JOURNAL OF VIROLOGY, Oct. 1989, p. 4417–4421
0022-538X/89/104417-05$02.00/0
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Vol. 63, No. 10

NOTES

The E6 and E7 Genes of the Human Papillomavirus Type 16 Together Are Necessary and Sufficient for Transformation of Primary Human Keratinocytes

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Received 2 May 1989/Accepted 22 June 1989

The early human papillomavirus type 16 genes that directly participate in the in vitro transformation of primary human keratinocytes have been defined. In the context of the full viral genome, mutations in either the E6 or E7 open reading frame completely abrogated transformation of these cells. Mutations in the E1, E2, and E2-E4 open reading frames, on the other hand, had no effect. Thus, both the full-length E6 and E7 genes were required for the induction of keratinocyte immortalization and resistance to terminal differentiation. The E6 and E7 genes expressed together from the human β-actin promoter were sufficient for this transformation; mutation of either gene in the context of this recombinant plasmid eliminated the ability to induce stable differentiation-resistant transformants.

More than 50 different types of human papillomaviruses (HPVs) have now been isolated from a variety of squamous epithelial lesions, and approximately 18 of them have been associated with anogenital tract lesions. Some of these, such as HPV type 6 (HPV-6) and HPV-11, are generally associated with benign proliferative lesions, including condyloma acuminata, which only infrequently progress to cancers. Others, such as HPV-16, HPV-18, HPV-31, HPV-33, and HPV-35, are associated with genital tract lesions, which are at risk for malignant progression, and with genital tract cancers (41).

HPV-16 or HPV-18 DNA has been found integrated in a high percentage of cervical carcinomas and in cell lines derived from these cancers (3, 8, 39). This is in contrast with the premalignant dysplastic lesions associated with HPV-16 and HPV-18, in which the viral DNA is usually found in an extrachromosomal state (9). In several cases in which the number of integrated viral genomes was low enough to permit a detailed analysis, the integration pattern revealed remarkable specificity with respect to the circular viral genome. Integration occurs in the E1-E2 region (1, 16, 24), disrupting the E2 viral transcriptional regulatory circuitry. The E2 open reading frame (ORF), as originally demonstrated with the bovine papillomavirus type 1, encodes both positive- and negative-acting transcriptional regulatory factors (14, 28). For HPV-16 and HPV-18, E2 appears to act principally as a repressor of the promoter from which the E6 and E7 genes are transcribed (5, 31; H. Romanczuk and P. M. Howley, manuscript in preparation). The HPV genomes in cervical carcinomas and in derived cell lines are transcriptionally active, and the patterns of viral mRNA species are specific, with regular expression of the E6 and E7 ORFs (1, 23, 27).

The E7 ORF of HPV-16 encodes a 21-kilodalton phosphoprotein (27), and the E7 genes of HPV-16 and HPV-18 are sufficient for focus formation of established rodent fibroblasts such as NIH 3T3 cells (12, 20, 30, 32, 34). The E7 protein is functionally and structurally related to the adenovirus E1A proteins (AdE1A); it can transactivate the AdE2 promoter and can cooperate with an activated *ras* oncogene to transform primary rat cells (20, 29). The amino-terminal 38 amino acids of E7 are strikingly similar to portions of conserved domain 1 (amino acids 37 to 49) and domain 2 (amino acids 116 to 137) of the AdE1A proteins (20) as well as to portions of the large tumor antigens (T) of papovaviruses. The AdE1A, simian virus 40 (SV40) T, and HPV-16 E7 proteins form specific complexes with the product of the retinoblastoma tumor suppressor gene (p105-RB) (6, 10, 35), and complex formation with p105-RB is mediated through these conserved sequences for AdE1A and SV40 T (6, 36) as well as for HPV-16 E7 (K. Münger, B. Werness, W. C. Phelps, and P. M. Howley, unpublished observations). The transforming potential of the E6 gene has been less well defined. In NIH 3T3 fibroblasts, it may contribute to characteristics of the transformed phenotype such as anchorage independence (2; K. Münger, W. C. Phelps, and P. M. Howley, unpublished observation) or tumorigenicity in nude mice (40).

HPV-16 and HPV-18 DNA can immortalize human keratinocytes in vitro. These immortalized cell lines exhibit altered characteristics of cellular proliferation and differentiation but are not tumorigenic in nude mice. They contain integrated copies of HPV DNA and express viral mRNAs and proteins (7, 13, 21, 22, 37, 38). In epithelial cells grown in organotypic culture, HPV-16 DNA can induce histological abnormalities that resemble those observed in cervical intraepithelial neoplasia in vivo (17). It has been shown recently that both the E6 and E7 ORFs are necessary for the extension of the life span of human diploid fibroblasts (33).

In this study, we analyzed the HPV-16 early genes in-

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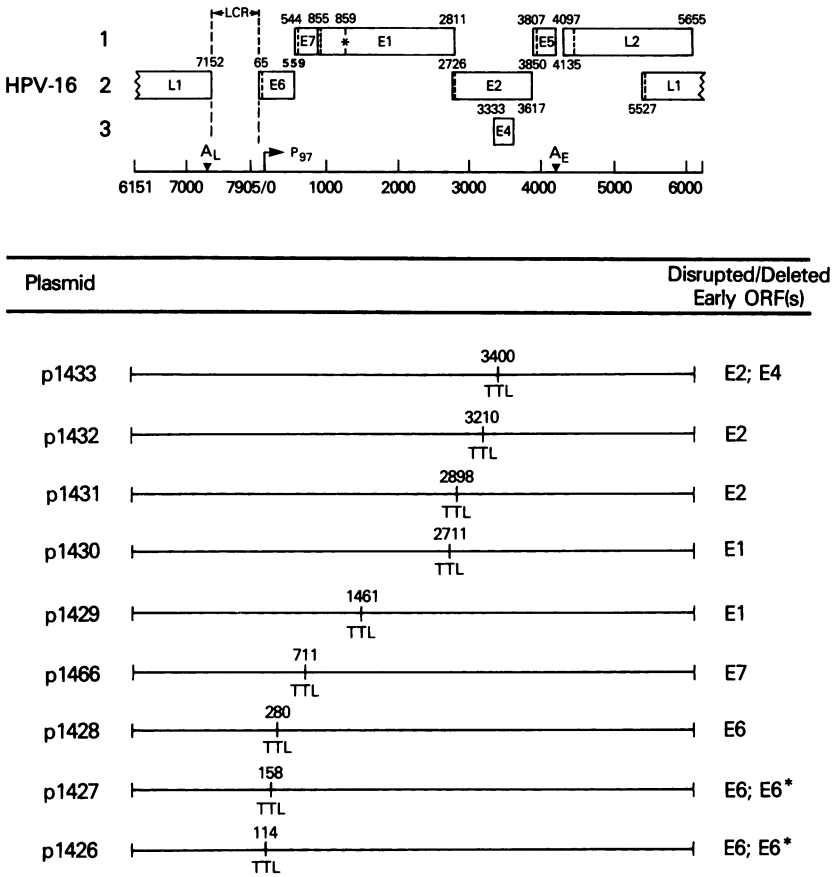

FIG. 1. HPV-16 mutants used in the transformation of primary human foreskin keratinocytes. The HPV-16 genome is shown schematically at the top, with the long control region (LCR) and the major ORFs indicated (the prefixes E and L indicate early and late ORFs, respectively). The approximate position of the first methionine residue in each ORF is indicated by the broken vertical line, and the sequence positions of the first and last nucleotides are indicated. Positions of the promoter P_{97} and the early (A_E) and late (A_L) polyadenylation sites are given. The parental plasmid is the original isolate described by Dürst et al. (8), which contains a frameshift mutation at sequence position 1138 (*), leading to disruption of the E1 ORF. Plasmids p1426 to p1433 and p1466 were constructed by introduction of a self-complementary TTL oligonucleotide (TTAGTTAACTAA) (19) at the indicated sequence position by restriction endonuclease digestion, T4 DNA polymerase fill-in, and blunt-end ligation. TTLs were inserted at restriction sites for *Nla*IV (nucleotide [nt] 114; p1426), *Fnu*4HI (nt 158; p1427), *Nde*I (nt 280; p1428), *Ban*II (nt 711; p1466), *Spe*I (nt 1461; p1429), *Tth*111I (nt 2711; p1430), *Bst*XI (nt 2898; p1431), *Hin*cII (nt 3210; p1432), and *Bal*I (nt 3400; p1433). The ORF(s) disrupted by TTL insertion is given on the right. These plasmids were all cloned in pUC18 at the unique *Bam*HI site located in the L1 ORF at nt 6151.

volved in the transformation of the normal host cell, the genital keratinocyte, by using an assay that evaluates both cellular immortalization and the response to inducers of terminal differentiation (22). We transfected human foreskin keratinocytes with a series of plasmids containing translation termination linkers (TTLs) inserted into individual viral ORFs in the context of the full-length cloned HPV-16 genome, using electroporation (22) or lipofection (11). For liposome-mediated DNA transfection, keratinocytes in 10-cm-diameter plates were exposed to 5 μg of plasmid DNA and 50 μg of liposomes (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) in 3 ml of KGM (Clonetics, San Diego, Calif.) for 18 h and then passaged into 150-cm^2 flasks. When the cultures became 80% confluent, they were transferred to medium containing 2 mM Ca^{2+}, 10% fetal calf serum, and 1 μg of hydrocortisone per ml to select for differentiation-resistant colonies as previously described (22). Duplicate flasks of cells were either stained with 0.1% methylene blue for quantitative analysis or passaged to evaluate cellular immortalization. In this assay, no significant quantitative or qualitative differences were noted be-

TABLE 1. Effects of mutations in individual HPV-16 ORFs on keratinocyte transformation

Plasmid	Disrupted ORF	No. of transformed colonies/10^6 cells per 10 μg of DNA[a]
p769.1	None	20, 4
p1433	E2 and E4	33, 30
p1432	E2	53, 30, 21
p1431	E2	10, 24, 28
p1430	E1	43, 44
p1429	E1	24, 16, 14
p1428	E6	0, 0, 0, 0, 0, 0
p1427	E6 and E6*	0, 0, 0, 0, 0, 0
p1426	E6 and E6*	0, 0, 0, 0, 0, 0
p1466	E7	0, 0, 0, 0, 0, 0
pUC18	NA[b]	0, 0, 0, 0

[a] Numbers correspond to individual experiments.
[b] NA, Not applicable.

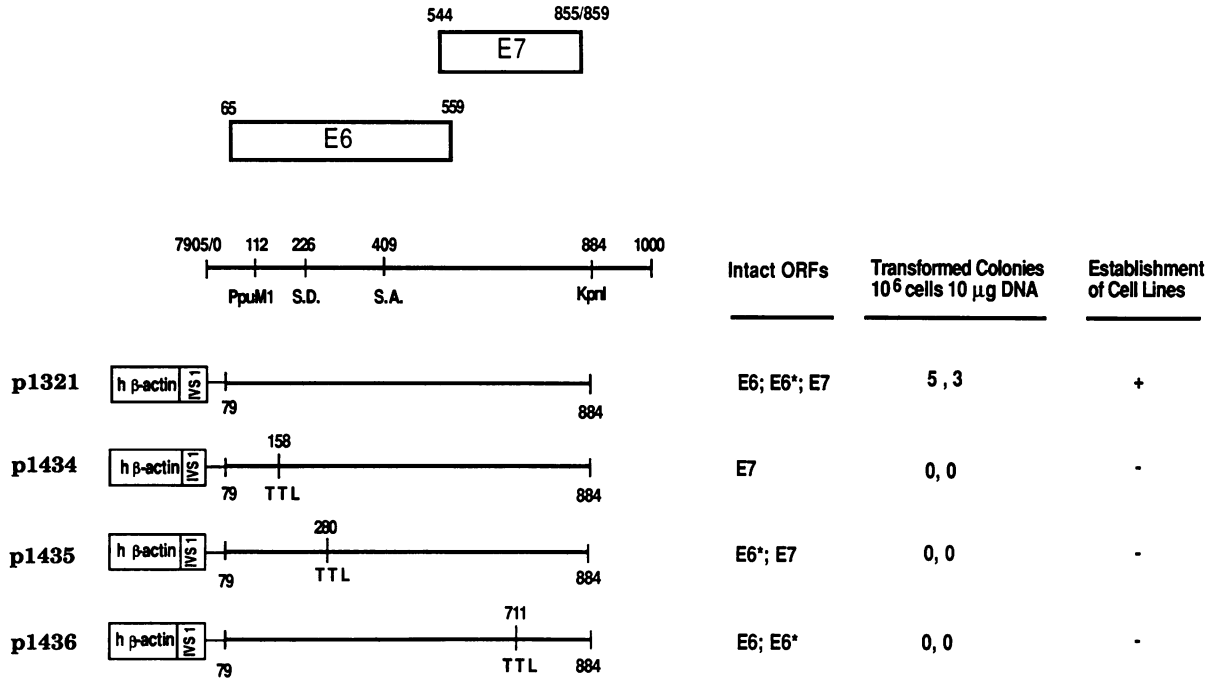

FIG. 2. Plasmids containing the subgenomic HPV-16 E6 and E7 DNA fragments and the human β-actin promoter. The E6-E7 region of HPV-16 is shown at the top. The sequence positions of the first and last nucleotides and of the major splice donor (S.D.) and acceptor (S.A.) sites located within the E6 ORF (27) are indicated. Plasmid p1321 was created by cloning a *Ppu*M1 (nt 112)-to-*Kpn*I (nt 884) fragment and a synthetic oligonucleotide linker (GATCCTTTTATGCACCAAAAGAGAACTGCAATGTTTCAG), reconstructing HPV-16 sequences up to nucleotide 79 into the human β-actin expression plasmid p1318. Plasmid p1318 contains the 4.3-kilobase *Eco*RI-*Alu*I human β-actin promoter fragment derived from p14Tβ-17 (15, 18) and the human β-actin 3′ untranslated region, poly(A) site, and 3′ flanking region (15). TTLs were inserted at restriction enzyme sites for *Fnu*4H (nt 158; p1434), *Nde*I (nt 280; p1435), and *Ban*II (nt 711; p1436). The intact ORFs, number of transformed keratinocyte clones, and ability to establish keratinocyte cell lines are indicated for each plasmid on the right.

tween the HPV-16 prototype plasmid (p769) and any of the plasmids with TTLs inserted into the E1 (p1429 and p1430), E2 (p1431 and p1432), or E2-E4 (p1433) ORFs (Fig. 1 and Table 1). In contrast, plasmids with TTL insertions in either the E6 ORF (p1426, p1427, and p1428) or the E7 ORF (p1466) were all transformation negative. Thus, in the context of the full-length cloned viral genome, translational integrity of the full-length E6 and E7 genes was necessary for the induction of keratinocyte transformation. This result is in agreement with the observation that in cervical carcinoma and in cervical carcinoma cell lines, the consistent pattern of viral transcription involves both the E6 and E7 genes (1, 23, 27). These mRNAs have been mapped and shown to encode a full-length E7 protein, a full-length E6 product, and internally spliced versions of E6 designated E6* (23, 27). Only those HPV types that have been associated with an increased risk of malignant progression, such as HPV-16 and HPV-18, have the capacity to encode the truncated forms of E6. The genital HPVs, which are not associated with an increased risk of malignant progression, do not have this internal E6 splice (23). It is as yet unresolved whether the E6* gene products have a function per se or whether the mRNA-splicing event is important for efficient expression of the E7 protein (26). In the keratinocyte transformation assay, it is notable that E6* did not provide the function of the full-length E6, since plasmid p1428, with a TTL inserted downstream of the 5′ splice site in E6, which could still express E6* but not E6, was transformation defective (Table 1).

Since E2 can act as a transcriptional repressor for the genital HPVs in human keratinocytes (4, 5, 31; Romanczuk and Howley, in preparation), disruption of this ORF might have been expected to lead to an increased number of transformed keratinocyte cell clones. This was not observed. It is conceivable that an indirect effect by E2 could have been masked in these experiments by a frameshift mutation in the E1 ORF in the prototype HPV-16 DNA clone used in this study (p769), originally described by Dürst et al. (1, 8, 25). A full-length HPV-16 DNA plasmid with an intact E1 ORF, however, did have the same transformation capacity in the keratinocyte assay as p769 (data not shown). Further studies are needed to fully assess whether either or both of the E1 and E2 mutations may have indirect effects on transformation of human keratinocytes in the context of this reconstructed HPV-16 plasmid. One can conclude from the mutational studies presented here, however, that the E1 and E2 genes do not have a direct effect on keratinocyte transformation.

To examine whether the HPV-16 E6 and E7 genes were sufficient for transformation of keratinocytes, the E6-E7 region of HPV-16 was cloned and expressed from the human β-actin promoter in a plasmid designated p1321 (Fig. 2). This DNA was capable of inducing immortalized, differentiation-resistant keratinocyte cell lines, although at a frequency lower than that of the cloned full-length HPV-16 genome (p769) (Fig. 2 and Table 1). There could be several reasons for the consistently decreased transformation efficiency of the subgenomic plasmids. There may be *cis* elements in the 3′ end of the HPV-16 early region that affect levels of expression of the E6 and E7 genes or viral mRNA stability. It is also a formal possibility that ORFs downstream of E7 in the HPV-16 early region such as E5 encode a factor(s) that

indirectly affects the frequency of keratinocyte transformation. Regardless, the E6 and E7 genes by themselves are sufficient for keratinocyte transformation, indicating that the 3′ portion of the HPV-16 early region does not encode a factor(s) necessary for the transformed phenotype defined by this assay.

TTLs were inserted in the E6, E6*, or E7 ORFs in the subgenomic plasmid p1321 (p1434 to p1436) to assess whether any of these gene products, when expressed from a strong heterologous promoter, was sufficient for keratinocyte transformation. Each of the TTL insertion mutants was transformation negative (Fig. 2). Of note is the finding that the E6*- and E7-encoding plasmid p1435 was transformation negative, further supporting the conclusion that the E6 and E6* genes are not able to provide the same function in the transformation of primary human keratinocytes. Thus, the full-length E6 and E7 genes are sufficient as well as necessary for transformation of primary human keratinocytes, and the direct participation of any other viral gene is not required.

Primary human cells provide the first evidence for the necessity of the full-length E6 gene product for cellular transformation. Further experiments are needed to delineate the specific functions and cellular mechanisms by which E6 and E7 participate in the transformation of human keratinocytes. Both E6 and E7 are low-molecular-weight proteins for which no enzymatic activities have been defined. It is therefore conceivable that the transformation functions of both E6 and E7 are mediated by either an association with or modification of cellular proteins involved in the control of cellular proliferation, differentiation, or both. Such a mechanism has recently been proposed for the HPV-16 E7 protein which, similar to AdE1A and SV40 T, can form a complex with the product of the retinoblastoma tumor suppressor gene (p105-RB) (6, 10, 35). In addition, the SV40 T and AdE1A proteins form complexes with a series of other cellular proteins. Of particular interest are 107- and 300-kilodalton proteins that interact with AdE1A sequences which are conserved in HPV-16 E7 (36). Experiments have been initiated to identify additional cellular proteins interacting with the E7 protein and to test the significance of complex formation between HPV-16 E7 and p105-RB, as well as other host proteins, for cellular transformation. Similarly, cellular targets for the E6 protein will need to be identified.

We thank Robin Levis for suggesting liposome-mediated DNA transfections, Lex Cowsert for helpful discussions during the progress of this research, Janet Byrne for oligonucleotide synthesis, and Jesse Quintero and Susan Gamble for excellent technical assistance. We are grateful to Lex Cowsert, Helen Romanczuk, and Françoise Thierry for critically reading the manuscript.

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