JOURNAL OF VIROLOGY, Mar. 1989, p. 1247–1255
0022-538X/89/031247-09$02.00/0

Vol. 63, No. 3

Identification of Human Papillomavirus Type 18 Transforming Genes in Immortalized and Primary Cells

MARY A. BEDELL,[1] KATHERINE H. JONES,[2] STEVEN R. GROSSMAN,[1] AND LAIMONIS A. LAIMINS[1,2]*

Department of Molecular Genetics and Cell Biology[1] and the Howard Hughes Medical Institute,[2] University of Chicago, Chicago, Illinois 60637

Received 22 September 1988/Accepted 18 November 1988

The selective retention and expression of the E6-E7 region of human papillomavirus (HPV) types 16 and 18 in cervical carcinomas suggests that these viral sequences play a role in the development of genital neoplasia. Each of three possible gene products, E6, E6*, and E7, from this region of HPV-18 were examined for transforming properties in several types of rodent cells. We have found that in immortalized fibroblasts, both E6 and E7 (but not E6*) are capable of inducing anchorage-independent growth. In rat embryo cells, the HPV-18 E7 open reading frame was an effective immortalizing agent and complemented an activated *ras* oncogene for transformation. In both immortalized and primary cells, transformation was observed when the HPV-18 sequences were expressed from either the HPV-18 promoter or a heterologous promoter. The E6-E7 region is not, however, the sole transforming domain of HPV-18, since another portion of the early region, possibly E5, also exhibited transforming capability in immortalized fibroblasts. The development of human cervical carcinomas may therefore involve a series of steps involving multiple viral and cellular gene products.

Papillomaviruses are a group of small DNA viruses which induce a variety of proliferative lesions in the skin and internal mucosa. More than 50 types of human papillomaviruses (HPV) have been identified, with the majority found in benign lesions such as warts and papillomas (7, 29, 44). However, a subset of these viruses are found in over 90% of malignant carcinomas of the genital tract. Of these HPV-positive carcinomas, the majority contain type 16 (HPV-16), and HPV-18 is the next most common type (6, 14, 30). Although this evidence strongly suggests a role for HPV infection in the development of genital cancer, other genetic or environmental factors are also probably involved (7, 29).

In cervical tumor cell lines, the viral genome is frequently integrated into random sites of host chromosomes, whereas benign lesions usually contain multiple copies of viral episomes (3, 15, 34, 35, 37). In the majority of these tumors, the viral integration site is within or just upstream of the E2 open reading frame (ORF). Since E2 products of a number of papillomaviruses, including HPV-16 and HPV-18 have been shown to be both positive and negative regulators of viral transcription (8, 12, 18, 19, 22, 23, 27, 31, 39), the selective integration that has occurred in carcinomas may result in deregulation of HPV expression. The most abundant viral transcripts in carcinoma cells initiate within the noncoding region, terminate in cellular sequences, and have the potential to encode each of the E6 and E7 ORFs, as well as a splice product, E6* (3, 34, 37). The E6 and E7 proteins, but not E6*, have been identified in cervical carcinoma lines (1, 4, 36, 38), suggesting that either or both of these proteins are involved in the development of malignant disease.

Papillomavirus transforming functions have been best characterized in bovine papillomavirus type 1, in which transforming activity has been localized to the E6 and E5 ORFs (33, 42). Recently, several groups have reported that HPV-16 sequences transform immortalized rodent cells in vitro (24, 40, 43). The E7 ORF of this virus cooperates with an activated *ras* gene in the transformation of primary cells (28, 32) and by itself is an effective immortalizing agent (25). We have previously reported that a major transforming function of HPV-18 in immortalized fibroblasts is encoded by the E6-E7 region (5). In the present study, we have examined each of the E6 and E7 ORFs of HPV-18 and the remainder of the early region for the ability to confer anchorage-independent growth and tumorigenesis in several types of cultured cells. Both of the E6 and E7 ORFs (but not E6*) were found to encode independent transforming functions in immortalized cells. We also demonstrate that HPV-18 E7, like E7 of HPV-16, cooperates with an activated *ras* oncogene in transforming primary cells. This suggests that the two most common HPV types found in genital carcinomas may function through a similar mechanism of transformation in vivo. Another portion of the HPV-18 early region, perhaps E5, is also shown to be capable of transforming immortalized fibroblasts.

MATERIALS AND METHODS

Plasmids. The plasmids used for transformation experiments are shown in Fig. 1 and 2. The construction and transforming activity of p18PEpolyA (Fig. 1B) has been previously reported (5). Mutations were created in the different ORFs of p18PEpolyA by insertion of oligonucleotide linkers into various restriction sites. Two types of linkers which result in different kinds of mutations were used: a translation termination linker (TTL), TTAAGTTAA CTTAA, was used to create nonsense mutations, while linkers which alter the reading frame were used to create frameshift mutations (FS). The restriction sites used, the affected ORFs, and the resulting plasmids which contain the mutagenized sequences are as follows: *Bam*HI, both E6 and E6*, p18PEBam$_{FS}$ and p18PEBam$_{TTL}$; *Sph*I, E6, p18 PESph$_{FS}$ and p18PESph$_{TTL}$; and *Hin*cII, E7, p18PEHinc$_{FS}$ and p18PEHinc$_{TTL}$. An 8-base-pair (bp) linker (CAGAT CTG) was used for p18PEBam$_{FS}$ and p18PEHinc$_{FS}$, and a 12-bp linker (GGAAGATCTTCC) was used for p18 PESph$_{FS}$. (The 12-mer was used because there are two adjacent *Sph*I sites which are only 7 bp apart). After digestion with *Bam*HI or *Sph*I, the ends were made flush with mung bean nuclease or T4 DNA polymerase, respectively,

* Corresponding author.

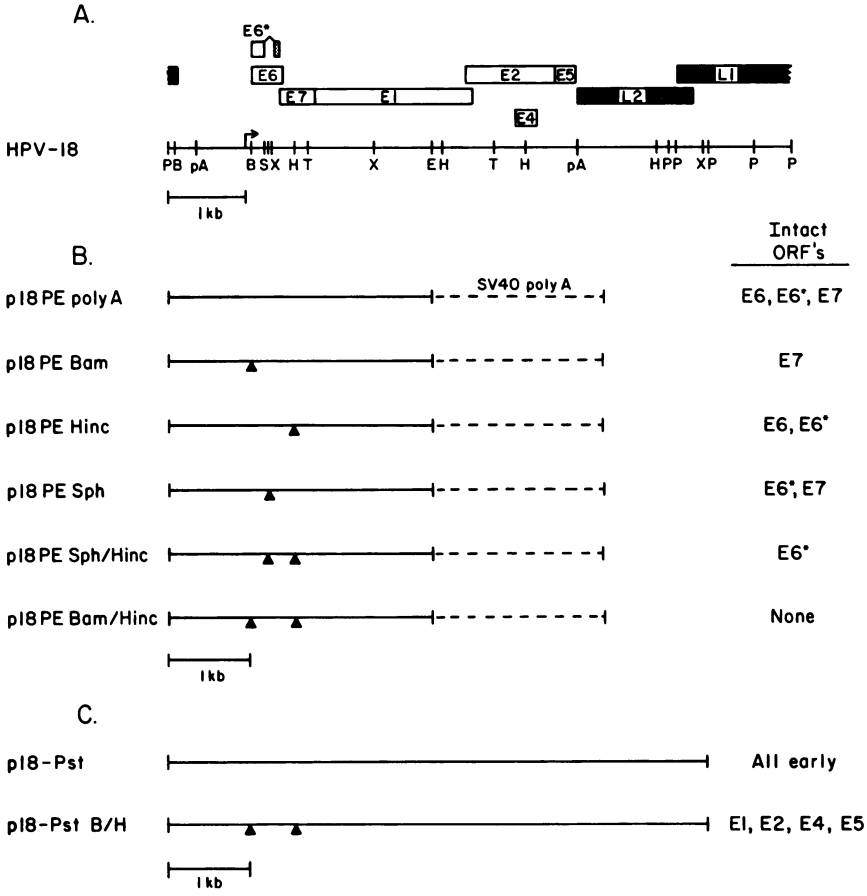

FIG. 1. (A) Genomic organization of HPV-18. The HPV-18 sequences (11) for the early (▭) and late (▬) ORFs and potential polyadenylation sites (pA) are shown. Also shown is E6* (▭◪), a spliced product of E6, and the start of transcription for the E6-E7 region (↱), both of which were characterized in viral cDNAs of cervical tumor cell lines (34). Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hinc*II; P, *Pst*I; S, *Sph*I; T, *Taq*I; X, *Xba*I. (B) Structure of a subgenomic portion of HPV-18 and different mutations in the E6-E7 region. The subgenomic construct, p18PEpolyA, was described previously (5). For each construct, the 3.4-kb *Pst*I-*Eco*RI fragment of HPV-18 (——) and a 2.1-kb *Eco*RI fragment containing SV40 polyadenylation sequences (- - -) are shown. Symbol: ▲, restriction sites into which oligonucleotide linkers were inserted (see Materials and Methods). Both TTL and FS linkers were inserted into the *Bam*HI, *Sph*I, and *Hinc*II sites (e.g., p18PEBam$_{TTL}$ and p18PEBam$_{FS}$ [Table 1]), whereas only FS insertions were made in p18PESph/Hinc and p18PEBam/Hinc. According to the sequence of Cole and Danos (11), the nucleotide coordinates for the linker insertions are 119, 287, and 656, respectively, for the *Bam*HI, *Sph*I, and *Hinc*II sites. The ORFs left intact in each construct are listed to the right of each line. (C) Structures of plasmids containing the entire early region of HPV-18. p18-Pst contains a 6.2-kb *Pst*I fragment of HPV-18 encompassing the entire noncoding region, all early-region ORFs, and the putative early polyadenylation sequence. The E6-E7 region of p18-PstB/H contains FS linker insertions identical to those of p18PEBam/Hinc, whereas all other sequences are the same as in p18-Pst.

and ligated to linkers. Linkers were ligated directly to the blunt ends of *Hinc*II-digested DNA. Plasmids which contain FS mutations in both of the E6 and E7 ORFs were constructed by exchanging appropriate fragments of p18PEBam$_{FS}$, p18PESph$_{FS}$, and p18PEHinc$_{FS}$. The affected ORF in each of the resulting double-site mutant plasmids is as follows: E6 and E7, p18PESph/Hinc; and E6 and E6*, and E7, p18PEBam/Hinc.

The 6.2-kilobase (kb) *Pst*I fragment of HPV-18, which contains the entire early region, was inserted into the *Pst*I site of pUC9 to generate p18-Pst (Fig. 1C). This plasmid carries an intact noncoding region and E6-E7 region identical to that of p18PEpolyA but also contains the entire E1, E2, E4, and E5 ORFs and putative early polyadenylation sequence. p18-PstB/H was constructed by replacing the *Eco*RI fragment of p18PEBam/Hinc, which carries the simian virus 40 (SV40) polyadenylation sequences, with a fragment of p18Pst from the *Eco*RI site in E1 to an *Eco*RI site in the polylinker of pUC9. The E6-E7 region of p18-PstB/H is thus identical to that of p18PEBam/Hinc with FS linker insertions in E6, E6*, and E7, whereas all other sequences are identical to those of p18-Pst.

Expression vectors for each of the E6 and E7 ORFs were constructed by inserting fragments with each ORF into a plasmid containing the human metallothionein II$_A$ transcriptional-regulatory region (Fig. 2). This plasmid, pHSI, consists of human metallothionein II$_A$ sequences from −770 to +69 and has a unique *Bam*HI site located 70 bp 3′ to the start of transcription (21). The E6 ORF was contained on a 540-bp *Ava*II fragment that had been filled in by using the Klenow fragment of *Escherichia coli* DNA polymerase I, whereas a 410-bp *Hae*III-*Sau*3A fragment was used for the E7 ORF. *Bgl*II oligonucleotide linkers were added to the blunt ends of each fragment for cloning into the *Bam*HI site of pHSI. An expression plasmid which contains both E6 and E7 was constructed by inserting an *Xba*I fragment of HPV-18 (Fig. 1A) into the *Xba*I site of p18MTE6. Translation termination linkers, as described above, were inserted into the *Bam*HI

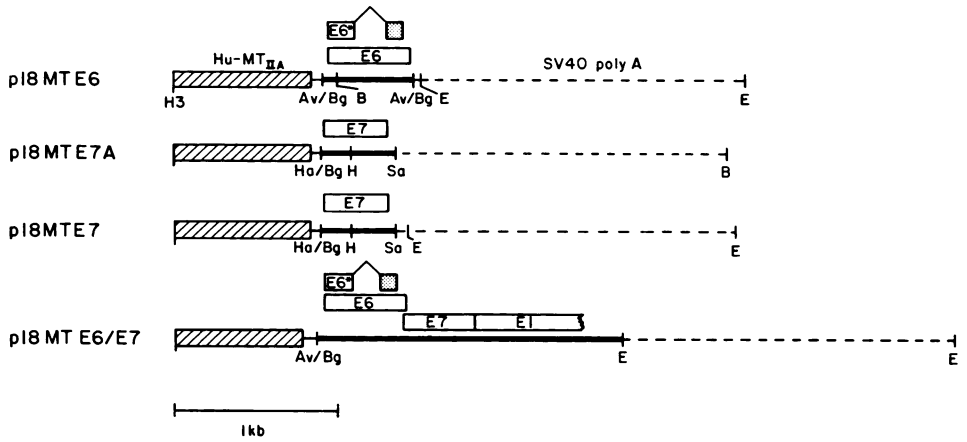

FIG. 2. Structures of expression plasmids for the HPV-18 E6 and E7 ORFs. The human metallothionein II_A promoter (▨), HPV-18 sequences (▬), and SV40 polyadenylation (- - -) sequences are shown, whereas plasmid sequences are not shown. The E6 ORF was contained on an *Ava*II fragment (nucleotides 57 to 597 [11]), which was made blunt and ligated to *Bgl*II linkers for cloning. *Bgl*II linkers were added to a *Hae*III-*Sau*3A fragment (nt 510 to 920 [11]) containing the E7 ORF. Both fragments were cloned into the *Bam*HI site of pHSI (21). An *Xba*I fragment from HPV-18 (Fig. 1A) was inserted into p18MTE6 to make p18MTE6/E7. The SV40 early polyadenylation sequences of p18MTE6, p18MTE7, and p18MTE6/E7 are contained on a 2.1-kb *Eco*RI fragment of pSV2cat (20), whereas p18MTE7A carries SV40 early polyadenylation sequences on a *Bgl*II-*Bam*HI fragment of an SV40 expression vector (see Materials and Methods). Not shown are analogous expression vectors in which E6 and E7 were mutagenized by insertion of translation termination linkers into the *Bam*HI and *Hin*cII sites, respectively (Fig. 1). Restriction sites, in addition to those in Fig. 1: Av, *Ava*II; Bg, *Bgl*II; Ha, *Hae*III; H3, *Hin*dIII; Sa, *Sau*3A.

and *Hin*cII sites of E6 and E7, respectively, to generate p18MTE6Bam and p18MTE7Hinc, respectively. Early SV40 polyadenylation sequences on an *Eco*RI fragment from pSV2Cat (20) were cloned into all expression plasmids except p18MTE7A. In p18MTE7A, the polyadenylation sequences were contained on a *Bgl*II-*Bam*HI fragment of pKS, an SV40 expression vector derived from pKSV10 (Pharmacia, Inc., Piscataway, N.J.) by deletion of a 0.5-kb *Hin*dIII fragment in T antigen sequences (not shown).

Cell culture, transfections, and transformation assays. NIH 3T3 and Rat-1 cells were maintained and transfected as previously described (5). Briefly, HPV-18 plasmids or control (pUC9 or pHSI) plasmids were transfected at a 10:1 ratio with either pRSVneo or pSV2gpt. First-passage rat embryo cells from Fisher rats (REFs) were obtained from M. A. Bioproducts, Walkersville, Md. REFs were transfected after the second passage as described for NIH 3T3 and Rat-1 cells (5), except that a total of 55 μg of DNA was used (25 μg each for HPV-18 plasmids and either pEJras or pUC9 and 5 μg of pRSVneo) and the G418 concentration was 300 μg/ml. After 1 to 2 weeks in selective media, resistant colonies were either pooled from an entire plate or individually cloned and expanded into cell lines. After the first passage of these lines, anchorage-independent growth was determined by plating the cells in 0.3% agarose. In most experiments, growth in soft agar was scored after 2 to 3 weeks for NIH 3T3 and Rat-1 cells and 1 week for REFs. To determine the life span of REF lines, we passaged cultures twice weekly at a ratio of 1:5. Representative NIH 3T3 transfected cell lines were injected (2×10^6 cells) into two to four subcutaneous sites of 3- to 6-week-old *nu/nu* mice, and the animals were monitored for the appearance of tumors.

DNA and RNA analysis. Total cellular RNA was isolated (9) from subconfluent cultures of either pooled or individual colonies, whereas high-molecular-weight DNA of individual transfected lines was prepared as described previously (5). Both Southern and Northern (RNA) blots were prepared and probed as described previously (5).

RESULTS

Mutational analysis of the HPV-18 E6-E7 transforming region in immortalized cells. In cervical tumor cells, the most abundant HPV transcripts originate from the E6-E7 region and terminate with cellular polyadenylation signals (3, 15, 35, 37). We have previously demonstrated that a plasmid (p18PEpolyA) which mimics this state is sufficient to transform NIH 3T3 and Rat-1 cells to an anchorage-independent state in the absence of morphological transformation or focus formation (5). p18PEpolyA (Fig. 1B) contains the entire noncoding region, intact E6 and E7 ORFs, and truncated E1 ORF of HPV-18, as well as heterologous polyadenylation sequences. To identify which of three possible gene products (E6, E6*, or E7) from this region of HPV-18 encodes transforming activity, we introduced translation termination and frameshift mutations into each ORF. The resulting plasmids were then transfected with a selectable marker into NIH 3T3 and Rat-1 cells, and their transforming capabilities were determined in a manner identical to that described previously (5). This assay involves random selection, cloning, and expansion of individual drug-resistant colonies into cell lines which are then tested for their ability to grow in 0.3% agarose. We have used the incidence of anchorage-independent lines induced by each construct as a measure of transforming ability.

The frequency at which NIH 3T3 and Rat-1 lines exhibited anchorage independence following transfection of p18 PEpolyA and its derivatives is shown in Table 1. In both cell types, p18PEpolyA transformed nearly all of the lines tested, with 15 of 18 NIH 3T3 and 18 of 18 Rat-1 lines exhibiting growth in soft agar. The total transformation frequency for p18PEpolyA in both types of cells was thus 33 of 36 (92%). The cloning efficiency in soft agar of individual p18PEpolyA anchorage-independent lines ranged from 15 to 89%, with an average of 35% (not shown). Although p18PEpolyA derivatives which contain FS or TTL mutations at the same site gave similar results in NIH 3T3 cells, TTL mutants appeared

TABLE 1. Transformation of immortalized cells by mutants of the E6-E7 region of HPV-18

Plasmid	Intact ORFs	Mutations[a]	No. of anchorage-independent lines[b]	
			NIH 3T3	Rat-1
p18PEpolyA	E6, E6*, E7	None	15/18	18/18
p18PESph	E6*, E7	FS; TTL	6/11; 8/9 (14/20)[c]	8/11; 4/4 (12/15)
p18PEBam	E7	FS; TTL	8/13; 7/9 (15/22)	9/10; 3/3 (12/13)
p18PEHinc	E6, E6*	FS; TTL	9/15; 3/8 (12/23)	5/9; 4/4 (9/13)
p18PESph/Hinc	E6*	FS	1/9	ND[d]
p18PEBam/Hinc	None	FS	2/11	1/7
p18-Pst	E1, E2, E4, E5, E6, E6*, E7	None	9/11	ND
p18-PstB/H	E1, E2, E4, E5	FS	4/5	ND
pUC9	None		2/16	1/9

[a] Two kinds of linkers were used: linkers which alter the reading frame (FS) and those which have translation termination codons in all reading frames (TTL).
[b] Each plasmid (see Fig. 1) was cotransfected with a selectable marker into each cell line. After selection for drug resistance, individual colonies were chosen at random, expanded into cell lines, and plated in 0.3% agarose. The lines were considered positive for anchorage independence if more than 10% of the plated cells formed colonies of more than eight cells after 2 to 3 weeks in soft agar. The number of lines that were positive out of the total number tested are shown for each plasmid.
[c] The total number of cell lines transfected with FS and TTL mutations in each site are shown in parentheses.
[d] ND, Not determined.

to give higher transformation frequencies than FS mutants in Rat-1 cells. This apparent difference between the two types of mutants in Rat-1 cells may reflect only the smaller number of lines tested with the TTL mutants. The results from transfection of all mutants in both cell types indicate that plasmids which contain mutations in the E6 or the E7 ORF retain transforming capability; however, the frequency of anchorage-independent lines varies depending on the ORF which was mutated (Table 1).

After transfection of p18PESph$_{FS}$ and p18PESph$_{TTL}$, in which only E6* and E7 are intact, anchorage-independent growth was observed in a total of 14 of 20 NIH 3T3 and 12 of 15 Rat-1 cell lines tested. Mutants whose only intact ORF is E7, p18PEBam$_{FS}$ and p18PEBam$_{TTL}$, were similarly effective, with a total of 15 of 22 and 12 of 13 NIH 3T3 and Rat-1 cell lines, respectively, capable of growth in soft agar. The combined frequency of anchorage-independent lines in the two cell types induced by all of the E6 mutants was thus 74 and 77%, compared with the 92% transformants observed with p18PEpolyA. The transformation frequency observed after transfection with E7 mutants was even lower than that of the E6 mutants, but was not abolished. Only 21 of 36 (58%) Rat-1- and NIH 3T3-derived cell lines were anchorage independent after transfection of p18PEHinc$_{FS}$ and p18PEHinc$_{TTL}$. The cloning efficiency of cell lines growing in soft agar after transfection of either E6 mutants or E7 mutants was slightly lower than p18PEpolyA lines, ranging from 13 to 52% and averaging 21, 28, and 25%, respectively, for FS and TTL mutations at the *Sph*HI, *Bam*HI, and *Hin*cII sites (data not shown).

To confirm that the transforming activity of individual ORFs was abolished by the linker insertions, we have also constructed and tested two plasmids which contain FS mutations in both of the E6 and E7 ORFs. In p18PESph/Hinc, the only complete and intact ORF is E6*, whereas p18PEBam/Hinc contains no complete, intact ORFs. Neither plasmid appears to be capable of transforming these cells, since only 1 of 9 p18PESph/Hinc and 3 of 18 p18PEBam/Hinc cell lines were anchorage independent. This frequency is similar to that observed after transfection of pUC9 control DNA, for which 3 of 25 lines were positive in the two cell types. These results indicate that E6* by itself is not sufficient for anchorage-independent growth and that the transforming capability of single-site mutants is due to the action of the intact ORFs.

Expression of individual E6 and E7 ORFs in immortalized cells. To confirm that each of the E6 and E7 ORFs encodes transforming activity and to more directly compare their transforming capabilities, we cloned each ORF into an expression vector which uses the human metallothionein II_A promoter and SV40 polyadenylation signals. As controls, the TTL mutations described above were constructed in the *Bam*HI and *Hin*cII sites, respectively, of the E6 and E7 expression vectors. An expression vector which contains both the E6 and E7 ORFs, p18MTE6/E7, was constructed for comparison with p18PEpolyA. Each of these plasmids (Fig. 2), as well as the parental plasmid, pHSI, bearing only the metallothionein transcriptional regulatory sequences, was transfected into Rat-1 and NIH 3T3 cells, and the ability of individually derived lines to grow in soft agar was determined.

Consistent with the results of the mutational analysis, each ORF induced anchorage-independent growth in both Rat-1 and NIH 3T3 cells (Table 2). Transformation by p18MTE6/E7 was observed at a frequency similar to that of p18PEpolyA, with 20 of 23 lines (87%) from both types of cells capable of growth in soft agar. Similarly, a total of 19 of 22 lines (86%) from both cell types transfected with the E7-expressing plasmids, p18MTE7A and p18MTE7, were

TABLE 2. Transformation of immortalized cells by expression vectors for the HPV-18 E6 and E7 ORFs

Plasmid	Intact ORFs	No. of anchorage-independent lines[a]	
		NIH 3T3	Rat-1
p18MTE6/E7	E6, E6*, E7	10/10	10/13
p18MTE7A[b]	E7	9/10	5/6
p18MTE7	E7	5/6	ND[c]
p18MTE7Hinc[d]	None	1/6	ND
p18MTE6	E6, E6*	10/15	11/18
p18MTE6Bam[d]	None	1/6	ND
pHSI	None	1/12	2/8

[a] See footnote *b* of Table 1. The transfected plasmids are shown in Fig. 2, except pHSI, which contains only the human metallothionein promoter and no coding sequences.
[b] p18MTE7A differs from all of the other expression plasmids in the source of SV40 polyadenylation sequence (see Materials and Methods).
[c] ND, Not determined.
[d] A TTL was inserted into the *Bam*HI and *Hin*cII sites, respectively, of p18MTE6 and p18MTE7 to mutate their E6 and E7 sequences.

anchorage independent. In soft agar, the cloning efficiencies of individual lines transfected with each of these three plasmids were nearly identical and averaged 30% (data not shown), like that observed with p18PEpolyA. The use of different polyadenylation sequences in p18MTE7 and p18MTE7A did not appear to affect the transforming capability of E7, since the two plasmids had similar transformation frequencies (5 of 6 for p18MTE7 and 9 of 10 for p18MTE7A in NIH 3T3 cells). Compared with the E7 expression vectors, the transformation frequency observed after transfection of p18MTE6 was reduced, with only 21 of 33 (64%) transfected lines forming anchorage-independent colonies. The p18MTE6-containing lines had a slightly lower cloning efficiency in soft agar than did lines which contained p18MTE7 and averaged 22% (not shown). Neither p18MTE6Bam nor p18MTE7Hinc was capable of transforming NIH 3T3 cells, since only one of six cell lines transfected with each plasmid exhibited growth in soft agar.

Physical state and expression of E6 and E7 sequences in NIH 3T3 cell lines. Three or four representative NIH 3T3 lines that had been transfected with each of the plasmids in Fig. 1 and 2 were examined by Southern analysis for the state of viral DNA and by Northern analysis for steady-state viral RNA levels. All cell lines examined had intact, integrated E6-E7 sequences at roughly 2 to 50 copies per cell (data not shown). No correlation between the copy number of integrated sequences and transformation phenotype was observed. The sizes of unspliced transcripts that should be expressed from each set of HPV-18 plasmids are as follows: a 3.2-kb mRNA for p18PEpolyA and its derivatives; a 1.9-kb and a 1.8-kb transcript, respectively, for p18MTE6 and p18MTE7 and their mutants; and a 3.3-kb mRNA for p18MTE6/E7. Northern blots of total cellular RNA that were probed with an E6-E7-specific fragment revealed that all transfected lines examined expressed viral RNA of the expected size (data not shown).

To determine whether the mutant derivatives of p18PEpolyA and all of the expression vectors expressed E6-E7 RNA at similar levels, RNA from pooled colonies of three separate transfections with each plasmid was examined, and representative Northern blots are shown in Fig. 3. The expression of E6-E7 sequences in cell lines transfected with plasmids containing each type of single- or double-site mutation was decreased two- to threefold relative to p18PEpolyA (Fig. 3A). However, cell lines containing each p18PEpolyA mutant expressed comparable levels of RNA, and there was no obvious correlation between a mutated sequence and diminished expression. The reason for the increased expression of p18PEpolyA over that of its mutant derivatives is presently unknown. In pooled cell lines transfected with the expression vectors (Fig. 3B), maximal expression was observed with p18MTE6/E7, whereas cells containing each of the single ORF plasmids, p18MTE6 and p18MTE7 and their respective mutants, expressed slightly reduced levels of viral RNA.

Tumorigenicity of transformed NIH 3T3 cells. NIH 3T3 cell lines transfected with the HPV-18 E6-E7 region have previously been shown to be tumorigenic in nude mice (5). To determine whether the HPV-18 E6 and E7 ORFs differ in their ability to induce tumorigenic growth, representative anchorage-independent NIH 3T3 transfected cell lines were injected subcutaneously into nude mice. Tumors of roughly 2 to 3 cm in diameter were observed within 4 weeks for 10 lines transfected with either p18PEpolyA or p18MTE6/E7. Of seven cell lines transfected with either of the E7-expressing plasmids, p18PEBam$_{TTL}$ or p18MTE7A, five were also able to form tumors of about the same size as p18PEpolyA or p18MTE6/E7. In contrast, only two of five p18PEHinc$_{TTL}$ and p18MTE6 transformants, which both express only E6, were tumorigenic, and these tumors grew to only 1 to 2 cm in diameter. No such tumors were observed after injection of six different lines transfected with pUC9 DNA. These experiments suggest that anchorage-independent lines which contain either both E6 and E7 or E7 alone are more tumorigenic than transformed lines which carry only E6.

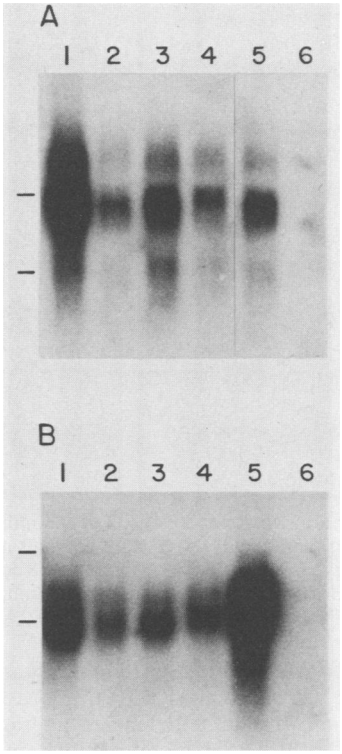

FIG. 3. Expression of E6-E7 sequences in transfected NIH 3T3 cells. Total cellular RNA was isolated from pooled transfected NIH 3T3 colonies and Northern blots probed with a *Bam*HI-*Taq*I fragment encompassing most of the E6-E7 region (Fig. 1A). Both panels are from film exposed overnight with intensifying screens. The marks indicate the positions of 28S and 18S rRNA. (A) Viral RNA expressed in lines transfected with p18PEpolyA and derivative mutants. The transfected plasmids in each lane are as follows: 1, p18PEpolyA; 2, p18PEBam$_{TTL}$; 3, p18PESph$_{TTL}$; 4, p18PEHinc$_{TTL}$; 5, p18PEBam/Hinc; 6, pUC9. (B) E6 and E7 RNA expressed in cell lines transfected with the metallothionein expression vectors. The transfected plasmids in each lane are as follows: 1, p18MTE6; 2, p18MTE6Bam; 3, p18MTE7; 4, p18MTE7Hinc; 5, p18MTE6/E7; 6, pHSI.

Transformation of pooled NIH 3T3 colonies. In addition to the analysis of individual transfected colonies described above, we determined the anchorage-independent growth of cells that were pooled from drug-resistant colonies of two to four transfections of NIH 3T3 cells. In this pooled assay, the ability to grow in soft agar is less likely to be the result of any selective pressures which might occur during the expansion of individual colonies. In control transfections with either pUC9 or pHSI, no colonies of eight cells or more were observed after 3 weeks in 0.3% agarose. After transfection of the plasmids which express both E6 and E7 (p18PEpolyA and p18MTE6/E7, respectively), a cloning efficiency in soft agar of 18 and 20% occurred after the same period, and

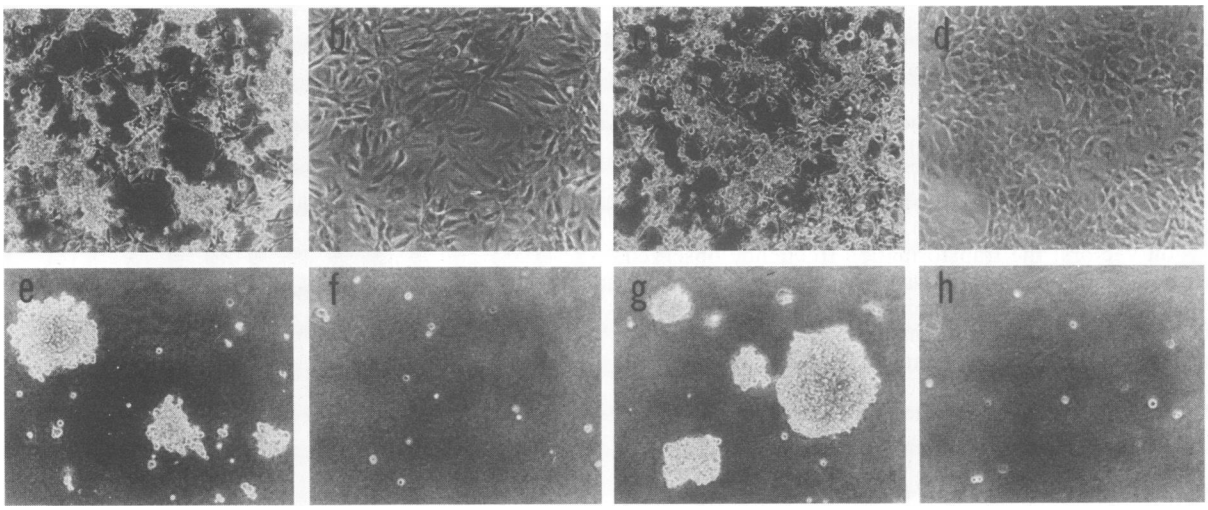

FIG. 4. Anchorage-independent growth and morphological transformation of rat embryo fibroblasts induced by HPV-18 E7 and the EJras oncogene. Representative G418-resistant colonies in media at the first passage after cloning (a to d) or after 1 week of growth in 0.3% agarose (e to h) are shown. Each of the following HPV-18 constructs was cotransfected with EJras and pRSVneo: panels a and e, p18PEpolyA; panels b and f, p18PEHinc$_{FS}$; panels c and g, p18PEBam$_{FS}$; panels d and h, pUC9. Magnification, ×100.

typical colonies were estimated to contain at least 50 cells. Similar values were observed with the E7-expressing plasmids, for which cloning efficiencies averaged 19% after transfection of p18PEBam$_{FS}$ and p18PEBam$_{TTL}$, 16% with p18MTE7A, and 19% with p18MTE7. In contrast, an average of only 9% growth was observed after transfection of the plasmids containing E6 (p18PEHinc$_{FS}$, p18PEHinc$_{TTL}$, and p18MTE6). Mutagenesis of both E6 and E7 in p18PEpolyA or either of the single ORFs in the expression vectors resulted in an average of 4 and 3%, respectively. Because pooled cells transfected with different constructs contain comparable levels of E6-E7 RNA (Fig. 3), the differences in soft-agar cloning efficiencies are not likely to be the result of differential expression. These results therefore provide further evidence that the E6 and E7 ORFs encode independent transforming functions but may differ in their oncogenic potency.

The E6-E7 region is not the sole transforming domain of HPV-18 in immortalized cells. To determine whether E6 and E7 are the only transforming genes present in the HPV-18 early region, we constructed and tested two additional plasmids p18-Pst and p18-PstB/H (Fig. 1C). Although both of these plasmids contain a 6.2-kb fragment which encompasses the entire noncoding region, all early ORFs, and the putative early polyadenylation sequence of HPV-18, p18-PstB/H differs from p18-Pst only in the presence of linker insertions in the E6, E6*, and E7 ORFs. When p18-Pst was transfected into NIH 3T3 cells, 9 of 11 individual lines were transformed to anchorage independence (Table 1). Interestingly, mutagenesis of the E6-E7 region in p18-PstB/H did not significantly affect transforming function, since four of five transfected lines exhibited anchorage independence (Table 1). It is unlikely that the mutagenized E6-E7 sequences in p18-PstB/H encode residual transforming activity, since they contain the identical linker insertions which abolished the activity of p18PEBam/Hinc (see above). The E6-E7 region therefore does not appear to be the only transforming domain expressed from the intact early region. By analogy to the transforming function of bovine papillomavirus type 1 E5 (33, 42), we suspect that an HPV-18 E5 gene may encode this additional transforming activity. In support of this, eight p18Pst and p18PstB/H lines examined contained an intact E5 ORF, and E5-specific RNA was expressed (data not shown).

Cooperation of HPV-18 sequences with a *ras* oncogene in the transformation of primary rodent fibroblasts. The E7 ORF of HPV-16, when expressed from strong heterologous promoters, has recently been shown to cooperate with an activated *ras* gene in the transformation of primary rodent cells (28, 32). To determine whether the HPV-18 E6-E7 sequences also complement *ras*, second-passage REFs were cotransfected with p18PEpolyA, pEJras (a plasmid which contains an activated human *c*-Ha-ras gene), and a plasmid carrying a selectable marker in four different experiments. After selection, a high frequency of the drug-resistant colonies from these transfections exhibited morphological transformation. In contrast, no morphologically altered colonies were observed in over 200 colonies derived from control transfections with pUC9 and EJras or when p18PEpolyA was cotransfected with pUC9. The colonies transformed by p18PEpolyA and *ras* were elongated, highly refractile, and poorly adherent, whereas pUC9-plus-*ras* or p18PEpolyA-plus-pUC9 transfectants displayed a morphology similar to that of untransfected cells (Fig. 4). Eight of the p18PEpolyA-plus-*ras* morphological transformants were cloned and tested for anchorage-independent growth, and all formed large colonies (>100 cells) within 1 week after plating in 0.3% agarose (Table 3). In addition, all five transformants tested have been passaged 10 or more times and appear to be established cell lines. In contrast, none of six randomly chosen colonies derived from pUC9 and pEJras cotransfections were anchorage independent, and four colonies tested for immortalization all senesced before passage 5.

To identify which HPV-18 sequence mediates *ras*-cooperating activity, the mutant and expression constructs shown in Fig. 1 and 2 were cotransfected with pEJras and a selectable marker into REFs. When pEJras was cotransfected with any of the plasmids bearing an intact E7 ORF (p18PEBam$_{FS}$, p18PEBam$_{TTL}$, p18PESph$_{FS}$, p18PESph$_{TTL}$, p18MTE7A, and p18MTE7), morphologically transformed colonies were observed at a frequency similar to that observed after cotransfection of pEJras and p18PEpolyA. In contrast, morphologically transformed colonies were not

TABLE 3. Cooperation of HPV-18 E7 with the EJras oncogene in the transformation of rat embryo fibroblasts

Plasmid[a]	Intact HPV ORFs	Anchorage independence[b]	No. of colonies immortalized[c]
p18PEpolyA	E6, E6*, E7	8/8	5/5
p18PEBam[d]	E7	8/10	7/8
p18PEHinc	E6, E6*	0/6	0/4
p18PESph	E6*, E7	6/6	6/6
p18PESph/Hinc	E6*	0/2	0/2
p18PEBam/Hinc	None	0/2	0/2
pUC9	None	0/6	0/4
p18MTE7A	E7	2/2	ND[e]
p18MTE7	E7	6/6	6/6
p18MTE7Hinc	None	0/6	0/6
p18MTE6	E6, E6*	0/4	ND

[a] Each plasmid was cotransfected with pEJras and pRSVneo. Transfected colonies were selected with G418 and then cloned and expanded.

[b] Expanded colonies were plated in 0.3% agarose at the first passage after cloning. If more than 1% of the plated cells were able to form foci in soft agar within 1 week, the clones were considered positive. The number of expanded colonies which were positive for anchorage-independent growth, versus the number tested with each pair of plasmids, is shown.

[c] Expanded colonies were passaged twice a week at a ratio of 1:5 and were considered immortalized if they survived 10 passages.

[d] The results with plasmids containing TTL and FS mutations in each site are combined.

[e] ND, Not determined.

observed when pEJras was cotransfected with either plasmids containing mutated E7 sequences (p18PEHinc$_{FS}$, p18PEHinc$_{TTL}$, p18PESph/Hinc, p18PEBam/Hinc, and p18 MTE7Hinc) or p18MTE6. Each of these plasmids was tested at least three times, and 20 to 50 colonies were examined in each experiment.

Anchorage-independent growth was observed in a total of 22 of 24 colonies derived from cotransfection of pEJras and either p18PEBam$_{FS}$, p18PEBam$_{TTL}$, p18PESph$_{FS}$, p18PES ph$_{TTL}$, p18MTE7A, or p18MTE7 (Table 3). The morphology of these transformants on plastic and in soft agar was similar to that of p18PEpolyA-plus-pEJras transformants (Fig. 4). Of 20 E7-plus-EJras transformants, representing at least three independent lines isolated following transfection of p18MTE7 and each kind of mutation in the *Bam*HI and *Sph*I sites, 19 have been continuously passaged more than 10 times without senescence or loss of morphological alterations (Table 3). To ensure that these transformants contained and expressed both HPV-18 sequences and pEJras DNA, Southern and Northern analyses were performed on 14 different anchorage-independent lines. In all 14 lines, both the E6-E7 sequences of the HPV-18 plasmids and the coding sequences of EJras were intact and expressed (data not shown).

The ability of REFs expressing both EJras and HPV-18 E7 sequences to survive continuous passage suggests that they had become immortalized. To determine whether the HPV-18 E7 sequences alone were capable of immortalizing these cells, we cloned and continuously passaged six colonies each from transfections of p18MTE7, p18MTE7Hinc, or pUC9. None of the colonies transfected with either E7 plasmid exhibited a morphology substantially different from pUC9 transfectants. All six p18MTE7 colonies survived at least 10 passages, whereas senescence occurred before passage five in all p18MTE7Hinc colonies and pUC9 colonies (data not shown). Northern and Southern analyses were performed on five of the p18MTE7 immortalized lines, and the E7 sequences were intact and expressed in all of them (data not shown). These results demonstrate that the HPV-18 E7 sequence, like HPV-16 E7, encodes an immortalizing activity which is capable of complementing the activity of a *ras* oncogene in the transformation of primary cells.

The absence of morphological transformation after cotransfection of either E7 mutant plasmids or p18MTE6 with pEJras (Fig. 4) suggests that none of these plasmids are able to cooperate with *ras*. To determine whether these plasmids were capable of inducing anchorage-independent growth or immortalization, a total of 20 colonies were chosen at random from transfections of pEJras with either p18PEHinc$_{FS}$, p18PEHinc$_{TTL}$, p18PESph/Hinc, p18PEBam/Hinc, p18MTE7Hinc, or p18MTE6. None of these colonies exhibited growth in 0.3% agarose, and all senesced before passage 5 (Table 3). These experiments demonstrate that HPV-18 E6 is not capable of cooperating with *ras*. Whether this ORF may cooperate with other oncogenes in primary cell transformation is unclear.

DISCUSSION

We have examined the E6-E7 region of HPV-18 to identify specific ORFs which possess transforming ability in vitro and may thus contribute to the development of cervical cancer. A series of plasmids which express only one or two of the three possible products from this region were constructed, and their transforming capabilities in immortalized and primary cells were determined. Transfection of immortalized cells, such as NIH 3T3 and Rat-1 cells, with HPV-18 sequences does not produce morphologically altered foci; however, the majority of randomly chosen transfected colonies exhibit anchorage-independent growth (5). In the present investigation, both E6 and E7 were independently able to confer anchorage independence in immortalized cells; however, a higher frequency of anchorage-independent lines was consistently observed with E7 alone than with E6. Similarly, E7-expressing lines appear to be more tumorigenic than E6-expressing lines. The effects of different HPV-18 gene products in primary cells were also examined. We demonstrate that the HPV-18 E7 ORF was able to immortalize REFs and cooperate with an activated *ras* oncogene in causing both morphological transformation and anchorage-independent growth. These results with primary cells are in agreement with two recent reports of the cooperation of the HPV-16 E7 with an activated *ras* oncogene (28, 32) and suggest a mechanism of transformation which may be common to genital cancers induced by HPVs.

The transforming properties of E6 and E7 in vitro, as well as the continuous presence of both viral proteins in cervical carcinoma cell lines (1, 4, 36, 38), suggest that each may play a role in oncogenesis in vivo. The significance of E6* transcripts in tumor cell lines is presently unclear, since E6* protein has not been detected in these cell lines (1, 4) and our data indicate that E6* by itself has no transforming capability in NIH 3T3 cells. However, we cannot rule out the possibility that transformation by E6 either requires or is augmented by E6*, since none of the constructs tested contained E6 in the absence of E6*. Since splice sites for E6* are found in HPV types associated with malignancies and not in benign genital HPVs (10, 34), the possibility that E6* is in fact involved in transformation in vivo cannot be completely excluded.

Sequence analysis of the E6 and E7 sequences of all papillomaviruses suggests that the two ORFs may have arisen by duplication of a portion of an ancestral gene (11). Each protein includes a repeating motif of cysteine doublets (Cys-X2-Cys) that bears some similarity to a class of nucleic

acid-binding proteins (16). However, the two proteins appear to reside in entirely different cellular compartments and so may carry out different functions. The E6 proteins of both bovine papillomavirus type 1 (2) and HPV-18 (20a) have been localized to the nucleus and nonnuclear membranes, whereas the E7 protein of HPV-16 was found only in the cytoplasm (38). Consistent with its presence in the nucleus, a strong but nonspecific DNA-binding activity has been observed for the HPV-18 E6 protein (20a). Phelps et al. (32) have recently reported that the HPV-16 E7 encodes a transcriptional *trans*-activating activity, suggesting that at least a small fraction of the protein may localize to the nucleus. Interestingly, a small domain of E7 that is conserved in genital HPVs has been found to have significant homology to a sequence motif common to T antigen of papovaviruses, adenovirus E1a, and the v-*myc* and c-*myc* oncogenes (17, 32). Recently, both SV40 T antigen and adenovirus E1a have been shown to form a specific complex with the cellular retinoblastoma protein (13, 41), which is thought to be a tumor suppressor or regulator of cell growth (26). Significantly, mutations in this conserved sequence which abolish the transforming activity of these viral proteins also abolish complex formation. Since T antigen, E1a, *myc*, and E7 all share the ability to complement *ras* as well as a conserved sequence domain, it is tempting to speculate that the transforming functions of all four types of proteins involve a common pathway for cell growth.

Although the E6 and E7 sequences of HPV-18 are capable of transforming cells in vitro, they do not appear to be the sole transforming genes present in the HPV-18 genome. This is suggested by experiments in which the transforming activity of the 6.2-kb *Pst*I fragment was not affected by mutagenesis of the E6 and E7 sequences. Since the HPV-18 E5 ORF has some features in common with the bovine papillomavirus type 1 E5 sequence and the latter has transforming activity (33, 42), we speculate that this sequence may encode the additional transforming activity. Because sequences other than E6 and E7 are not conserved in cervical carcinoma cells, it is unlikely that any other gene products are required for maintenance of the malignant phenotype. However, the additional transforming functions could be involved in the development of benign or preneoplastic lesions, in which the entire viral genome is generally present (15, 29). In this case, the transforming activity of the E6-E7 sequences may be limited, perhaps by transcriptional repression by products of the E2 ORF. Consistent with this, Broker and co-workers have reported that the E6-E7 region of HPV-6 and HPV-11 in benign genital lesions is expressed at very low levels and that the HPV-11 E2 encodes a repressor of the E6 promoter (8, 10). Similarly, the E6-E7 region is poorly expressed from plasmids containing either a permuted viral genome (5) or the 6.2-kb *Pst*I fragment of the early region (M. Bedell, unpublished results). Once viral integration occurs and E2 is no longer expressed, E6-E7 transcription may be deregulated and the E6 and E7 proteins may play a significant role in the development of malignant lesions. However, epidemiological evidence demonstrates that other factors, which may involve the activation of cellular proto-oncogenes, are required in addition to HPV infection for the development of cervical carcinomas (7, 29). Thus, several events involving both viral and cellular genes may be required for the development of malignant lesions of the genital tract.

ACKNOWLEDGMENTS

We thank I. Blitz for making one of the expression plasmids and V. Sukhatme, A. Diamond, and members of the Laimins laboratory for comments on the manuscript.

This work was funded by the Howard Hughes Medical Institute. M.A.B. was supported by a training grant in Environmental Biology (CA 09273) from the National Institutes of Health and by a grant from the Lucille P. Markey Charitable Trust, Miami, Fla. S.R.G. is a trainee of a Medical Scientist Training Program and is supported by Public Health Service training grant 5T32 GM0782 from the National Institutes of Health.

LITERATURE CITED

1. **Androphy, E. J., N. L. Hubbert, J. T. Schiller, and D. R. Lowy.** 1987. Identification of the HPV-16 E6 protein from transformed mouse cells and human cervical carcinoma cell lines. EMBO J. **6**:989–992.
2. **Androphy, E. J., J. T. Schiller, and D. R. Lowy.** 1985. Identification of the protein encoded by the E6 transforming gene of bovine papillomavirus. Science **230**:442–445.
3. **Baker, C. C., W. C. Phelps, V. Lindgren, M.J. Braun, M. A. Gonda, and P. M. Howley.** 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J. Virol. **61**:962–971.
4. **Banks, L., P. Spence, E. Androphy, N. Hubbert, G. Matlashewski, A. Murray, and L. Crawford.** 1987. Identification of human papillomavirus type 18 E6 polypeptide in cells derived from human cervical carcinomas. J. Gen. Virol. **68**:1351–1359.
5. **Bedell, M. A., K. H. Jones, and L. A. Laimins.** 1987. The E6-E7 region of human papillomavirus type 18 is sufficient for transformation of NIH 3T3 and Rat-1 cells. J. Virol. **61**:3635–3640.
6. **Boshart, M., L. Gissmann, H. Ikenberg, A. Kleinheinz, W. Scheurlen, and H. zur Hausen.** 1984. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J. **3**:1151–1157.
7. **Broker, T. R., and M. Botchan.** 1986. Papillomaviruses: retrospectives and prospectives. Cancer Cells (Cold Spring Harbor) **4**:17–36.
8. **Chin, M. T., R. Hirochika, H. Hirochika, T. R. Broker, and L. T. Chow.** 1988. Regulation of human papillomavirus type 11 enhancer and E6 promoter by activating and repressing proteins from the E2 open reading frame: functional and biochemical studies. J. Virol. **62**:2994–3002.
9. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
10. **Chow, L. T., M. Nasseri, S. M. Wolinsky, and T. R. Broker.** 1987. Human papillomavirus types 6 and 11 mRNAs from genital condylomata acuminata. J. Virol. **61**:2581–2588.
11. **Cole, S. T., and O. Danos.** 1987. Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. Phylogeny of papillomaviruses and repeated structure of the E6 and E7 gene products. J. Mol. Biol. **193**:599–608.
12. **Cripe, T. P., T. H. Haugen, J. P. Turk, F. Tabatabai, P. G. Schmid III, M. Durst, L. Gissmann, A. Roman, and L. P. Turek.** 1987. Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 trans-activator and repressor gene products: implications for cervical carcinogenesis. EMBO J. **6**:3745–3753.
13. **DeCaprio, J. A., J. W. Ludlow, J. Figge, J.-Y. Shew, C.-M. Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston.** 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell **54**:275–283.
14. **Durst, M., L. Gissman, H. Ikenberg, and H. zur Hausen.** 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc. Natl. Acad. Sci. USA **80**:3812–3815.
15. **Durst, M., A. Kleinheinz, M. Hotz, and L. Gissmann.** 1985. The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumours. J. Gen. Virol. **66**:1515–1522.
16. **Evans, R. M., S. M. Hollenberg.** 1988. Zinc fingers: gilt by association. Cell **52**:1–3.

17. **Figge, J., and T. F. Smith.** 1988. Cell-division sequence motif. Nature (London) **334**:109.
18. **Giri, I., and M. Yaniv.** 1988. Study of the E2 gene product of the cottontail rabbit papillomavirus reveals a common mechanism of transactivation among papillomaviruses. J. Virol. **62**:1573–1581.
19. **Gius, D., S. Grossman, M. A. Bedell, and L. A. Laimins.** 1988. Inducible and constitutive enhancer domains in the noncoding region of human papillomavirus type 18. J. Virol. **62**:665–672.
20. **Gorman, C.M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
20a. **Grossman, S. R., R. Mora, and L. A. Laimins.** 1989. Intracellular localization and DNA-binding properties of human papillomavirus type 18 E6 protein expressed with a baculovirus vector. J. Virol. **63**:366–374.
21. **Haslinger, A., and M. Karin.** 1985. Upstream promoter element of the human metallothionein-II_A gene can act like an enhancer element. Proc. Natl. Acad. Sci. USA **82**:8572–8576.
22. **Hirochika, H., T. R. Broker, and L. T. Chow.** 1987. Enhancers and *trans*-acting E2 transcriptional factors of papillomaviruses. J. Virol. **61**:2599–2606.
23. **Hirochika, H., R. Hirochika, T. R. Broker, and L. T. Chow.** 1988. Functional mapping of the human papillomavirus type 11 transcriptional enhancer and its interaction with the *trans*-acting E2 proteins. Genes Dev. **2**:54–67.
24. **Kanda, T., A. Furuno, and K. Yoshiike.** 1988. Human papillomavirus type 16 open reading frame E7 encodes a transforming gene for rat 3Y1 cells. J. Virol. **62**:610–613.
25. **Kanda, T., S. Watanabe, and K. Yoshiike.** 1988. Immortalization of primary rat cells by human papillomavirus type 16 subgenomic DNA fragments controlled by the SV40 promoter. Virology **165**:321–325.
26. **Klein, G.** 1987. The approaching era of the tumor suppressor genes. Science **238**:1539–1545.
27. **Lambert, P. F., B. A. Spalholz, and P. M. Howley.** 1987. A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator. Cell **50**: 69–78.
28. **Matlashewski, G., J. Schneider, L. Banks, N. Jones, A. Murray, and L. Crawford.** 1987. Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. EMBO J. **6**:1741–1746.
29. **McCance, D. J.** 1986. Human papillomaviruses and cancer. Biochim. Biophys. Acta **823**:195–205.
30. **McNab, J. S., J. Walkinshaw, J. Cordiner, and J. B. Clements.** 1986. Human papillomavirus in clinically and histologically normal tissue of patients with genital cancer. N. Engl. J. Med. **315**:1052–1058.
31. **Phelps, W. C., and P. M. Howley.** 1987. Transcriptional transactivation by the human papillomavirus type 16 E2 gene product. J. Virol. **61**:1630–1638.
32. **Phelps, W. C., C. L. Yee, K. Muenger, and P. M. Howley.** 1988. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. Cell **53**:539–547.
33. **Schiller, J. T., W. C. Vass, and D. R. Lowy.** 1984. Identification of a second transforming region in bovine papillomavirus DNA. Proc. Natl. Acad. Sci. USA **81**:7880–7884.
34. **Schneider-Gaedicke, A., and E. Schwarz.** 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. **5**: 2285–2292.
35. **Schwarz, E., U. K. Freese, L. Gissmann, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) **314**:111–114.
36. **Seedorf, K., T. Oltersdorf, G. Krammer, and W. Rowekamp.** 1987. Identification of early proteins of the human papilloma viruses type 16 (HPV 16) and type 18 (HPV 18) in cervical carcinoma cells. EMBO J. **6**:139–144.
37. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83**:4680–4684.
38. **Smotkin, D., and F. O. Wettstein.** 1987. The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. J. Virol. **61**:1686–1689.
39. **Thierry, F., and M. Yaniv.** 1987. The BPV1-E2 trans-acting protein can be either an activator or a repressor of the HPV18 regulatory region. EMBO J. **6**:3391–3397.
40. **Tsunkokawa, Y., N. Takebe, T. Kasamatsu, M. Terada, and T. Sugimura.** 1986. Transforming activity of human papillomavirus type 16 DNA sequences in a cervical cancer. Proc. Natl. Acad. Sci. USA **83**:2200–2203.
41. **Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow.** 1988. Association between an oncogene and an antioncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature (London) **334**:124–129.
42. **Yang, Y.-C., H. Okayama, and P. M. Howley.** 1985. Bovine papillomavirus contains multiple transforming genes. Proc. Natl. Acad. Sci. USA **82**:1030–1034.
43. **Yasumoto, S., A. L. Burkhardt, J. Doniger, and J. A. DiPaolo.** 1986. Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. J. Virol. **57**:572–577.
44. **zur Hausen, H.** 1985. Genital papillomavirus infections. Prog. Med. Virol. **32**:15–21.