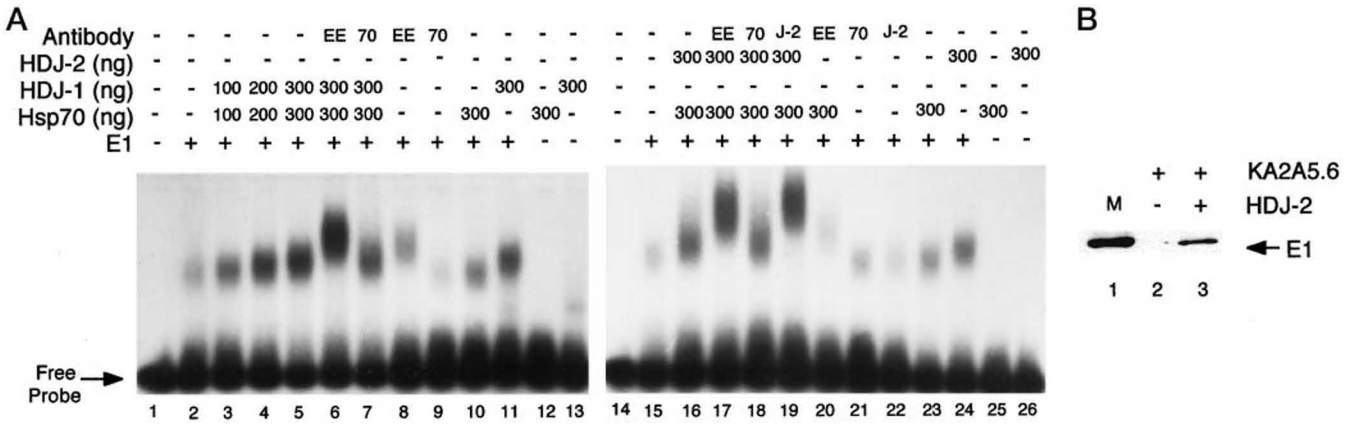


FIG. 2. **Hsp70 and Hsp40 independently enhance HPV-11 E1 protein binding to DNA.** *A*, 10 fmol of $^{32}$P-end-labeled 130-bp DNA fragment containing one E1BS was used as a probe in this and subsequent EMSAs. The inclusion of each reaction component is indicated by a *plus sign* (80 ng of E1) or in amounts (Hsp70, HDJ-1, or HDJ-2, in ng), while omission is indicated by a *minus sign above* each *lane*. Monoclonal antibody to the epitope tag of the E1 protein (labeled *EE*), Hsp70 (SPA815, labeled *70*), or HDJ-2 (KA2A5.6, labeled *J-2*) was added 10 min into the binding reaction. *Lanes 1–13* and *14–26* were separate experiments. *B*, co-immunoprecipitation of HDJ-2 and E1 proteins. E1 protein was immunoprecipitated from a mixture containing 100 ng of E1 and 500 ng of HDJ-2 by anti-HDJ-2 antibody KA2A5.6 and protein A-Sepharose beads (*lane 3*) but not when HDJ-2 was omitted (*lane 2*). *Lane 1* shows 50 ng of input E1 protein.

reflecting the inclusion of the DNA mass. Measurement of the length of the protein-free 600-bp DNA and comparison with the length of the DNA associated with E1 complexes showed no consistent or significant foreshortening of the DNA due to E1 binding, suggesting that the DNA does not significantly wrap about the protein particle. Some indication of DNA bending about the E1 complex was noted (Fig. 1) with a bending angle measuring 60 ± 30°.

*Human Chaperone Proteins Hsp70 and Hsp40 Enhance HPV-11 E1 Protein Binding to the Origin of Replication Independently and Additively*—The sequence-specific DNA binding activity of HPV E1 protein, compared with HPV E2 protein, was relatively weak (46). To test whether host chaperone proteins play a role in the binding of E1 to DNA, human Hsp70, HDJ-1, and HDJ-2 proteins were purified from *E. coli* harboring expression vectors, and then their effects on E1-*ori* complex formation were examined by EMSA (Fig. 2*A*). A 130-bp restriction fragment containing the minimal origin with one E1BS and one E2BS suitable for such assays was used as a substrate in this and subsequent EMSA. Representative results are shown. The addition of Hsp70 and Hsp40 (HDJ-1 or HDJ-2) (in a 1:2 molar ratio) dramatically stimulated the formation of slower migrating complexes as compared with those formed in their absence (compare *lanes 3–5* to *lane 2*; and *lane 16* to *lane 15*). In the presence of 300 ng each of Hsp70 and Hsp40 (*lanes 5* and *16*), the amount of DNA fragment shifted was 400% of that shifted in their absence (*lanes 2* and *15*). E1 was essential for this complex formation in the presence or in the absence of chaperones, since no slow migrating complexes were detected when E1 was omitted (*lanes 1*, *12*, *13*, *14*, *25*, and *26*). Furthermore, antibody to the EE tag of the E1 protein supershifted the E1-DNA complexes (*lanes 8* and *20*) (46) as well as the complexes formed in the presence of E1 and the chaperone proteins (*lanes 6* and *17*).

We also examined E1 protein binding to the *ori* in the presence of each chaperone protein alone. Unexpectedly, Hsp70 or Hsp40 independently stimulated E1-*ori* complex formation, although not as effectively as when both were present. At 300 ng, Hsp70 (approximately a 4:1 molar ratio of Hsp70 to E1) produced a maximal enhancement of 140% as compared with its absence. The migration of the complexes, however, was not altered (Fig. 2*A*, compare *lane 10* with *lane 2* and *lane 23* with *lane 15*). Thus, we conclude that Hsp70 is not retained in E1-DNA complexes and that Hsp70 enhances the association of E1 and DNA without altering the stoichiometry in the complex. Relative to Hsp70, increasing amounts of Hsp40 alone produced a more dramatic enhancement of E1-DNA complex formation. At 300 ng, the stimulation was 250% over E1 alone, and the effect did not yet reach a plateau (data not shown). The migration rate was slower than for complexes formed in its absence (Fig. 2*A*, compare *lane 11* with *lane 2* and *lane 24* with *lane 15*) and was similar, if not identical, to that when both chaperone proteins were present (compare *lane 11* with *lanes 3–5* and *lane 24* with *lane 16*). These quantitative analyses indicate that the stimulatory effects of Hsp70 and Hsp40 are additive (compare *lanes 10* and *11* with *lane 5* and *lanes 23* and *24* with *lane 16*) rather than synergistic, as previously observed in other reactions with these chaperone proteins.

*Hsp40 Remains Associated with the E1-DNA Complexes*—Because the HDJ-1 polyclonal antibodies interacted with E1-DNA complexes nonspecifically (data not shown), it was not possible to determine whether HDJ-1 remained in the complexes. In contrast, neither the Hsp70- nor the HDJ-2-specific antibodies (SPA-815 and KA2A5.6) showed any nonspecific interaction with E1-DNA complexes (Fig. 2*A*, *lanes 9*, *21*, and *22*). We therefore performed supershifts by using these antibodies. In the presence of Hsp70 and Hsp40, the addition of Hsp70-specific antibody SPA-815 did not alter the mobility of the complexes (*lanes 7* and *18*). Thus, Hsp70 was not associated with the E1-*ori* complexes in the presence of Hsp40, nor was it in the absence of Hsp40 (*lanes 10* and *23*). Most informatively, the addition of the EE antibody, the HDJ-2-specific antibody KA2A5.6, or both further retarded the migration rates of the protein-DNA complexes (Fig. 2*A*, *lanes 6*, *17*, and *19*; data not shown, but see Fig. 3). These data demonstrate the association of HDJ-2 with the E1-DNA complexes. Because of the similarity in the mobility of the complexes, we suggest that HDJ-1 was also present in the slower migrating complexes. Because of the presence of HDJ proteins in the E1-DNA complex, it was not possible to determine whether the stoichiometry of E1 to DNA was altered.

Since Hsp40 did not bind the DNA substrate (Fig. 2*A*, *lanes 13* and *26*), the EMSA results just described suggest a direct interaction between Hsp40 and E1 proteins. We demonstrated this association by co-immunoprecipitation assays (Fig. 2*B*). E1 was precipitated by HDJ-2-specific antibody on protein A-Sepharose beads when HDJ-2 protein was present, but not when HDJ-2 was omitted (Fig. 2*B*, compare *lanes 3* and *2*). This

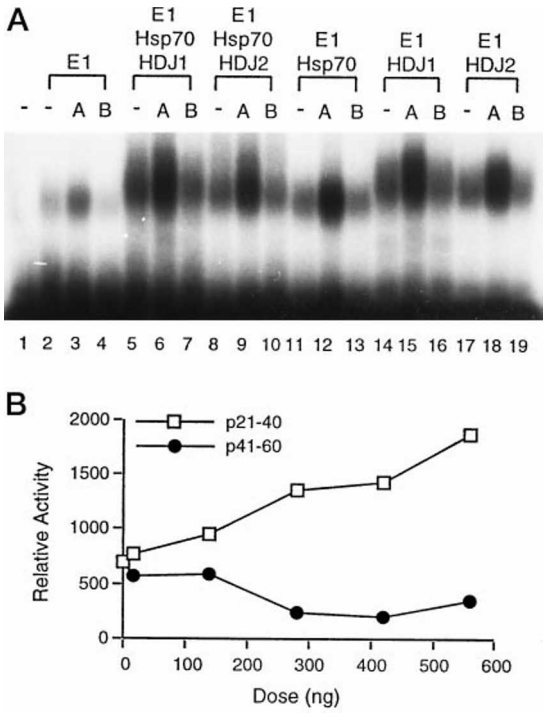


FIG. 3. **J domain peptide stimulates E1 DNA binding activity.** *A*, 700 ng of J domain peptide p21–40 (marked *A*) and the adjacent peptide p41–60 (marked *B*) derived from YDJ-1 were incubated with 80 ng of E1 and 300 ng of Hsp70, HDJ-1, or HDJ-2, or combinations thereof as indicated *above lanes 3–19*. *Lane 1*, free probe; *lane 2*, E1 and the probe. *B*, PhosphorImager quantification of E1-DNA complexes based on EMSA in the presence of increasing amounts of peptides p21–40 (*open squares*) or p41–60 (*filled circles*).

protein-protein interaction was independent of the presence or absence of *ori* DNA or ATP (data not shown).

*J Domain Peptide Enhances E1 Binding to the DNA Origin*—At higher concentrations, the yeast Hsp40 protein YDJ-1 also stimulated E1 binding to the *ori* (data not shown). To investigate whether the J domain plays any role in the interaction between Hsp40 proteins and E1, we examined the effects on E1 EMSA of two previously characterized peptides from YDJ-1. Peptide p21–40 corresponds to helix II and the HPD loop, and p41–60 spans helix III and the nonstructured tail (27). The inclusion of 700 ng of p41–60 (Fig. 3*A*, *lanes B*) alone or in combinations with Hsp70, HDJ-1, or HDJ-2 did not significantly alter the E1 DNA binding activity (*lanes 4*, *7*, *10*, *13*, *16*, and *19*) relative to complex formation in its absence (*lanes 2*, *5*, *8*, *11*, *14*, and *17*). In contrast, the p21–40 peptide (Fig. 3*A*, *lanes A*) alone stimulated the E1 DNA binding activity to 225% relative to that in its absence (Fig. 3*A*, compare *lanes 3* and *2*). A titration experiment confirmed the specific stimulation by p21–40 and a slight repression by p41–60 at very high doses (Fig. 3*B*). The effects of p21–40 in conjunction with Hsp70 and Hsp40 were also additive, reaching an activity of 440–560% relative to that achieved in the presence of E1 alone (compare *lanes* 6 and *9* with *lane 2*) or about 160–200% relative to stimulation observed in the presence of individual chaperone proteins (compare *lane 12* with *lane 11*, *lane 15* with *lane 14*, and *lane 18* with *lane 17*). Furthermore, contrary to the inhibitory effect of the J domain peptide in the Hsp70/Hsp40 chaperone activity (28), the p21–40 peptide enhanced rather than suppressed the stimulatory effects of Hsp70 and Hsp40, reaching an activity of 530 or 675% relative to E1 alone (compare *lanes 6* and *9* to *lane 2*) or 150% relative to those observed in the presence of Hsp70 and Hsp40 (*compare lane 6* with *lane 5* and *lane 9* with *lane 8*). These results support the conclusion that

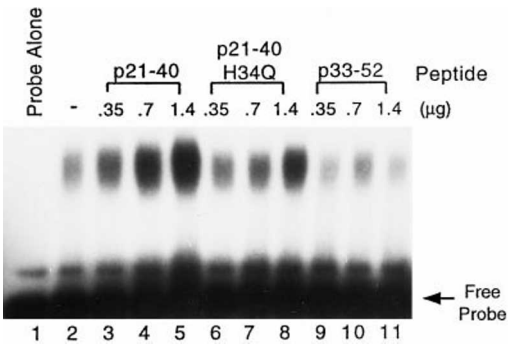


FIG. 4. **Differential effects of J domain peptides on E1 EMSA.** E1 EMSA was conducted in the absence (*lane 2*) or in the presence of increasing amounts of p21–40 (*lanes 3–5*), p21–40 H34Q (*lanes 6–8*), or p33–52 (*lanes 9–11*).

the preponderance of the stimulatory effects of Hsp70 and Hsp40 or the J peptide originates from independent activities rather than from a collaboration between them. We believe that the stimulatory effect of the p21–40 peptide in the presence of Hsp40 exists because the activity of Hsp40 was not yet at a plateau at the concentrations used. Although the J peptide increased the amount of E1-DNA complexes, it did not alter the migration rate of the complex (Fig. 3*A*, compare *lane 3* with *lane 2*, *lane 15* with *lane 14*, and *lane 18* with *lane 17*) as the full-length protein did (Fig. 2). These results demonstrate that the stoichiometry of the E1 protein and the DNA molecule in the complexes was not modified by the J peptide.

*Both Helix II and the HPD Loop of the Hsp40 Protein Are Required for Function*—To delineate further the region in Hsp40 proteins that binds E1, we tested the activities of two additional peptides of YDJ-1 in parallel with the functional p21–40. The overlapping peptide p33–52 spans the HPD loop and the helix III region, while p21–40 H34Q contains the same amino acid sequence as p21–40 except for a histidine to glutamine change at residue 34 in the highly conserved HPD motif. This H34Q mutation in YDJ-1 abolishes its ability to interact with Hsp70 (28). Neither peptide alone was able to bind to the *ori* DNA in the absence of E1 (data not shown). p33–52 had little effect on E1-DNA complex formation (Fig. 4, compare *lanes 9–11* with *lane 1*). Stimulation of complex formation by p21–40 was dose-dependent and, at 1.4 $\mu$g, the E1 DNA binding was stimulated to 425% (*lanes 3–5*). At the same dose, the stimulatory activity of the mutated peptide p21–40 H34Q was reduced to 220% (*lanes 6–8*) relative to complex formed in its absence. These results demonstrate the importance of both the helix II and HPD loop and support the interpretation that the stimulatory effects observed are due to specific interactions between the J domain and the E1 protein.

HDJ-2-specific antibody KA2A5.6 recognized peptide p21–40 but not p33–52, p41–60, or p21–40 H34Q in a dot blot assay (data not shown, but see Fig. 5). This antibody was able to supershift the E1-*ori* complex formed in the presence of p21–40 (Fig. 5, compare *lane 9* with *lane 3*). The addition of KA2A5.6, however, reduced the amount of complexes formed, suggesting that the antibody partially destabilized the complexes. The addition of both E1 and HDJ-2 antibodies further retarded the migration of the complexes (Fig. 5, compare *lane 3* with *lanes 5* and *6* and *lanes 11* and *12*). In contrast, the presence of p41–60 had no effect on complex formation in the presence or absence of either antibody (*lanes 3*, *7*, *10*, and *13*). We conclude that, similar to HDJ-2 and HDJ-1, p21–40 remained bound to the E1-DNA complexes and that the helix II and HPD loop constitute the minimal domain required for E1 association.

*Hsp70 and Hsp40 Stimulate E1 DNA Binding via Different Mechanisms*—Binding of HPV-11 E1 proteins to the *ori* re-

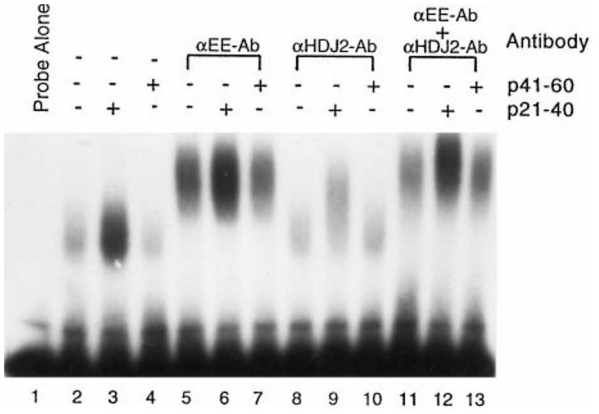


FIG. 5. **J domain peptide p21–40 remains associated with E1-DNA complexes.** E1 EMSA was conducted in the absence (−) or in the presence (+) of 700 ng of p21–40 or p41–60 as indicated *above* each *lane*. In some reactions, anti-EE antibody (*lanes 5–7*), anti-HDJ-2 antibody (*lanes 8–10*), or both antibodies (*lanes 11–13*) were added 10 min into the reaction. *Lanes 1* and *2* show the free probe and DNA binding by E1 protein alone.

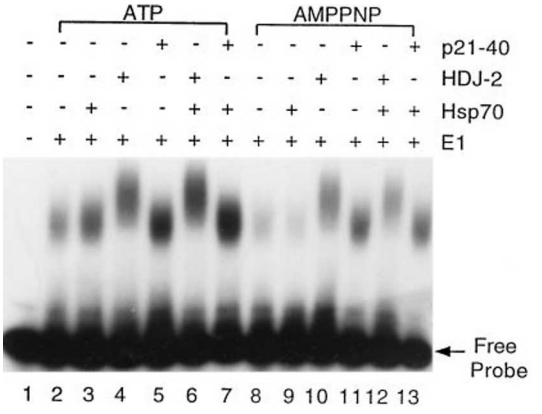


FIG. 6. **Hsp70 but not HDJ-2 requires ATP hydrolysis to enhance E1 protein binding to DNA.** E1 EMSA was performed in the absence (−) or in the presence (+) of 300 ng of Hsp70, 300 ng of HDJ-2, 700 ng of p21–40, Hsp70 plus HDJ-2, or Hsp70 plus p21–40 as indicated *above* each *lane*. *Lanes 1* and *2* show the free probe and DNA binding by E1 protein alone. The DNA binding reactions contained 4 mM ATP (*lanes 2–7*) or AMP-PNP (*lanes 8–13*). The Hsp70 protein used in these experiments was eluted from the ATP agarose column by 1 mM ADP.

quires the presence of ATP, but the hydrolysis of ATP is not essential. Replacement of ATP with AMP-PNP, ATPγS, or ADP resulted in a reduced activity, while AMP conferred no activity (47) (Fig. 6, compare *lane 2* with *lane 8*)[4] On the basis of these observations, we tested the role of ATP hydrolysis in the Hsp70- and Hsp40-stimulated DNA binding activity of E1. To eliminate ATP in the Hsp70 protein preparation, the Hsp70 used in these experiments was eluted from ATP-agarose with ADP. As shown in all of the previous experiments, in the presence of 4 mM ATP, Hsp70, HDJ-2, or p21–40, each stimulated E1 binding to DNA to about 150, 220, or 270%, respectively, relative to the control (Fig. 6, compare *lanes 3–5* with *lane 2*), and an additive effect between Hsp70 and HDJ-2 or the J peptide p21–40 was observed (compare *lanes 6* and *7* with *lane 2*), reaching an activity of 375% (*lane 7*). In contrast, in the presence of 4 mM AMP-PNP, there was no stimulation by Hsp70, while a similar extent of enhancement, which ranged from 240 to 280%, by HDJ2 or p21–40 in the presence or absence of Hsp70 was evident (compare *lanes 9–13* with *lane 8*). These results demonstrate that the Hsp70 function requires the hydrolysis of ATP, whereas the Hsp40 or the J peptide activity does not, consistent with the notion that Hsp40 proteins and the J peptide function independently of Hsp70 in this E1-*ori* binding assay.

*Hsp40 Stimulates the Formation of E1 Dihexamer on the Origin DNA*—We examined the effect of chaperone proteins on E1 binding to the *ori* sequence by electron microscopy. Incubations were carried out with the 600-bp *ori*-containing DNA fragment and E1 protein in the presence of Hsp70, Hsp40 (HDJ-2), or p21–40. The complexes were then prepared for EM as described above. When Hsp70 or p21–40 was added, the size or structure of the DNA-bound E1 complex was not altered by visual inspection of the micrographs (data not shown), but the fraction of DNA molecules containing an E1 complex appeared to increase, in agreement with the EMSA (Figs. 2–6). Interestingly, the addition of HDJ-2 resulted in a shift of the protein complexes from single E1 hexamer on the DNA to the bilobed complex (Fig. 1, *G–I*). The frequency of bilobed complexes present on the 600-bp fragment increased to 86% (54 out of 63), with 14% single spheres. Measurement of the projected areas of the single spheres on the DNA and the individual halves of the bilobed complexes showed that they were 1.2–1.3 times the mass of the apoferritin marker. These mass measurements were not sensitive enough to detect the possible presence of the Hsp40 protein in the complexes. No particles were seen associated with DNA when E1 was omitted from the incubation, demonstrating that the bilobed particles were not composed entirely of Hsp40. These experiments reveal that Hsp40 promotes the formation of dihexamers of E1 bound to the *ori*.

*Chaperone Proteins Reduce the Lag Time and Stimulate the HPV-11 ori Replication in a Cell-free System*—Purified HPV-11 E1 and E2 proteins and human 293 cell extracts support replication of HPV *ori*-containing plasmids in reactions containing all eight nucleoside triphosphates. Western blot assays with Hsp70/Hsc70- and Hsp40-specific antibodies showed highly abundant chaperone proteins in the 293 cell extracts, and attempts to deplete these proteins from the 293 extracts were unsuccessful (data not shown). Besides, other related chaperone proteins may also have similar functions. To test whether the stimulatory effects of the chaperone proteins on E1 binding to the *ori* have any functional significance in HPV-11 *ori* replication, we used a reduced amount of cell extracts to minimize the endogenous chaperone proteins and then added increasing amounts of purified Hsp70 and HDJ-1 to the replication reactions. The template pUC7874–99 with one E1BS and three E2BS was chosen because replication efficiency increases with the copy number of E2BS.[4] The incorporation of [α-$^{32}$P]dCTP into the slowly migrating replication intermediates and fast migrating form I product was stimulated by the addition of the chaperone proteins (data not shown, but see Fig. 7). In a parallel experiment, purified chaperone proteins did not alter the extent of replication from the SV40 *ori* in the presence of Tag. This latter result is not entirely unexpected, since the Tag already possesses a J domain, and it had a much shorter lag time during cell-free replication than HPV *ori* replication in a side by side comparison (data not shown).

In our cell-free replication assay system, there is invariably a 45–60-min lag time before detectable [α-$^{32}$P]dCTP is incorporated into slowly migrating replication intermediates (42). Since the chaperone proteins enhanced the E1 binding to the *ori*, we tested the hypothesis that preincubation of E1, *ori* plasmid, and the chaperone proteins could shorten the lag time. After preincubation in a buffer with ATP at room temperature for 15 min, the balance of a standard replication reaction mixture and [α-$^{32}$P]dCTP was then added, and repli-

[4] J.-S. Liu, S.-R. Kuo, T. R. Broker, and L. T. Chow, unpublished results.

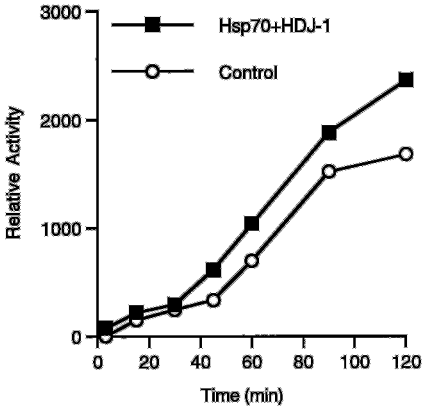


FIG. 7. **Hsp70 and HDJ-1 shorten the lag time and stimulate HPV-11 *ori* replication.** HPV-11 E1 protein, an efficient *ori* template pUC7874–99, and ATP were preincubated at room temperature for 15 min in the presence (*squares*) or in the absence (*circles*) of 100 ng each of purified Hsp70 and HDJ-1 proteins. 8 ng of E2, the balance of the replication reaction mixture, and 2.5 $\mu$Ci of [$\alpha$-$^{32}$P]dCTP were then added at the end of the preincubation and incubated for various lengths of time as indicated. Replication products were quantified by PhosphorImager.

cation was allowed to proceed for different lengths of time before determination. The lag time was shortened by 10–15 min (Fig. 7, *squares*) when compared with the control experiment in which bovine serum albumin rather than chaperones was included during preincubation (*circles*). The extent of replication was increased by a small amount. Both observations were highly reproducible in repeat experiments. These results are consistent with the interpretation that the E1-*ori* complexes formed in the presence of the chaperone proteins were functional and that Hsp70 and Hsp40 facilitated the binding of E1 to the *ori*. We suggest that there are at least two rate-limiting steps in the initiation of replication. One is the binding of E1 protein to the *ori* and the formation of dihexamer. The other is the recruitment of the DNA polymerase $\alpha$ and other replication protein such as replication protein-A to the *ori* marked by the viral proteins. The preincubation with chaperone proteins may have circumvented the first but not the second rate-limiting step.

## DISCUSSION

In this study, we have demonstrated that the Hsp70 and Hsp40 chaperone proteins independently and additively enhance the E1 protein binding to the HPV origin of replication via different mechanisms. The interaction between E1 and Hsp70 is transient and requires ATP hydrolysis. Hsp70 is not retained in the complexes, nor does it alter the ratio of E1 protein to *ori* DNA in the complexes as assessed by EMSA (Figs. 2*A*, 3, and 6). In contrast, the function of Hsp40 does not depend on ATP hydrolysis, and the chaperone protein remains stably bound to the E1-DNA complexes (Figs. 2, 5, and 6). The association of E1 and Hsp40 also occurs in the absence of *ori* (Fig. 2*B*), and the 20-amino acid J domain peptide p21–40 from YDJ-1 consisting of the highly conserved HPD loop and helix II is necessary and sufficient for stimulating E1 binding to *ori*. A H34Q mutation in p21–40 or in the full-length YDJ-1 protein reduced this activity (Figs. 3 and 4 and data not shown).

One of our most intriguing findings is that the intact Hsp40 altered the physical state of the E1 bound to *ori*. The purified HPV-11 E1 protein exists as a mixture of monomers and oligomeric complexes in a dynamic equilibrium when assayed under a variety of conditions such as native gel electrophoresis,[4] velocity sedimentation through a sucrose gradient, gel filtration,[3] and EM visualization of negatively stained protein (data not shown). Measurement of shadow-cast E1 protein showed that the majority of the large particles had a size corresponding to a hexamer. The majority of E1 bound to *ori* DNA was also hexameric in size in the presence or absence of Hsp70 or the J peptide (Fig. 1*A*, *B*, and *D–F*, and data not shown). This result is different from a previous conclusion that BPV-1 E1 protein binds to the *ori* as a trimer (61). However, more recent data indicate the BPV-1 E1 helicase is a hexamer (62). If the HPV E1 hexamer-*ori* complex is replication-competent, replication would be unidirectional. Most interestingly, Hsp40 promoted efficient formation of bilobed E1 complexes on *ori* with each half having the mass of a single hexamer (Fig. 1, *C* and *G–I*). Such a dihexameric E1-DNA complex would be expected of a bidirectional replication complex, and indeed, bidirectional replication has been demonstrated for HPV-11 in laryngeal papillomas and for BPV-1 in transformed cells (41, 44, 53, 63). The low percentage of dihexamers formed in the absence of Hsp40 may be due to a small amount of J proteins of Sf9 cells that might be present in the purified E1 preparation. We propose that dihexamer formation is mediated by virtue of the fact that Hsp40 functions as a dimer. Together with the binding data with YDJ-1 peptides (Figs. 3–5), our results also suggest that the dimerization domain of the J proteins lies partially or completely outside the p21–40 peptide because the peptide does not alter the E1:DNA ratio. However, since dihexamer also formed in the absence of purified Hsp40, the possibility cannot be ruled out that the function of Hsp40 is to stabilize E1 dihexamers that form inefficiently in the absence of Hsp40 but that Hsp40 is not responsible for the formation of the dihexamer. We do not favor this interpretation, since it would suggest the existence of two populations of dihexamers, one without Hsp40 and another with Hsp40.

The replication-competent polyomavirus and SV40 Tag form a dihexamer that surrounds the DNA strand at the origin or at the replication fork (64–66). Incubation of Tag with ATP-$Mg^{2+}$ in the absence of *ori* DNA promotes the formation of hexamers that are replication-incompetent because circular DNA cannot thread through the preformed hexamers (66). Although the distribution of monomers and oligomers of the HPV-11 E1 was not altered upon the addition of ATP-$Mg^{2+}$ when analyzed by gel electrophoresis,[4] we assume that to become replication-competent the multimeric E1 complexes must assemble from monomers or smaller oligomers upon binding to the origin, but not before. Based on this premise, we offer several possible explanations for the stimulation of E1 binding to *ori* DNA by the chaperone proteins.

The first interpretation of our observations is that the chaperone proteins merely help refold denatured or otherwise nonfunctional E1 aggregates in the E1 protein preparation. We consider this possibility unlikely, since Hsp70 did not alter the proportions of monomeric and oligomeric E1 proteins in the native gel electrophoresis, nor did the incubation with Hsp40 enhance the ATPase activities of the E1 protein (our unpublished results). Furthermore, the J domain peptide p21–40, which does not function as a chaperone and cannot dissociate protein complexes (20), nevertheless stimulated E1 binding to the *ori* (Figs. 3–6). A second possibility is that the stimulatory effects of Hsp40 are fortuitous in that the positively charged helix II of the J domain happens to bind to the negatively charged EE tag of the E1 protein. This interpretation is not supported by several observations. 1) Although the YDJ-1 H34Q protein and the p21–40 H34Q peptide contain the same negatively charged helix II and the mutated peptide had no known chaperone activity (28), both were able to stimulate E1 binding to *ori*, albeit at a reduced activity (Fig. 4 and data not shown). 2) The binding activity of another HPV-11 E1 protein, which is tagged by both polyhistidine and the FLAG peptide

(IBI) at the N terminus, was also similarly stimulated.[4] 3) The antibodies against HDJ-2 and EE can simultaneously bind to the complexes of E1-*ori*-Hsp40 or the J peptide as shown by supershifts in EMSA (Figs. 2 and 5). 4) Neither Hsp70 nor Hsp40 affected the binding of HPV-11 E2C purified from Sf9 cells to E2BS as a dimer (data not shown). E2C is an amino terminus-truncated E2 protein that binds to E2BS as does the full-length E2 protein (46). These results strongly suggest that the interactions between E1 and chaperone proteins have a considerable degree of specificity and, in particular, that Hsp40 does not function as a conventional chaperone protein.

The third interpretation, which we favor, is that chaperones are integral to the pathway of forming replication-competent dihexameric E1 complexes on the origin. In support of this hypothesis, preincubation of E1 protein with *ori* in the presence of Hsp70 and Hsp40 and ATP for 15 min correspondingly reduced the lag time and increased the extent of replication (Fig. 7). We propose that the Hsp70 chaperone protein may somehow alter the conformation of monomeric or oligomeric E1 protein or that it may possibly dissociate the preformed oligomers to facilitate reassembly on the *ori* as a hexamer, a role somewhat related to the chaperone functions proposed for the assembly of bacterial or phage replication initiators on each respective *ori* (7, 11, 14).

However, the manner by which HPV E1 protein takes advantage of the attributes of host HDJ proteins has not been described before. In its normal role as co-chaperone, the amino-terminal portion of Hsp40 containing the J domain interacts with Hsp70, whereas the carboxyl terminus binds the substrate (20, 67). In contrast, the J domain of Hsp40 binds to the E1 protein independent of Hsp70 (Figs. 2*B* and 3–6). Although our data do not rule out the possibility that the carboxyl-terminal peptide binding domain of the Hsp40 also interacts with the E1 protein, the occupancy by E1 of the J domain may have prevented further interaction between Hsp40 and Hsp70 and abolished the synergistic effect between these two chaperone proteins. All of the results presented indicate that Hsp40 protein does not function as a conventional co-chaperone of Hsp70 during E1 binding to *ori*. Rather, E1 is bound to Hsp40 in its capacity as an ATPase. To our knowledge, this is the first example of an association of Hsp40 via the J domain with an ATPase that is not a chaperone protein. This interpretation explains the independent and additive effects of Hsp70 and Hsp40 on the binding of E1 to the *ori*. It is also consistent with a reduced association between E1 and the p21–40 H34Q peptide or the full-length YDJ1 H34Q protein (Fig. 4 and data not shown), in agreement with the structural information that suggests helix II and the HPD motif to be the minimal sequences required for Hsp40 in interaction with Hsp70 (25). Thus, the effects of Hsp40 on E1 binding to *ori* appear to be 2-fold. First, the highly conserved J domain interacts with E1 and alone can stimulate or stabilize E1 binding to DNA. Second, Hsp40 functions as an assembly factor for the formation of dihexamer E1 on the *ori*. The dihexameric E1-DNA complex may have an increased stability relative to a single hexameric E1-DNA complex, accounting for the much more significant stimulatory effect of Hsp40 on E1 binding to *ori* compared with the effects of Hsp70 or the J peptide (Fig. 3).

In conclusion, using EMSA, EM, and cell-free replication, we have demonstrated that the human chaperone proteins Hsp70 and particularly Hsp40, including HDJ-1 and HDJ-2, play important roles in promoting the binding of an E1 hexamer to and the formation of an E1 dihexamer on the HPV-11 origin of replication prior to interactions with the host DNA polymerase to initiate DNA replication. At this juncture, we do not know whether these particular host proteins or other related members actually participate in these interactions *in vivo*. Thus, unlike the SV40 or polyomaviruses, which contain an endogenous J domain in their T antigens, the papillomavirus utilizes the host Hsp40 to assist the E1 protein function.

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