Induction of Anchorage-Independent Growth of Human Embryonic Fibroblasts with a Deletion in the Short Arm of Chromosome 11 by Human Papillomavirus Type 16 DNA

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Human embryonic fibroblasts with a large deletion (11p11.1p15.1) in the short arm of one chromosome 11 (del-11 cells) appeared to be susceptible to transformation by early human papillomavirus type 16 (HPV-16) DNA, whereas diploid human embryonic fibroblasts were not. This difference in susceptibility might be explained by the absence of a tumor suppressor gene located within the deleted part on the short arm of chromosome 11. The presence of abundant viral early-gene transcripts in transformed cells suggests that transformation was induced by an elevated level of an HPV-16 early-gene product(s). The low transcriptional activity of HPV-16 in diploid cells may indicate that cellular genes affect viral transcription. Interruption of the HPV-16 E2 early open reading frame is probably required for high-level HPV-16 early-gene expression driven from the homologous enhancer-promoter region.

Among the human papillomaviruses (HPVs) suspected to be involved in the development of cervical cancer, HPV type 16 (HPV-16) DNA is most frequently found associated with cervical tumors (9, 16, 18, 35, 42, 52, 59). HPV-16 DNA is observed not only in tumor cells, but also at relatively high frequency in apparently normal cervical tissue and in lesions of the cervix classified as dysplasia (13, 19, 32, 36). Fortunately, such lesions progress to malignancy only after a long latency period and at a low frequency, indicating that HPV infection is not the only risk factor involved (6). Neoplastic development of the cervical epithelium may in addition require the activation of a cellular proto-oncogene(s) and failing control of the suppressor gene(s). This may be reflected by chromosomal abnormalities often present in primary cervical carcinoma cells, most frequently affecting chromosomes 11, 1, 7, and 3 (2, 3). Loss of heterozygosity for genetic markers overlapping the p13 region on the short arm of chromosome 11 has been associated with Wilms' tumor development (40), suggesting the localization of a tumor suppressor gene on 11p13. Further evidence in support of this conclusion was provided by the suppression of the tumorigenic phenotype of Wilms' tumor cells and HeLa cells upon microcell transfer of a normal chromosome 11 into these cells (17, 27, 41, 48). We previously reported that human fibroblasts derived from four individuals, each showing a deletion overlapping p13 of one chromosome 11, were highly susceptible to transformation by early-region BK virus DNA, whereas diploid human fibroblasts were not (15). This difference may be explained by a suppressor locus situated within the deleted region. In order to obtain a system to determine the transforming potential of HPV for human cells and to analyze the cellular and viral requirements, we selected the cells of one of these individuals. The cells of this patient had the karyotype 46,XX,del(11)(p11.1p15.1) and are referred to as del-11 cells.

MATERIALS AND METHODS

Cell culture, transfection procedure, and anchorage independence test. Cells were cultured in Eagle minimal essential medium (Gibco) supplemented with streptomycin (100 µg/ml), neomycin (100 µg/ml), fungizone (100 µg/ml), and 8% fetal calf serum. Semiconfluent cultures of secondary cells (passage 15 to 20) were transfected with 4 µg of HPV recombinant DNA together with 1 µg of pRSVneo as a selectable marker gene by the calcium phosphate precipitation method (21). G418^r (200 µg/ml) selection was begun 2 days after the transfection.

G418-resistant colonies were tested for anchorage-independent growth by the protocol of MacPhearson and Montagnier (34). Briefly, 2.5×10^5 cells were suspended in 7.5 ml of semisolid medium consisting of 0.3% (wt/vol) agar or agarose in Dulbecco modified Eagle medium containing 8% fetal calf serum. Colonies were counted 4 and 6 weeks later, and the number of expanding colonies was expressed as a percentage of cells seeded.

DNA and RNA analysis. Purified DNA was digested with restriction enzymes, separated by electrophoresis through a 1% agarose gel, and blotted to nitrocellulose filters as described by Southern (46). DNA immobilized on filters was hybridized to vector DNA-free HPV-16 DNA labeled with ³²P by nick translation to a specific activity of 0.5×10^8 cpm/µg of DNA. Hybridization was performed in $3 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)– $10 \times$ Denhardt solution–0.1% sodium dodecyl sulfate (SDS)–2% dextran sulfate–50 µg of denatured salmon sperm DNA per ml at 64°C for 18 h. Subsequent washing was done in $1 \times$ SSC–1% SDS at 64°C, followed by two washes in $0.1 \times$ SSC–0.1% SDS at 64°C.

RNA was purified by pelleting of guanidinium thiocyanate–N-lauryl sarcosyl lysates of confluent cell cultures through a CsCl cushion as described by Chirgwin et al. (12). Polyadenylated [poly(A)⁺] RNA was selected by oligo(dT)-cellulose affinity chromatography (4). Poly(A)⁺ RNA was fractionated in a 1.2% agarose-formaldehyde gel (43) and blotted to nitrocellulose filters by capillary blotting in $20 \times$

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FIG. 1. HPV-16 genome organization and DNA recombinants. (Top) Genomic organization of HPV-16 and partial restriction map (40). Early (e) and late (l) ORFs, relevant restriction sites for *Bam*HI (b), *Eco*RI (e), *Mst*II (m), and *Pst*I (p), and potential polyadenylation site (arrow) are indicated. (Bottom) HPV-16 recombinants used in the present study. Vector sequences (pSP65) are not shown. Insertion of a TTL at the *Pst*I cleavage site (nt 3692) is indicated.

SSC. The filters were dried, baked for 2 h at 80°C, and hybridized to HPV-16 DNA probes exactly as described for Southern blot analysis.

Cytogenetic analysis. Chromosome preparations were made from monolayer cell cultures by standard techniques. Metaphases were stained with 0.5% atebine to obtain a Q-banding pattern (QFA) and photographed. A minimum of 30 cells were analyzed from each culture, and karyotypes are described according to the standard nomenclature (24).

RESULTS

The complete early region of HPV-16, including the putative early polyadenylation site (44) contiguous with the complete or only the 3' part of the long control region (LCR), was cloned into the polylinker of pSP65. From these constructs two other recombinants were derived with a translation termination linker (TTL), dCCTAGTCTAGACTAG, inserted into the E2 open reading frame (ORF) (Fig. 1). We used the latter constructs in our experiments, as the HPV DNA present in cervical carcinoma cell lines is often disrupted within the E1/E2 ORFs due to integration within the cellular genome (34) and the E2 ORF encodes a protein which probably modulates viral early-gene expression (29, 47) and thus could affect transformation. The position of the TTL was chosen in the 3' end of the E2 ORF, as this part of the E2 ORF appears to be essential for E2 ORF repressor function (29).

None of these recombinants appeared to transform either diploid fibroblasts or del-11 cells in a transformation assay which scores for the formation of dense foci of morphologically transformed cells. In order to obtain HPV-16 DNA containing cells which we subsequently could examine for growth in soft agar (anchorage-independent growth), we cotransfected HPV recombinants with pRSVneo as a selectable marker gene. G418 selection was started 2 days after transfection, and individual resistant colonies appearing 4 to 6 weeks postselection were expanded. From a total of nine del-11 cell cultures obtained upon transfection with HPV-16 DNA with an intact E2 ORF (recombinants pHPV-16-1 and pHPV-16-6), only one appeared to be anchorage independent for growth. Of the other cultures, less than 1% of the cells formed small colonies which did not expand further. In

contrast, most of the del-11 cell cultures obtained upon transfection with HPV-16 DNA in which a TTL was inserted into the E2 ORF at nucleotide (nt) 3692 (pHPV-16-18 and pHPV-16-10) were anchorage independent, as up to 30% of the cells formed large expanding cell colonies in semisolid medium (Table 1). These results contrast with the results obtained for normal diploid human embryonic fibroblasts, as induction of anchorage-independent growth was not observed in these cells with any of the HPV-16 recombinants. These results demonstrate that compared with diploid cells, del-11 cells are less resistant to transformation by HPV-16, suggesting that a deletion encompassing region 11p11.11p15.1 in the short arm of one chromosome 11 facilitated the transformation of primary human embryonic fibroblasts by HPV-16 DNA.

Modified or increased viral transcription could be responsible for the induction of the transformed phenotype in del-11 cells transfected with an HPV DNA recombinant containing a disrupted E2 ORF. To examine this possibility, we analyzed anchorage-independent del-11 cell cultures as well as several anchorage-dependent del-11 cell cultures for the presence of HPV transcripts (Fig. 2A). All anchorage-independent cultures transfected with either pHPV-16-18 or pHPV-16-10 contained abundant amounts of viral transcripts ranging in size from 1.0 to 4.4 kilobases (kb). The composition of the viral transcripts in these cells was more complex and the levels were higher than in the cervical carcinoma cell line CaSki. In contrast, no or a very low level of viral transcripts was present in untransformed anchorage-dependent HPV-16 DNA-containing del-11 cell cultures obtained after transfection with HPV-16 DNA containing an intact E2 ORF. Only one culture obtained after transfection with

TABLE 1. Anchorage-independent growth of del-11 cells and diploid human embryonic fibroblast HPV-16 transfectants

Plasmid	No. of positive cultures/no. tested	
	del-11 cells	Diploid cells
pHPV-16-1	1/6	0/3
pHPV-16-6	0/3	0/3
pHPV-16-18	11/16	0/3
pHPV-16-10	6/9	0/3

FIG. 2. Detection of viral mRNAs in transfected del-11 cells and diploid human fibroblasts. Poly(A)-selected RNA (1 μ g) was run on a formaldehyde-agarose gel, blotted to nitrocellulose, and hybridized to a DNA probe prepared from the 7.9-kb HPV-16 genome. The length of the transcripts (in kilobases) is shown to the left. (A) HPV-16-transfected del-11 cell cultures. Lanes 1, 2, and 3, pHPV-16-10-transfected cultures 1, 2, and 3, respectively; lanes 4, 5, and 6, pHPV-16-18-transfected cultures 1, 2, and 3, respectively; lanes 7 and 8, pHPV-16-6-transfected cultures 1 and 2, respectively; lane 9, CaSki cells; lanes 10–15, pHPV-16-1-transfected cultures 1, 2, 3, 4, 5, and 6, respectively. The pHPV-16-10-transfected cultures, the pHPV-16-18-transfected cultures, and pHPV-16-1-transfected culture 2 were anchorage independent. The other cultures were anchorage dependent. (B) HPV-16-transfected diploid cell cultures. Lanes 1 and 2, pHPV-16-10 transfected cultures 1 and 2, respectively; lanes 3 and 4, pHPV-16-18-transfected cultures 1 and 2, respectively; lane 5, CaSki cells.

pHPV-16-1 contained viral transcripts at a level comparable to that in the anchorage-independent cultures obtained after transfection with pHPV-16-10 or pHPV-16-18 (Fig. 2A, lane 11). This culture appeared to be moderately anchorage independent (5% growth in semisolid medium). It is possible that in the latter culture, the HPV DNA was disrupted due to integration or rearrangement, leading to the absence of a functional E2 product.

The absence of a substantial level of viral transcripts in the normal diploid human fibroblasts transfected with pHPV-16-10 or pHPV-16-18 indicated that these fibroblasts are not able to support efficient viral transcription even if the E2 ORF is disrupted. This could explain the resistance of these diploid cells to transformation. To see whether the level of transcription was dependent on the viral copy number, we examined the presence of HPV-16 DNA in transfected del-11 and diploid cell cultures (Fig. 3). In all cell cultures examined, the HPV DNA was present at high copy numbers (up to 200 copies per cell were estimated to be present in some cultures), presumably integrated within the cellular genome, as indicated by the presence of extra bands. No correlation was found between the copy number of HPV-16-DNA and the level of transcription (compare lanes 6 and 7 with lanes 8 to 12 in Fig. 3A).

If HPV-16 transcription is initiated at the putative transcription promoter (p97) within the 3' part of the LCR (45), the expected size of an unspliced mRNA from the entire early region would be about 3.8 kb. Consistent with this, major mRNAs of 3.8 and 4.4 kb were observed in anchorage-independent del-11 cell cultures. Hybridization with subgenomic probes showed that the smallest 1.0-kb mRNA was derived from the E6/E7 region, as this band hybridized with the 1,776-base-pair (bp) *Pst*I fragment of HPV-16 encompassing the E6 and E7 ORFs (Fig. 4). The 1.8- and 2.5-kb mRNAs presumably represent splice products between the E6/E7 and the E2/E4 region, as they hybridized with subgenomic probes containing these ORFs but not with the E1 probe. No major transcripts could be detected which hybrid-

ized with the E2/E4 probe but not with the E6/E7 probe, indicating that no major transcripts are initiated from a viral promoter located within the early region.

Cytogenic analysis of three individual transformed del-11 cell cultures revealed additional chromosome abnormalities apart from the original 11p deletion (not shown). Each culture examined has its own specific chromosomal aberrations. In the pHPV-16-10-transfected del-11 cell culture 1, this was a deletion of the arm of chromosome 3 [del(3)(p21)], a 14p+, and monosomy of chromosome 13. In the pHPV-16-10-transfected del-11 cell culture 2, a structurally abnormal chromosome 12 was present resulting from an unbalanced translocation with chromosome 14, i.e. 12qter $\rightarrow$ 12p11::14p11 $\rightarrow$ 14qter, and monosomy of chromosome 14. In the pHPV-16-18-transfected del-11 cell culture 3, chromosomes 1 and 4 were involved in an unbalanced translocation resulting in a 4p+ chromosome (4qter $\rightarrow$ 4p16:1p34 $\rightarrow$ 1pter); two normal chromosomes 1 were also present. Whether these cytogenetic changes are also involved in the transformation process itself remains to be elucidated. It may be significant that three of the chromosomes involved are also known or presumed to carry tumor suppressor genes; chromosome 13 bears the suppressor gene for retinoblastoma (11), region p21 of chromosome 3 carries the gene involved in small cell lung carcinoma (28), and the short arm of chromosome 1 is thought to carry a putative suppressor gene(s) associated with neuroblastoma (10).

DISCUSSION

The use of human embryonic fibroblasts carrying a deletion on one chromosome 11 overlapping the p13 region enabled us to demonstrate transforming activity of HPV-16 DNA in human embryonic fibroblasts in vitro as revealed by the ability to grow in semisolid medium. No loss of contact inhibition could be demonstrated, however, and at present it is not known whether the transfected cells have an extended life span. Therefore, HPV-16 induces a partially transformed

FIG. 3. HPV-16 DNA present in transfected primary human fibroblasts. *Pst*I digests of cellular DNA as well as of plasmid DNA were subjected to Southern blot hybridization with the 7.9-kb HPV-16 genome as the probe. Some markers (in kilobases) of *Hind*III-digested lambda DNA are shown to the left. (A) del-11 cells. Lanes 1-3, 50, 100, and 200 pg of pHPV-16-6 DNA, respectively; lane 4, 200 pg of pHPV-16-10; lane 5, 200 pg of pHPV-16-18 DNA; lanes 6 and 7, 1 μ g of DNA isolated from pHPV-16-6-transfected cell cultures; lanes 8 and 9, 1 μ g of DNA from pHPV-16-10-transfected cultures 1 and 2, respectively; lanes 10-12, 1 μ g of DNA from pHPV-16-18-transfected cultures 1, 2, and 3, respectively. (B) Diploid cells. Lanes 1-3, 50, 100, and 200 pg, respectively, of pHPV-16-10 DNA digested with *Pst*I; lane 4, 200 pg of pHPV-16-18 DNA digested with *Pst*I; lanes 5-7, 1 μ g of DNA isolated from pHPV-16-10-transfected cultures 1, 2, and 3, respectively; lanes 8-10, 1 μ g of DNA from pHPV-16-18-transfected cultures 1, 2, and 3, respectively.

phenotype only. In contrast, diploid cells appeared to be resistant to transformation. This difference might be explained by the presence of a repressor locus within the deleted region on the short arm of chromosome 11. A suppressor locus within 11p13 has been implicated in the development of Wilms' tumor (8).

The del-11 cells which we have used were diploid except for the deletion overlapping p13 on the short arm of one chromosome 11. Whether the second chromosome 11 which appeared to be cytogenetically intact contains a functional Wilms' tumor locus is presently unknown. Submicroscopic deletions, mutations, or genomic imprinting may have rendered the second allele inactive. Alternatively, a gene dosage effect (26) may be sufficient to cause the increased susceptibility of del-11 cells to transformation by BK virus DNA (14) and HPV-16 early-region DNA.

Some indication of the mechanism of transformation involved came from studying viral expression. The transforming activity of HPV-16 presumably maps to the 5' part of the early region of the HPV genome (5, 7, 25, 31, 45, 51), of which abundant transcripts of varying length could be detected in transformed anchorage-independent del-11 cells. In contrast, no or only low levels of HPV-specific transcripts were detected in transfected anchorage-dependent diploid cell cultures with the same HPV constructs. This difference in viral transcriptional activity did not appear to be a result of different viral copy number. Although we cannot exclude the possibility that viral transcripts are less stable in normal cells than they are in del-11 cells, we think that this difference is more likely due to a difference in the presence of cellular factors involved in the regulation of gene expression. At present we cannot discriminate between the possibilities that del-11 cells provide a cellular factor(s) required for activation of viral gene expression or that diploid cells

FIG. 4. Transcription of HPV ORFs by pHPV-16-10-transfected del-11 cell cultures. Northern blots of poly(A)⁺-selected RNA isolated from pHPV-16-10-transfected del-11 cell culture 1 (lanes 1) and 2 (lanes 2) were hybridized with a full-length HPV-16 genomic probe (A) or with subgenomic probes encompassing the E6/E7 ORFs (B), the E1 ORF (C), and the E2/E4 ORFs (D). The 1,776-bp *Pst*I fragment (E6/E7 ORFs), the 1,838-bp *Pst*I-*Ava*II fragment (E1 ORF), and the 979-bp *Ava*II-*Pst*I fragment (E2/E4 ORFs) of HPV-16 were used as subgenomic probes. The lengths of the transcripts (in kilobases) are shown to the left. Transcripts present are indicated by lines.

provide a factor(s) suppressing viral gene transcription. HPV-16-specific transcripts previously have been detected in HPV-16-transfected human foreskin keratinocytes (16, 39) and fibroblasts (39), which had obtained an extended life span. Our data indicate that low-level viral early-gene transcription is not sufficient to induce anchorage-independent growth of normal diploid cells or del-11 cells. Elevated levels of viral transcription, as detected in pHPV-16-18- and pHPV-16-10-transfected del-11 cells, are apparently required to induce this change in growth characteristic.

We observed high-level viral gene transcription in del-11 cells transfected with HPV recombinants containing a disrupted E2 ORF, while with only one exception del-11 cell cultures transfected with an HPV recombinant containing an intact E2 ORF showed no or low transcriptional activity. The E2 ORF of bovine papillomavirus type 1 (BPV-1) encodes a *trans*-acting factor(s) which regulates the initiation of viral early-gene transcripts from the enhancer-promoter region in the LCR 5' to the early region (23). Accordingly, *cis*-acting E2-responsive elements have been identified in the LCR of BPV-1 (1, 37). The LCR of HPV-16 also contains *cis*-acting elements which can be *trans*-activated by the E2 products of HPV-1, HPV-11, and BPV-1 (24, 38). The HPV-18 LCR contains an enhancer which is also stimulated in the presence of the E2 *trans*-activating protein of BPV-1. However, the BPV-1 E2 protein repressed transcription from the genuine HPV-18 enhancer-promoter sequences (50). The absence of E2 ORF *trans*-regulating activity in cervical carcinoma cell lines (50) containing HPV-16-specific transcripts is consistent with a repressor function of the HPV-16 E2 ORF. An HPV-16 E2 ORF *trans*-repressor function would explain the low transcriptional activity in del-11 cells containing an intact E2 ORF.

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