The Carboxyl-Terminal Region of the Human Papillomavirus Type 16 E1 Protein Determines E2 Protein Specificity during DNA Replication

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The mechanism of DNA replication is conserved among papillomaviruses. The virus-encoded E1 and E2 proteins collaborate to target the origin and recruit host DNA replication proteins. Expression vectors of E1 and E2 proteins support homologous and heterologous papillomaviral origin replication in transiently transfected cells. Viral proteins from different genotypes can also collaborate, albeit with different efficiencies, indicating a certain degree of specificity in E1-E2 interactions. We report that, in the assays of our study, the human papillomavirus type 11 (HPV-11) E1 protein functioned with the HPV-16 E2 protein, whereas the HPV-16 E1 protein exhibited no detectable activity with the HPV-11 E2 protein. Taking advantage of this distinction, we used chimeric E1 proteins to delineate the E1 protein domains responsible for this specificity. Hybrids containing HPV-16 E1 amino-terminal residues up to residue 365 efficiently replicated either viral origin in the presence of either E2 protein. The reciprocal hybrids containing amino-terminal HPV-11 sequences exhibited a high activity with HPV-16 E2 but no activity with HPV-11 E2. Reciprocal hybrid proteins with the carboxyl-terminal 44 residues from either E1 had an intermediate property, but both collaborated more efficiently with HPV-16 E2 than with HPV-11 E2. In contrast, chimeras with a junction in the putative ATPase domain showed little or no activity with either E2 protein. We conclude that the E1 protein consists of distinct structural and functional domains, with the carboxyl-terminal 284 residues of the HPV-16 E1 protein being the primary determinant for E2 specificity during replication, and that chimeric exchanges in or bordering the ATPase domain inactivate the protein.

Human and animal papillomaviruses contain a double-stranded circular DNA genome. Replication of plasmids containing a papillomaviral origin sequence (ori) in cell-free systems is dependent on virus-encoded E1 and E2 proteins and the host DNA replication machinery (17, 43). Transfection of E1 and E2 expression plasmids into mammalian cells can support transient replication of an ori-containing plasmid (7, 42). The ori sequences are highly conserved among all papillomavirus types and consist of a known or putative E1 protein binding site and multiple copies of E2 protein binding sites in close proximity (for reviews, see references 8 and 37). The E1 protein binding sites are similar but not identical in sequence among different virus types, whereas all E2 proteins bind to the consensus sequence ACAN₅GGT. Of all papillomavirus proteins, E1 is the most conserved. It is an ATPase and helicase (5, 15, 16, 25, 36, 44) and has sequence homology to the ATPase domain of the simian virus 40 (SV40) T antigen (9, 26), the initiator for SV40 ori replication (see Fig. 2A). As does the T antigen, the E1 protein binds to the ori and unwinds DNA in the presence of the host single-stranded DNA binding protein RPA and topoisomerase I. The human papillomavirus (HPV) and bovine papillomavirus type 1 (BPV-1) E1 proteins are thought to function as a helicase at the replication fork, since each is required during elongation (20). The BPV-1 E1 protein is known to interact with the 180-kDa catalytic subunit of the host DNA polymerase α, thereby bringing host replication proteins to the unwound ori (3, 30). The E2 protein is a transcription factor but also serves multiple functions in ori replication. It interacts with E1, stabilizing its binding to the ori (12, 24, 28, 34, 38, 43), and it also helps recruit host replication proteins into the initiation complex (20). In addition, E2 may prevent nucleosome formation around the ori in vivo (19). By virtue of its strong and specific affinity to the E2 protein binding site and its interaction with the E1 protein, the E2 protein is critical to the initiation of replication from ori (4, 6, 17, 22, 31, 33, 40), but it appears to be dispensable during elongation (20).

Because of the homologous nature of the ori and of E1 and E2 proteins, proteins from one virus type can efficiently replicate either a homologous or heterologous viral ori. In contrast, mixed pairs of proteins support ori replication with various levels of efficiency (2, 7, 10, 13, 39). These observations indicate that a degree of specificity in E1-E2 protein interactions must exist. Binding studies in vitro and the yeast two-hybrid system have yielded conflicting results with regard to the E1 domain which interacts with the E2 protein. Some investigators have reported that the amino-terminal portion of the E1 protein of BPV-1 and HPV types 31 (HPV-31) contains the major DNA binding and E2 interaction domains (1, 11, 18, 41). However, others have demonstrated that the C-terminal portion of the BPV-1 E1 protein (residues 162 to 605), the HPV-16 E1 protein (residues 144 to 649), or the HPV-33 E1 protein (residues 312 to 644) was necessary for E2 binding (23, 29, 32, 45) (see Fig. 2A). A study to examine the functional interactions between E1 and E2 proteins during replication would be of significant interest. Here we report such an investigation.

To examine the interactions between the E1 and E2 proteins, we tested combinations of HPV-11 and HPV-16 E1 and E2 proteins for their ability to support ori replication in trans-
electrophoretically in a 0.8% agarose gel and transferred to a nitrocellulose which linearized the three plasmids (DpnI was digested to small fragments and migrated ahead of the linearized, newly Hin linearized all three plasmids, or with both. A Southern blot hybridization with the replicated DNA. The digestion products were separated which eliminated all unreplicated input DNA and linearized was resistant to recovered DNA from one 100-mm plate was subjected to Low-molecular-weight DNA was harvested 48 h posttransfection. Half of the and E2 expression vector plasmids used are indicated above each pair of lanes. The ori plasmid contained either the HPV-11 ori (nt 7730 to 4337), whereas the unreplicated DNA was digested to small fragments and migrated ahead of the linearized, newly replicated ori plasmid (see Fig. 3 and 4).

FIG. 1. Transient replication by combinations of HPV-11 and HPV-16 E1 and E2 proteins. Replication assays of an HPV-11 ori plasmid (nt 7730 to 7933/1 to 99) (lanes 1 to 8) or an HPV-16 ori plasmid (nt 7455 to 7906/1 to 111) (lanes 9 to 12) were conducted with human 293 cells as described in the text. The E1 and E2 expression vector plasmids used are indicated above each pair of lanes. Low-molecular-weight DNA was harvested 48 h posttransfection. Half of the recovered DNA from one 100-mm plate was subjected to HindIII and DpnI double digestion (DpnI -). The other half was subjected to HindIII digestion, which linearized the three plasmids (DpnI +). The products were separated electrophoretically in a 0.8% agarose gel and transferred to a nitrocellulose membrane. The membrane was probed with the [α-32P]dCTP-labeled, origin-containing plasmid and exposed to X-ray film overnight. Only replicated DNA was resistant to DpnI digestion (marked Ori), whereas the unreplicated DNA or replication by matched or mixed pairs of HPV-11 and HPV-16 E1 and E2 proteins. Transient replication assays were conducted as previously described (7). Briefly, 48 h after electroporation of 5 μg each of the expression vectors and 0.5 μg of the ori plasmid, low-molecular-weight DNA was harvested by alkaline Hirt lysis and digested with HindIII alone, which linearized all three plasmids, or with both HindIII and DpnI, which eliminated all unreplicated input DNA and linearized the replicated DNA. The digestion products were separated electrophoretically in a 0.8% agarose gel and then revealed by Southern blot hybridization with [α-32P]dCTP-labeled ori plasmid probes generated by random-priming reactions. These results showed that the matched protein pairs were able to replicate both HPV-11 and HPV-16 ori plasmids efficiently (Fig. 1, lanes 1 and 2, 7 and 8, and 11 and 12; see also Fig. 4B, lane 5). Cotransfection of the HPV-11 E1 expression vector with the HPV-16 E2 expression plasmid supported ori replication with a slightly reduced efficiency relative to that achieved with either homologous protein pair (Fig. 1, compare lanes 5 and 6 to 1 and 2 and to 7 and 8). In contrast, the HPV-16 E1 protein consistently failed to replicate either HPV ori plasmid in the presence of HPV-11 E2 (Fig. 1, lanes 3 and 4 and lanes 9 and 10; see also Fig. 3, lane 7, and Fig. 4A, lane 1). Furthermore, these results were reproducible when the cells were transfected with E1 and E2 expression plasmids in a wide range of relative quantities (data not shown). The stringent discrimination between the two E2 proteins exhibited by HPV-16 E1 and the lack of specificity by the HPV-11 E1 provided us with an opportunity to investigate the domains involved in functional interactions during replication by using chimeric E1 proteins.

**Reciprocal HPV-16/11 and HPV-11*/16 hybrid proteins.** Nine hybrid E1 protein genes were constructed (Fig. 2). Each hybrid E1 gene encodes a protein identical in length (649 amino acids) to that encoded by the wild-type HPV-11 or HPV-16 gene or a modified HPV-11 E1 gene (designated 11*; described below). The junctions of the hybrid proteins were selected based on one of two criteria or both. Either they were located near a boundary of functional domains inferred from the homologous BPV-1 E1 protein or they were within a highly conserved region to minimize the probability of generating a misfolded, nonfunctional protein (Fig. 2B). Five HPV-16/11 (16/11) hybrid E1 genes contained HPV-16 E1 sequences at the 5′ portion and the balance from HPV-11 E1 sequences at the 3′ portion. We prepared four HPV-11/16 (11/16) hybrid E1 protein genes in which the sequences encoding the amino-terminal portion were derived from HPV-11, with the balance from HPV-16. To facilitate a quantitative comparison among various wild-type and hybrid E1 genes, we modified the 5′ untranslated sequence and the first 5 amino acids of the 11/16 hybrid gene to those of HPV-16 E1 (Fig. 2A, designated HPV-11*/16 E1H1, E1H2, E1H4.5, and E1H5). This modification resulted in a net change of 2 amino acids from MADDs of HPV-11 to MADPA of HPV-16. These two altered residues are not conserved among different HPVs. The reason for making the HPV-11*/16 hybrid genes is the following. We note that HPV-16 proteins always supported a higher level of replication than HPV-11 proteins (Fig. 1, compare lanes 7 and 8 to lanes 1 and 2). This distinction could in part be due to differences intrinsic to the viral proteins, to differences in levels of protein expression, or both. By constructing clones that had identical sequences in the 5′ untranslated region and around the translation initiation codon, we could at least reduce differences in replication that may have originated from variations in protein translation efficiency among the different E1 genes. To provide a reference protein, we also prepared an HPV-11 E1 gene with the same modification and designated it HPV-11*E1. In transient replication assays, HPV-11*/16 E1H1, E1H2, E1H4.5, and E1H5. This modification involved in functional interactions during replication by using chimeric E1 proteins.

**Hybrid genes** 16/11 E1H1 through E1H4 were generated by PCR amplification followed by exchanging a restriction fragment with the wild-type HPV-16 E1 gene in the expression vector. Hybrid gene 16/11 E1H5 was prepared similarly except that a HindIII site was introduced downstream of the hybrid E1 gene during PCR amplification. Using a similar strategy, we constructed hybrid genes containing the amino terminus of HPV-11 E1 and the balance from HPV-16 E1 except for the extra steps to replace their 5′ ends with that of the HPV-16 E1
gene as follows. We took advantage of a PstI site located in the fifth codon of the HPV-16 E1 gene. A PstI site was generated by PCR amplification at the comparable site in HPV-11 E1 or 11/16 hybrid E1, and an EcoRI site was introduced at the 3' flanking sequence. After digestion with PstI and EcoRI, the fragment containing the bulk of the E1 gene was ligated to the vector fragment of similarly digested pMTX-16E1. Hence, all of the E1 sequences were located at the same site in the pMTX vector and had identical sequences at the 5' ends of the mRNAs. All junction sequences or the entire sequences generated by PCR were confirmed by sequencing.

The domain of HPV-16 E1 protein which confers E2 protein specificity during replication. The 16/11 E1H1 and 11/16 E1H2 hybrid proteins contain amino acids 1 to 237 and 1 to 365, respectively, from HPV-16 E1 and the balance from HPV-11 E1. The junctions are located in the middle or at the carboxyl-terminal boundary of the putative DNA and E2 binding domains by analogy to BPV-1 (Fig. 2A). Figure 3 presents the results of transient replications of an HPV-11 ori with the expression vectors of 16/11 hybrid E1 proteins in combination with the HPV-11 or HPV-16 E2 expression vector. Unlike the wild-type HPV-16 E1 protein, chimeric proteins 16/11 E1H1 and E1H2 supported efficient replication in the presence of either E2 protein, a property shared with HPV-11 E1 (Fig. 3, compare lanes 1 to 3 and 7 to lanes 9 to 11 and 14). Similar results were observed when the HPV-16 ori plasmid was used in the test (data not shown). These results demonstrate that the amino-terminal region up to residue 365 of HPV-16 E1 is not responsible for functional discrimination of the two E2 proteins during replication. By inference, the carboxyl-terminal domain of the HPV-16 E1 protein is expected to constitute the determinant. We then tested the reciprocal 11/16 hybrid E1 genes. Transient replication assays of an HPV-11 or HPV-16 ori showed that neither 11/16 E1H1 nor 11/16 E1H2 had detectable replication activity with HPV-11 E2 while both supported efficient replication of either viral ori in the presence of HPV-16 E2 (Fig. 4A, compare lanes 1, 2, and 5 to lanes 6, 7, and 10). These properties are similar to those of the HPV-16 E1 protein and support the previous conclusion that the carboxyl-terminal region of the HPV-16 E1 is responsible for E2 specificity. This conclusion is supported by the replication properties of reciprocal H5 hybrid proteins described below.

Inactivation of the E1 protein by a chimeric junction in the ATPase domain. For further delineation of functional domains, we tested 16/11 E1H3, E1H4, and E1H5, which contain amino acid residues 1 to 443, 1 to 526, and 1 to 605 from HPV-16 E1, respectively, and the balance from HPV-11 E1. The junctions reside within or border on the putative ATPase domain (Fig. 2A). 16/11 E1H3 showed a very low replication activity with HPV-11 E2, and the activity with HPV-16 E2 was comparable to that of HPV-16 E1 or HPV-11* E1 in the presence of HPV-11 E2. 16/11 E1H4 had no detectable activity with either E2 protein (Fig. 3, lanes 4 and 6, 7, and 10). These properties are similar to those of the HPV-11 E1 protein and support the previous conclusion that the carboxyl-terminal region of the HPV-16 E1 is responsible for E2 specificity. This conclusion is supported by the replication properties of reciprocal H5 hybrid proteins described below.
the presence of HPV-16 E2 (Fig. 3, compare lane 13 to lanes 8 and 14). Interestingly, this hybrid now gained a moderate activity in collaboration with HPV-11 E2 (Fig. 3, lane 6).

Two 11*/16 hybrid genes with a junction near the carboxyl terminus were tested. The exchange in 11*/16 E1H4.5 occurs between residues 559 and 560, whereas the 11*/16 E1H5 is the reciprocal hybrid of 16/11 E1H5 (Fig. 2). Transient replication assays of the HPV-11 or HPV-16 ori by 11*/16 E1H4.5 revealed no detectable activity with either HPV-11 E2 or HPV-16 E2 (Fig. 4A, lanes 4 and 10; Fig. 4B, lanes 3 and 8). Only after extended exposure was a very weak replication signal observed, and only with HPV-16 E2 (data not shown). Transient replication assays of 11*/16 E1H5 showed a low activity with HPV-11 E2 and a higher activity with HPV-16 E2 (Fig. 4A, compare lanes 5 and 11; Fig. 4B, compare lanes 4 and 9). Therefore, our results showed that 16/11 E1H3, 16/11 E1H4, and 11*/16 E1H4.5 were severely crippled in their function in transient replication assays. Since the reciprocal H5 hybrids have a property intermediate between the HPV-11 E1, HPV-11*/16 E1 (or 16/11 E1H1 or E1H2), and HPV-16 E1 (or 11*/16 E1H1 or E1H2), we suggest that the carboxyl-terminal 44 amino acids of HPV-16 contribute to but do not comprise the entire determinant for E2 specificity.

Multiple structural and functional domains of the E1 protein. The extent of replication shown in Fig. 3 and 4A was quantified by PhosphorImager and compared to the activities achieved by HPV-16*E1 and HPV-11 E2. The results are shown in Fig. 2A. Several conclusions can be drawn. (i) The HPV-11 E1 protein is more flexible in its collaboration with a heterologous E2 protein than is the HPV-16 E1 protein, which does not function in the presence of HPV-11 E2 (Fig. 1, 3, and 4). (ii) The E1 protein can be divided into at least three structural and functional domains on the basis of the ability of hybrid proteins to function in transient replication assays. The amino-terminal residues 1 to 237 or 1 to 365 (E1H1 and E1H2) and the carboxyl-terminal 44 amino acids (E1H5) can be exchanged between the two viral proteins and result in hybrid proteins that exhibit low (5 to 10%), moderate (10 to 50%), or high (50 to 90%) replication activity in the presence of one or
both E2 proteins. However, residues 366 to 605, which comprise the putative ATPase domain (H3, H4, and H4.5), are very sensitive to changes within the domain or in the flanking regions, since the hybrid proteins are largely or entirely nonfunctional in the presence of either E2 protein (Fig. 3 and 4). Whether this loss of function is due to the inactivation of the ATPase and helicase activities or to other defects is not known.

(iii) The amino-terminal 56% of the HPV-16 E1 protein (residues 1 to 365) does not play a role in E2 protein specificity, as neither of the two E2 proteins with the HPV-11 E2 protein is due to steric interference. This work was supported by USPHS grant CA36200.

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REFERENCES


