Papilloma Formation by Cottontail Rabbit Papillomavirus Requires E1 and E2 Regulatory Genes in Addition to E6 and E7 Transforming Genes

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The present study used the cottontail rabbit papillomavirus DNA-rabbit system to evaluate whether the regulatory genes E1 and E2 and the transforming gene E6 are required for papilloma formation. Frameshift mutations were generated in the individual genes in the context of a full-length cottontail rabbit papillomavirus genome, and the mutant DNAs were intradermally inoculated into domestic rabbits. None of the mutants induced papillomas. Marker rescue experiments confirmed that the defects were due to mutations that we deliberately introduced. Marker rescue also confirmed our previous report that the upstream region of E7 around position 9 was critical for papilloma induction. These results demonstrate that the E1 and E2 regulatory genes as well as the E6 and E7 transforming genes are each required for papilloma formation. Each gene may provide molecular targets for therapeutic intervention.

Papillomaviruses induce benign lesions that can progress to squamous cell carcinoma, particularly at the uterine cervix (for a review, see reference 48). It is important to understand papillomavirus gene functions to identify molecular targets for therapeutic intervention. A partial understanding has been obtained using a variety of *in vitro* assays (for reviews, see references 14, 24, 29, and 31). It has been found, for instance, that the E1 and E2 genes are necessary and sufficient for episomal viral DNA replication in cultured cells and in a cell-free system (10, 13, 35, 55, 62). E2 protein additionally regulates transcription of early genes, including E6 and E7 (15, 21, 51, 53). Cell culture studies have also shown that the E6 and E7 genes of high risk human papillomavirus types (HPVs), *i.e.*, those associated with human cancer, mediate immortalization and transformation (11, 25, 26, 37, 39, 41, 44, 46).

Each papillomavirus protein appears to be multifunctional; however, it is not known which functions are involved in which aspects of viral pathogenesis. The only animal model that permits such issues to be addressed is the cottontail rabbit papillomavirus (CRPV) rabbit system (28, 58). The CRPV system is unique because papillomas and carcinomas can be induced experimentally with molecular clones of the viral genome (5, 6, 18, 38). Recombinant methods allow the ready generation of site-specific viral mutants, whose biological activity can then be tested in whole animals (6, 7, 33, 38). The first step in investigating the mechanisms of papillomavirus-mediated disease is to identify viral genes involved in the induction of papillomas. By mutational analysis, we showed previously that the E7 gene of CRPV is required (6). Another group reported that E7 and E6 are both involved (33). In contrast, a requirement for the putative E5 open reading frame (ORF) has not been found, at least not in domestic rabbits (7, 33). We report here that the E1 and E2 regulatory genes, in addition to the E6 and E7 transforming genes, are required for induction of papillomas by CRPV. We also demonstrate that the lack of papilloma induction by the CRPV E7 p9Leu mutant

(6) is directly attributable to the single codon insertion in the 5' terminus of E7.

CRPV frameshift mutants were generated in CRPV-pLAI, a genomic clone derived from the Washington B strain of virus (CRPV-WA) (38, 59). The mutations are diagrammed in Fig. 1. Frameshift mutations were introduced into the E1 ORF upstream of the domains that encode putative DNA-binding (E1Nfs1 and E1Nfs2) and ATP-dependent helicase activities (E1Nfs1, E1Nfs2, and E1Hfs1) (47, 54, 63). Mutations were introduced into the E2 ORF in the hinge region (E2/E4Cfs1) that overlaps with the E4 ORF and in the DNA binding and dimerization region (E2Cfs1) (20). The E6 mutation was made in the carboxyl-terminal coding region common to CRPV long E6 and short E6 proteins (3).

During construction of the mutants, we found nine nucleotide differences in the E1, E2, and E4 ORFs of CRPV-WA compared with another CRPV genome that was cloned from a Kansas isolate (CRPV-KS) (19). The differences occurred at nucleotide (nt) 1531 (T instead of C), nt 1535 (C instead of T), nt 1951 (C instead of T), nt 3458 (G instead of T), nt 3516 (G insertion), nt 3533 (A deletion), nt 3913 (A instead of G), nt 3997 (T instead of C), and nt 4045 (C instead of T). However, these variations did not disrupt the ORFs and, like others previously identified in the E7 and E5 ORFs (6, 7), may represent the geographical diversity of the independent CRPV isolates.

The papilloma-inducing activity of the CRPV mutant genomes was tested by intradermal inoculation of New Zealand White rabbits using a jet injector, as previously described (6). In each experiment, wild-type CRPV DNA was included as control and induced papillomas at approximately 28% of inoculated sites (Table 1). Rabbits inoculated with CRPV E1Nfs2 and E1Hfs1 genomes, encoding 29 and 48% of wild-type E1 protein, respectively, did not form papillomas, indicating that E1 was required. Two rabbits inoculated with E1Nfs1, encoding just 5% of wild-type E1, also failed to form lesions, although a third animal developed two lesions that were determined to be typical viral papillomas by morphologic and histologic criteria (data not shown). Molecular analysis of the viral DNA in the unexpected lesions, using PCR, restriction

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FIG. 1. CRPV genes and mutations generated. The names of the mutant genome appear on the left, and the wild-type and mutated genes are diagrammed on the right. Filled bars indicate codons that are preserved. Open bars indicate out-of-frame codons that precede a stop codon. Dashed lines represent ATG start codons. Numbers refer to nucleotide positions (19). The location of each mutation is indicated above the bars. Deletions (del) and insertions (ins) were made at the following restriction sites: *Eco*NI at 1443 (E1Nfs1), *Sac*I at 1882 (E1Nfs2), *Xcm*I at 2230 (E1Hfs1), *Bst*EII at 3842 (E2/E4Cfs1), *Afl*II at 4066 to 4074 (E2Cfs1), *Avr*II at 741 (E6Cfs1), and *Hind*III at 1093 (E7p9Leu). The DNA sequences containing the mutations are as follows: CCTTGAGGAGG (E1Nfs1), GAGCTCACCA (E1Nfs2), CC AATGCCTGAATGG (E1Hfs1), GGTCACC (E2/E4Cfs1), CTTAAGTGCTTAAG (E2Cfs1), CCTAGCTAGG (E6Cfs1), and AGCTAGCTT (E7p9Leu). The underlined bases indicate either deleted or inserted bases shown in the figure.

analysis, and DNA sequencing, revealed a wild-type *Eco*NI sequence at the site that was mutated in E1Nfs1 from CCTTGAGGAGG to CCTTGAAGG (data not shown). The likelihood of reversion occurring by reinsertion of exactly two (underlined) G residues at nt 1445 to 1446 is extremely low. Thus, the results strongly suggest that these papillomas were a result of contamination with wild-type CRPV DNA and not of reversion in vivo. Thus, E1Nfs1, like E1Nfs2 and E1Hfs1, does not induce papillomas, and the E1 gene is required for papilloma formation (Table 1).

Inoculation of E2/E4Cfs1 DNA, encoding 63% of E2 (fused

in frame to the 3' end of E4), did not induce lesions, suggesting a requirement for E2 and/or E4. A second E2 mutant, E2Cfs1, that encodes 82% of E2 and an unaltered E4 ORF (Fig. 1), also failed to induce papillomas, indicating that E2 is essential (Table 1).

Four rabbits were inoculated with E6Cfs1 genomes, encoding 72% of long E6 and 56% of short E6 ORFs. On three of the rabbits, no lesions formed at sites inoculated with E6Cfs1. The fourth animal developed three papillomas at E6Cfs1-inoculated sites. Each was a typical viral papilloma and contained wild-type E6 sequences (data not shown). Since reversion in vivo would have required an exact 4-bp deletion following nt 741, the results suggest a contamination similar to the E1Nfs1 case and lead us to conclude that the E6 gene is required. This conclusion was also reached by another group using different E6 mutants (33). We show here, by marker rescue experiments, that the papilloma-inducing defect of E6Cfs1 was due to the mutation that we introduced (see below).

The possibility of inducing a papilloma as a consequence of contamination emphasizes the importance of characterizing viral DNA in a lesion. We have not yet successfully induced papillomas by jet injection when less than about 5 μ g of CRPV DNA was inoculated per site; however, this may be because much of the DNA is sheared during delivery. In the presence of a large quantity of mutant DNA (50 to 100 μ g per site), a small number of contaminating wild-type genomes may be protected and may be able to initiate papilloma formation. Once CRPV DNA is established, the infected cells are substantially amplified, and a papilloma may develop from a very small number of infected cells.

Marker rescue experiments were performed to exclude the possibility that inadvertent mutations outside the engineered mutations were responsible for the lack of papilloma induction. Rescue was accomplished by replacing a subgenomic fragment, containing the engineered mutation, with the homologous wild-type CRPV fragment. If the engineered mutation was responsible for the biological deficiency, then activity would accordingly be restored. The following CRPV segments were replaced: nt 1064 to 1448 for E1Nfs1, nt 3815 to 3982 for E2/E4Cfs1, nt 727 to 859 for E6Cfs1, and nt 1064 to 1882 for E7p9Leu. Four CRPV genomes that failed to induce lesions were thus repaired and then tested in rabbits. In each case, the frequency of papilloma induction was restored to the level of wild-type CRPV DNA, approximately 35% (Table 2). These results provide yet stronger evidence that E1, E2, E6, and E7 are each required for papilloma formation. They also indicate that inadvertent mutations are not commonly introduced by the cloning methods that we have employed. While rescue of the E2 gene in E2/E4Cfs1 also rescued the overlapping E4 gene, E4 appears not to be required because E4 mutants that do not disrupt the E2 ORF do induce papillomas (61).

Previous work had shown that the protein encoded by the CRPV E7p9Leu mutant was stable, bound retinoblastoma protein (pRB), and mediated functions associated with pRB binding in vitro but did not induce lesions on rabbits (6, 12). The papilloma-inducing defect of the E7p9Leu mutant was reexamined in the present study and was found to be reproducible (Table 1). Moreover, using the rescued CRPV genome E7p9Leu^{Rsc}, we obtained a wild-type rate of papilloma induction (Table 2), confirming the importance of this mutation.

In summary, this study extends our previous finding that the CRPV E7 gene is required for induction of rabbit papillomas (6) by showing that the CRPV genes E1, E2, and E6 are also necessary. Frameshift mutation of any of these genes in a full-length CRPV genome abolished the papilloma-inducing

TABLE 1. Papilloma induction by mutant CRPV genomes

Mutated gene	Expt no.	% of protein ^a	CRPV genome	Papilloma formation			Total no. of papillomas/ total no. of inoculated sites (%)
				No. of papillomas/no. of inoculated sites ^b			
				Rabbit 1	Rabbit 2	Rabbit 3	
None	1	100	Wild type ^c	8/50	35/50		
	2	100	Wild type	3/50	5/50	3/50	
	3	100	Wild type	11/50	18/50		
	4	100	Wild type	24/50	19/50		
	5	100	Wild type	16/60	15/60		
	6 ^d	100	Wild type	23/100			
	7	100	Wild type	18/50			198/720 (28)
E1	2	5	E1Nfs1	0/50			
	3	5	E1Nfs1	0/50	2/50		2/150 (1) ^e
E1	2	29	E1Nfs2	0/50			
	3	29	E1Nfs2	0/50	0/50	0/50	0/200 (0)
E1	4	48	E1Hfs1	0/50	0/50		
	4	48	E1Hfs1	0/50	0/50		0/200 (0)
E2	2	63	E2/E4Cfs1	0/50			
E4	3	74	E2/E4Cfs1	0/50	0/50		0/150 (0)
E2	1	82	E2Cfs1	0/50	0/50		
	3	82	E2Cfs1	0/50	0/50		0/200 (0)
LE6	4	72	E6Cfs1	0/50	0/50		
SE6	5	56	E6Cfs1	0/60	3/60		3/220 (1) ^e
E7	6	101	E7p9Leu	0/90	0/100	0/100	
	7	101	E7p9Leu	0/50			0/340 (0)

^a Predicted from the mutant gene.^b Each ratio is the number of papillomas that formed per number of sites inoculated in one rabbit.^c Wild-type control DNAs were CRPV-pLAII (for experiments 1, 2, 3, 6, and 7) or CRPV-pLAIIDRI (for experiments 4 and 5). Plasmid CRPV-pLAIIDRI is identical to CRPV-pLAII, except that the *EcoRI* site in the vector was abolished (7).^d Data from experiment 6 have been previously published (6).^e DNA sequence analysis showed this to be wild type, suggesting contamination of the inoculum with wild-type CRPV DNA.

ability of the virus, and marker rescue completely restored biological activity (Tables 1 and 2). We also confirmed the papilloma-inducing defect of the CRPV E7p9Leu mutant (6) and demonstrated that this defect could also be corrected by marker rescue (Table 2), reemphasizing that this region of unknown function merits further investigation.

Considering what is known about the activities of the E1, E2, E6, and E7 genes in vitro, we can speculate about what roles these genes play in forming and maintaining a papilloma. The E1 and E2 genes mediate plasmid DNA replication in cultured cells and in a cell extract (10, 13, 35, 55, 62). In rabbit epidermis, these functions are probably required to establish

and maintain the viral genome as multicopy extrachromosomes in infected basal keratinocytes. In quiescent basal cells that are neither dividing nor differentiating, the virus might be maintained in a latent state for a protracted period. This probably explains our observation that an occasional papilloma forms only several months after inoculation of wild-type CRPV (4). In the absence of either E1 or E2, the virus life cycle in rabbit skin is aborted or, at a minimum, severely impeded. A similar effect has been observed in vitro; BPV1 E1 mutants that do not replicate episomally are also impeded in their ability to transform (9). However, since transfection with E1 or E2 mutants may lead to viral DNA integration and thus may increase viral

TABLE 2. Papilloma induction by rescued CRPV genomes

Rescued gene ^a	Expt no. ^b	CRPV genome ^c	Papilloma formation			Total no. of papillomas/ total no. of inoculated sites (%)
			No. of papillomas/no. of inoculated sites ^d			
			Rabbit 1	Rabbit 2	Rabbit 3	
NA	1	Wild type ^e	24/50	19/50		
NA	2	Wild type	16/60	15/60		
NA	3	Wild type	9/50	9/50	25/50	
NA	4	Wild type	6/50	19/50		142/410 (35)
E1	1	E1Nfs1 ^{Rsc}	18/50	20/50		38/100 (38)
E2/E4	2	E2/E4Cfs1 ^{Rsc}	22/60	14/60		36/120 (30)
E6	3	E6Cfs1 ^{Rsc}	16/50	17/50		33/100 (33)
E7	4	E7p9Leu ^{Rsc}	18/50	12/50	38/50	68/150 (45)

^a NA, not applicable.^b Experiments 1 and 2 in this table are the same as experiments 4 and 5 in Table 1.^c The rescued (Rsc) CRPV constructs were derived from the corresponding CRPV mutants by replacing the mutant-containing region with wild-type sequence (see text).^d Each ratio is the number of papillomas that formed per number of sites inoculated in one rabbit.^e CRPV-pLAII was used as a control in experiment 3, and CRPV-pLAIIDRI was used as a control in experiments 1, 2, and 4.

transforming or immortalizing activities (30, 42, 43, 45), the requirement for E1 or E2 is probably less stringent in cultured cells than in vivo. In the whole animal, a small number of E1 or E2 mutant viral genomes may persist by integrating into the cellular genome, and viral effects could develop after a prolonged incubation period. Alternatively, there may be little potential for viral DNA to integrate as an immediate consequence of primary infection without E1 and/or E2. If so, a possible means to cure latency might be to thwart the action of the E1 and/or E2 proteins.

The E1 and E2 genes may be required to maintain CRPV DNA in suprabasal cells as they differentiate to a stage at which vegetative replication ensues. It is not inconceivable that the E1 and/or E2 genes might play a direct role in maintaining the papilloma as a benign lesion, since the loss of E1 and/or E2 in high-risk human lesions commonly coincides with the loss of a purely benign phenotype (for a review, see reference 48). Given its ability to transactivate transcription of the E6/E7 promoter in vitro (21, 51), the E2 protein could also be required to induce appropriate expression of E6 and/or E7 in basal and suprabasal cells. Finally, E1 and E2 proteins are likely to play key roles in driving vegetative viral DNA replication, although the requirement of any viral proteins for vegetative replication has not been assessed because of the lack of a suitable cell culture assay.

The E6 and E7 protein products of the high-risk HPVs interact with tumor suppressor proteins that control cell growth. E6 protein associates with and degrades the cellular tumor suppressor protein p53 (44, 57), and the E7 protein binds to the retinoblastoma tumor suppressor protein pRB, disrupting pRB-E2F complexes and allowing activation of promoters that contain E2F-binding sites (16, 60). These protein-protein interactions play key roles in E6- and E7-mediated immortalization of human keratinocytes and in E7-mediated transformation of established rodent cell lines (25, 26, 37, 46, 52, 56). CRPV is a high-risk rabbit papillomavirus, and its E6 and E7 genes can transform NIH 3T3 cells and also the cottontail rabbit epithelial cell line sf1EP (33). The CRPV E6 protein may interact with a rabbit homolog of p53, although this has not been shown. It is unclear whether such interaction would facilitate papilloma formation, since the low-risk HPVs do not efficiently bind p53, even though they obviously induce papillomas. We have previously shown that the CRPV E7 protein binds both rabbit and human pRB. CRPV E7-pRB binding causes disruption of pRB-E2F complexes and transactivation of the adenovirus E2 promoter (12). However, we have also shown that CRPV E7 protein binding to pRB was not required for the virus to induce papillomas (12). The converse is illustrated by the CRPV E7 p9Leu mutant. Although E7 p9Leu protein binds pRB almost as well as wild-type CRPV E7 protein (12), the presence of the E7 p9Leu gene in a CRPV genome abolishes its ability to induce rabbit papillomas (Table 1) (6). Apparently, the CRPV E7 protein performs functions other than binding to pRB that are critical for papilloma formation.

Whether papillomas are monoclonal or polyclonal, which is controversial (8, 17, 36), the infected cell(s) must be substantially amplified during expression of the papilloma phenotype. The advantage to the virus is that the size of the infected-cell population is directly proportional to the amount of infectious progeny that can be produced. Other viruses achieve this goal by cell-to-cell transmission after virus release through cytolysis or budding. However, papillomaviruses need an alternate strategy, because the basal keratinocytes that maintain the infection do not produce virus, and virions that are synthesized in the cornified layer do not normally contact the basal cell

layer. The population of papillomavirus-infected cells can grow only by proliferation. The known functions of E6 and E7 suggest that these genes are intimately involved in inducing or enhancing the process. Since the extent of division of infected cells probably determines the rate at which lesions grow, it may be desirable to target the E6 and/or E7 proteins to control rapidly proliferating lesions. The E6 and/or E7 genes may also mobilize the cellular DNA replication machinery in the differentiated epithelium; E6 and E7 transcripts increase in the differentiated cell layers in which replication takes place (49, 50). By inducing cellular DNA replicative functions, the virus could divert critical factors for its own use. Thus, to interrupt the synthesis of infectious virus and hence transmission, the E6 or E7 proteins may make good targets.

Although necessary, the E6 and E7 genes alone are not sufficient to induce papillomas. No effects were noted in rabbits inoculated with CRPV mutants that contained wild-type E6 and E7 genes when either of the large regulatory genes was inactivated by frameshift mutation. Similarly, transgenic mice that contained and expressed HPV 16 E6 and E7 in the absence of other transgenes did not develop typical viral papillomas (1, 23, 27, 31), whereas transgenic rabbits expressing full-length CRPV genomes developed extensive papillomatosis (40).

The number of papillomavirus genes is small, and virus-host cell interactions that cause a papilloma to form are likely to be complex. It is not surprising that the E1, E2, E6, and E7 genes are all required. HPV genes are homologous to those of CRPV and perform similar functions in vitro (2, 12, 22, 33). Thus, an implication of our work is that the HPV E1, E2, E6, and E7 genes are also all likely to be required to cause human disease. Future work will further characterize and map the critical pathogenic activities of papillomavirus genes. The knowledge that we gain should lead to identification of precise molecular targets for the development of vaccines and other antiviral and antitumor therapies.

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REFERENCES

- Arbeit, J. M., K. Munger, P. M. Howley, and D. Hanahan. 1993. Neuroepithelial carcinomas in mice transgenic with human papillomavirus type 16 E6/E7 ORFs. *Am. J. Pathol.* **142**:1187-1197.
- Baker, C. C. 1987. Sequence analysis of papillomavirus genomes, p. 321-385. *In* N. Salzman and P. M. Howley (ed.), *The Papovaviridae*, vol. 2. Plenum Publishing Corp., New York.
- Barbosa, M. S., and F. O. Wettstein. 1987. Transcription of the cottontail rabbit papillomavirus early region and identification of two E6 polypeptides in COS-7 cells. *J. Virol.* **61**:2938-2942.
- Brandsma, J. L. Unpublished data.
- Brandsma, J. L., and W. Xiao. 1993. Infectious virus replication in papillomas induced by molecularly cloned cottontail rabbit papillomavirus DNA. *J. Virol.* **67**:567-571.
- Brandsma, J. L., Z.-H. Yang, S. W. Barthold, and E. A. Johnson. 1991. Use of a rapid, efficient inoculation method to induce papillomas by cottontail rabbit papillomavirus DNA shows that the E7 gene is required. *Proc. Natl. Acad. Sci. USA* **88**:4816-4820.
- Brandsma, J. L., Z.-H. Yang, D. DiMaio, S. W. Barthold, E. Johnson, and W. Xiao. 1992. The putative E5 open reading frame of cottontail rabbit papillomavirus is dispensable for papilloma formation in domestic rabbits. *J. Virol.* **66**:6204-6207.
- Buscema, J., K. Shah, S. Hsu, N. Rosenshein, and J. D. Woodruff. 1987. Genetic investigation of the cellular origin of vulvovaginal

- condylomata acuminata, p. 245–247. *In* B. Steinberg, J. Brandsma, and L. Taichman (ed.), *Cancer cells 5: papillomaviruses*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Chiang, C. M., T. R. Broker, and L. T. Chow.** 1992. Properties of bovine papillomavirus E1 mutants. *Virology* **191**:964–967.
10. **Chiang, C.-M., M. Ustav, A. Stenlund, T. F. Ho, T. R. Broker, and L. T. Chow.** 1992. Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. *Proc. Natl. Acad. Sci. USA* **89**:5799–5803.
11. **Crook, T., J. P. Morgenstern, L. Crawford, and L. Banks.** 1989. Continued expression of HPV-16 E7 protein is required for maintenance of the transformed phenotype of cells co-transformed by HPV-16 plus EJ-ras. *EMBO J.* **8**:513–519.
12. **Defeo-Jones, D., G. A. Vuocolo, K. M. Haskell, M. G. Hanobik, D. M. Kiefer, E. M. McAvoy, M. Ivey-Hoyle, J. L. Brandsma, A. Oliff, and R. E. Jones.** 1993. Papillomavirus E7 protein binding to the retinoblastoma protein is not required for viral induction of warts. *J. Virol.* **67**:716–725.
13. **Del Vecchio, A. M., H. Romanczuk, P. M. Howley, and C. C. Baker.** 1992. Transient replication of human papillomavirus DNAs. *J. Virol.* **66**:5945–5958.
14. **DiMaio, D.** 1991. Transforming activity of bovine and human papillomaviruses in cultured cells. *Adv. Cancer Res.* **56**:133–159.
15. **Dong, G., T. R. Broker, and L. T. Chow.** 1994. Human papillomavirus type 11 E2 proteins repress the homologous promoter by interfering with the binding of host transcription factors to adjacent elements. *J. Virol.* **68**:1115–1127.
16. **Dyson, N., P. M. Howley, K. Münger, and E. Harlow.** 1989. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**:934–937.
17. **Friedman, J. M., and P. J. Fialkow.** 1976. Viral “tumorigenesis” in man: cell markers in condylomata acuminata. *Int. J. Cancer* **17**:57–61.
18. **Georges, E., G. Pehau-Arnaudet, and G. Orth.** 1992. Molecular and biological characterization of cottontail rabbit papillomavirus variant DNA sequences integrated in the VX7 carcinoma. *Virology* **186**:750–759.
19. **Giri, I., O. Danos, and M. Yaniv.** 1985. Genomic structure of the cottontail rabbit (Shope) papillomavirus. *Proc. Natl. Acad. Sci. USA* **82**:1580–1584.
20. **Giri, I., and M. Yaniv.** 1988. Structural and mutational analysis of E2 *trans*-activating proteins of papillomaviruses reveals three distinct functional domains. *EMBO J.* **7**:2823–2829.
21. **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.
22. **Griep, A. E., R. Herber, S. Jeon, J. K. Lohse, R. R. Dubielzig, and P. F. Lambert.** 1993. Tumorigenicity by human papillomavirus type 16 E6 and E7 in transgenic mice correlates with alterations in epithelial cell growth and differentiation. *J. Virol.* **67**:1373–1384.
23. **Ham, J., N. Dostatni, J. M. Gauthier, and M. Yaniv.** 1991. The papillomavirus E2 protein: a factor with many talents. *Trends Biochem. Sci.* **16**:440–444.
24. **Haskell, K. M., G. A. Vuocolo, D. Defeo-Jones, R. E. Jones, and M. Ivey-Hoyle.** 1993. Comparison of the binding of the human papillomavirus type 16 and cottontail rabbit papillomavirus E7 proteins to the retinoblastoma gene product. *J. Gen. Virol.* **74**:115–119.
25. **Hawley-Nelson, P., K. H. Vousden, N. L. Hubbert, D. R. Lowy, and J. T. Schiller.** 1989. HPV 16 E6 and E7 proteins cooperate to immortalize primary human foreskin keratinocytes. *EMBO J.* **8**:3905–3910.
26. **Hudson, J. B., M. A. Bedell, D. J. McCance, and L. A. Laimins.** 1990. Immortalization and altered differentiation of human keratinocytes in vitro by the E6 and E7 open reading frames of human papillomavirus type 18. *J. Virol.* **46**:519–526.
27. **Kondoh, G., Y. Murata, K. Aoza, M. Yutsudo, and K. Hakura.** 1991. Very high incidence of germ cell tumorigenesis (seminomagenesis) in human papillomavirus type 16 transgenic mice. *J. Virol.* **65**:3335–3339.
28. **Kreider, J. W., and G. L. Bartlett.** 1981. The Shope papilloma-carcinoma complex of rabbits: a model system of neoplastic progression and spontaneous regression. *Adv. Cancer Res.* **35**:81–110.
29. **Lambert, P. F.** 1991. Papillomavirus DNA replication. *J. Virol.* **65**:3417–3420.
30. **Lambert, P. F., and P. M. Howley.** 1988. Bovine papillomavirus type 1 E1 replication-defective mutants are altered in their transcriptional regulation. *J. Virol.* **62**:4009–4015.
31. **Lambert, P. F., H. Pan, H. C. Pitot, A. Liem, M. Jackson, and A. E. Griep.** 1993. Epidermal cancer associated with expression of human papillomavirus type 16 E6 and E7 oncogenes in the skin of transgenic mice. *Proc. Natl. Acad. Sci. USA* **90**:5583–5587.
32. **McBride, A. A., H. Romanczuk, and P. M. Howley.** 1991. The papillomavirus E2 regulatory proteins. *J. Biol. Chem.* **266**:18411–18414.
33. **Meyers, C., J. Harry, Y.-L. Lin, and F. O. Wettstein.** 1992. Identification of three transforming proteins encoded by cottontail rabbit papillomavirus. *J. Virol.* **66**:1655–1664.
34. **Meyers, C., and F. O. Wettstein.** 1991. The late region differentially regulates the *in vitro* transformation by cottontail rabbit papillomavirus DNA in different cell types. *Virology* **181**:637–646.
35. **Mohr, I. J., R. Clark, S. Sun, E. J. Androphy, P. MacPherson, and M. R. Botchan.** 1990. Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. *Science* **250**:1694–1699.
36. **Mounts, P., and K. V. Shah.** 1984. Respiratory papillomatosis: etiological relation to genital tract papillomaviruses. *Prog. Med. Virol.* **29**:90–114.
37. **Münger, K., W. C. Phelps, V. Bubbs, P. M. Howley, and R. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J. Virol.* **63**:4417–4421.
38. **Nasser, M., C. Meyers, and F. O. Wettstein.** 1989. Genetic analysis of CRPV pathogenesis: the L1 open reading frame is dispensable for cellular transformation but is required for papillomas formation. *Virology* **170**:321–325.
39. **Nishikawa, T., T. Yamashita, T. Yamada, H. Kobayashi, A. Ohkawara, and K. Fujinaga.** 1991. Tumorigenic transformation of primary rat embryonal fibroblasts by human papillomavirus type 8 E7 gene in collaboration with the activated H-ras gene. *Jpn. J. Cancer Res.* **82**:1340–1343.
40. **Peng, X., R. O. Olson, C. B. Christian, C. M. Lang, and J. W. Kreider.** 1993. Papillomas and carcinomas in transgenic rabbits carrying EJ-ras DNA and cottontail rabbit papillomavirus DNA. *J. Virol.* **67**:1698–1701.
41. **Phelps, W. C., C. L. Yee, K. Münger, 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.
42. **Romanczuk, H., and P. M. Howley.** 1992. Disruption of either the E1 or the E2 regulatory gene of human papillomavirus type 16 increases viral immortalization capacity. *Proc. Natl. Acad. Sci. USA* **89**:3159–3163.
43. **Sang, B. C., and M. S. Barbosa.** 1992. Increased E6/E7 transcription in HPV 18-immortalized human keratinocytes results from inactivation of E2 and additional cellular events. *Virology* **189**:448–455.
44. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63**:1129–1136.
45. **Schiller, J. T., E. Kleiner, E. J. Androphy, D. R. Lowy, and H. Pfister.** 1989. Identification of bovine papillomavirus E1 mutants with increased transforming and transcriptional activity. *J. Virol.* **63**:1775–1782.
46. **Sedman, S. A., M. S. Barbosa, W. C. Vass, N. L. Hubbert, J. A. Haas, D. R. Lowy, and J. T. Schiller.** 1991. The full-length E6 protein of human papillomavirus type 16 has transforming and *trans*-activating activities and cooperates with E7 to immortalize keratinocytes in culture. *J. Virol.* **65**:4860–4866.
47. **Seo, Y. S., F. Müller, M. Lusk, and J. Hurwitz.** 1993. Bovine papilloma virus (BPV)-encoded E1 protein contains multiple activities required for BPV DNA replication. *Proc. Natl. Acad. Sci. USA* **90**:702–706.

48. **Shah, K. V., and P. M. Howley.** 1990. Papillomaviruses, p. 1651–1676. *In* B. N. Fields, D. M. Knipe, et al. (ed.), *Virology*, 2nd ed. Raven Press, New York.
49. **Stoler, M. H., A. Whitbeck, S. M. Wolinsky, T. R. Broker, L. T. Chow, M. K. Howett, and J. W. Kreider.** 1990. Infectious cycle of human papillomavirus type 11 in human foreskin xenografts in nude mice. *J. Virol.* **64**:3310–3318.
50. **Stoler, M. H., S. M. Wolinsky, A. Whitbeck, T. R. Broker, and L. T. Chow.** 1989. Differentiation-linked human papillomavirus types 6 and 11 transcription in genital condylomata revealed by in situ hybridization with message-specific RNA probes. *Virology* **172**: 331–340.
51. **Szymanski, P., and A. Stenlund.** 1991. Regulation of early gene expression from the bovine papillomavirus genome in transiently transfected C127 cells. *J. Virol.* **65**:5710–5720.
52. **Takami, Y., T. Sasagawa, T. M. Sudiro, M. Yutsudo, and A. Hakura.** 1992. Determination of the functional difference between human papillomavirus type 6 and 16 E7 proteins by their 30 N-terminal amino acid residues. *Virology* **186**:489–495.
53. **Thierry, T., and P. M. Howley.** 1991. Functional analysis of E2-mediated repression of the HPV18 P105 promoter. *New Biol.* **3**:90–100.
54. **Thorner, L. K., D. A. Lim, and M. R. Botchan.** 1993. DNA-binding domain of bovine papillomavirus type 1 E1 helicase: structural and functional aspects. *J. Virol.* **67**:6000–6014.
55. **Ustav, M., and A. Stenlund.** 1991. Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. *EMBO J.* **10**:449–457.
56. **Watanabe, S., H. Sato, N. Komiya, T. Kanda, and K. Yoshiike.** 1992. The E7 functions of human papillomaviruses in rat 3Y1 cells. *Virology* **187**:107–114.
57. **Werness, B. A., A. J. Levine, and P. M. Howley.** 1990. Association of human papillomavirus type 16 and type 18 E6 proteins with p53. *Science* **248**:76–79.
58. **Wettstein, F. O.** 1987. Papillomaviruses and carcinogenic progression I: cottontail rabbit (Shope) papillomavirus, p. 167–186. *In* N. Salzman and P. M. Howley (ed.), *The Papovaviridae*, vol. 2. Plenum Publishing Corp., New York.
59. **Wettstein, F. O., and J. G. Stevens.** 1980. Distribution and state of viral nucleic acid in tumors induced by Shope papilloma virus. *Cold Spring Harbor Conf. Cell Proliferation* **7**:301–307.
60. **Wu, E. W., K. E. Clemens, D. V. Heck, and K. Munger.** 1993. The human papillomavirus E7 oncoprotein and the cellular transcription factor E2F bind to separate sites on the retinoblastoma tumor suppressor protein. *J. Virol.* **67**:2402–2407.
61. **Wu, X., and J. L. Brandsma.** Unpublished data.
62. **Yang, L., R. Li, J. Mohr, R. Clark, and M. R. Botchan.** 1991. Activation of BPV-1 replication in vitro by the transcription factor E2. *Nature (London)* **353**:628–632.
63. **Yang, L., I. Mohr, E. Fouts, D. A. Lim, M. Nohaile, and M. R. Botchan.** 1993. The E1-protein of bovine papilloma virus-1 is an ATP-dependent DNA helicase. *Proc. Natl. Acad. Sci. USA* **90**: 5086–5090.