JOURNAL OF VIROLOGY, Mar. 1996, p. 1912–1922 Vol. 70, No. 3
0022-538X/96/$04.00+0

The Primary Target Cells of the High-Risk Cottontail Rabbit Papillomavirus Colocalize with Hair Follicle Stem Cells

A. SCHMITT,[1] A. ROCHAT,[2] R. ZELTNER,[1] L. BORENSTEIN,[3] Y. BARRANDON,[2] F. O. WETTSTEIN,[3] AND T. IFTNER[1]*

Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, 91054 Erlangen, Germany[1]; Departement de Biologie, Ecole Normale Superieure, 75230 Paris Cedex 05, France[2]; and Department of Microbiology and Immunology, School of Medicine, University of California, Los Angeles, Los Angeles, California 90095[3]

Received 21 August 1995/Accepted 12 December 1995

Papillomaviruses are small DNA tumor viruses with a life cycle inseparably linked to the differentiation of the pluristratified epithelium. The infection of epithelial layers of the skin may remain latent or may result in the development of benign tumors. A certain number of distinct papillomavirus types, however, cause lesions which have a high risk of progression into carcinomas, and extensive efforts have been made to understand this process. Comparatively little is known about the initial events during the establishment of a persistent infection and papilloma development. Although it is generally accepted that the growth of a papilloma requires the infection of cells in the basal layer of the epithelium, it remains unknown which cells perform this task. We have analyzed by in situ hybridization biopsy samples taken at various time points after infection of domestic rabbits with cottontail rabbit papillomavirus. The first positive cells detected at a low frequency in biopsy samples taken after 11 days predominantly expressed high levels of E6 and E7 mRNA and were localized in the outer epithelial root sheath and in the bulbs of hair follicles. A clonal analysis of keratinocytes isolated from different subfragments of individual rabbit hair follicles demonstrated a clear colocalization of cottontail rabbit papillomavirus mRNA-positive cells with clonogenic cells in hair follicles. These data suggest that the cells competent to establish papillomatous growth represent a subpopulation of keratinocytes in hair follicles with properties expected of epithelial stem cells.

Papillomaviruses (PV) are highly species-specific DNA tumor viruses with a life cycle inseparably linked to differentiation processes in pluristratified epithelia (30). Regulatory and nonstructural viral genes are already expressed within the lower multiplying layers of the pluristratified epithelium, whereas massive DNA replication, expression of the structural genes, and viral capsid assembly are restricted to keratinocytes committed to terminal differentiation (3, 12, 30, 37). A PV infection of the skin may result in benign tumors with limited growth which tend to regress spontaneously (30). Some PV called high-risk types, however, cause lesions which have a high risk for conversion to malignancy. Among genital human PV (HPV), the DNAs of HPV type 16 (HPV16) and HPV18 are most frequently detected in high-grade intraepithelial lesions and in carcinomas of the anogenital tract (38), and DNA of HPV5 or HPV8 is present in skin carcinomas of patients with the disease epidermodysplasia verruciformis (19). The first PV suspected of involvement with a malignant development was the cottontail rabbit PV (CRPV). The virus induces strictly epithelial tumors in both cottontail and domestic rabbits (27). Cottontail rabbits are the natural host of CRPV and allow production of progeny virus. Domestic rabbits are equally susceptible to viral infection and papilloma formation; however, papillomas of domestic rabbits produce little if any virus (27), and in situ hybridization with a late viral mRNA probe revealed only an occasional positive cell (37). The transcripts of CRPV present in domestic rabbit papillomas have been characterized by S1 and Exo VII mapping and by primer extension (see Fig. 1) (36). Briefly, the two major transcripts are the mRNAs coding for the E7 protein and for a truncated E6 protein (SE6) initiated at the second ATG of open reading frame (ORF) E6. The two mRNAs contain a common noncoding downstream exon extending from a splice acceptor in the middle of ORF E4 to the early polyadenylation signal. The mRNA for SE6 also contains the sequences of ORF E7, but the E7 protein is not translated from this RNA (2). The E7 mRNA is initiated at a promoter located toward the 3′ end of ORF E6 (P3) (see Fig. 1). A minor mRNA contains an exon encoding the entire ORF E2, and this exon is spliced upstream to the exon encoding SE6 or E7 (35). Finally, primer extension analysis with a primer annealing to ORF E4 revealed only extension products compatible with mRNA containing, upstream of E4, the sequence of ORF E7 or ORF E6 and E7. No extension products indicative of an E1^E4 mRNA (18), which represents a major transcript in papillomas induced by several genital HPV, were detected. The tumors induced by CRPV are benign at first, but 8 to 14 months later carcinomas develop at the same site, particularly in domestic rabbits (14, 32, 35). The finding that carcinomas associated with PV always contain transcripts encoding the viral oncoproteins E6 and E7 was the first indication for an important role of these genes in the process of cancer development (38). Numerous experiments with cultured cells and with transgenic animals demonstrated the transforming and tumorigenic properties of the E6 and E7 proteins, and their mechanisms of action on the control of the cell cycle have been studied in great detail (15, 25, 26, 38). In contrast, nothing is known about the initial steps of virus infection, the nature of the target cells, and the cellular mechanisms that determine if a lesion will develop. Indeed, latent infections are more frequent than overt lesions (30, 31). This is indicated by the observations that (i) PV genomes can be detected in a certain percentage of normal tissue samples, (ii) tumors often reccur in the vicinity of surgically removed lesions

* Corresponding author. Mailing address: Schlossgarten 4, 91054 Erlangen, Germany. Phone: 9131-85-2104. Fax: 9131-85-2101. Electronic mail address: TSIFTNER@viro.med.uni-erlangen.de.

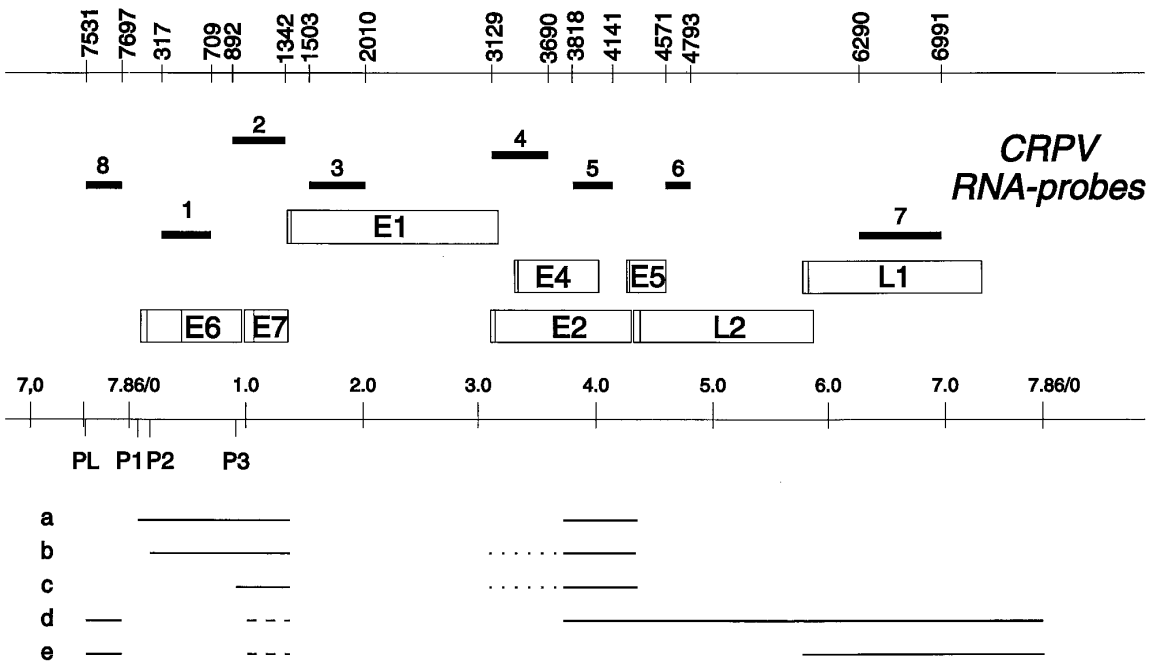


FIG. 1. CRPV-specific riboprobes. Genetic and transcriptional map of CRPV and localization of mRNA-specific probes 1 to 8 (37). The ORFs (open boxes) and the nucleotide positions of the probe boundaries (top) correspond to the published sequence (9). Vertical lines within the ORFs represent the translational start codons. The general transcription map with the major transcripts (a to e) is depicted in the lower part (35, 36). Dotted lines represent alternative splicing patterns, and dashed lines represent unknown 5′-exon boundaries. The early promoters (6) are indicated as P1, P2, and P3, and the late promoter (34) is indicated as PL.

(7), and (iii) endogenous latent PV genomes in animals can be reactivated by skin irritation (28). Furthermore, when rabbits are infected with a low dose of CRPV most infected sites do not develop papillomas, although viral DNA can be detected at the site of infection and irritation of these sites results in the development of papillomas (1). It is not known which cells of the epithelium have the ability to support early viral gene expression permitting, ultimately, the development of papillomas.

To experimentally address this question, we used high-pressure injection rather than scarification to infect New Zealand White rabbits with CRPV. This method of infection minimized destruction of the epithelium and permitted identification of the cells competent for early viral gene expression. The results indicate that primary target cells of CRPV are present in hair follicles, where they colocalize with clonogenic keratinocytes with properties of epidermal stem cells.

MATERIALS AND METHODS

Experimental infection and collection of specimens. Three New Zealand White rabbits (animals 344, 345, and 346) were injected by using a pressure injection apparatus (pedo-jet injector; Ventron Medical Products) with 50 μl of virus suspension in phosphate-buffered saline (PBS) (corresponding to 50 pg of viral DNA or 6×10^6 viral particles per site) at a total of 20 sites per rabbit. Two to four 4-mm-diameter punch skin biopsy specimens were obtained from each infected rabbit after 3, 11, 21, and 45 days and kept frozen at −70°C.

RNA probes used for in situ hybridization. The construction of the plasmids used to generate specific riboprobes for CRPV and the conditions for RNA hybridization and for DNA hybridization with a sense-strand riboprobe for CRPV (nucleotides [nt] 4571 to 6991) have been described previously (37). The map positions (nucleotide numbers are according to reference 9) of the eight small subgenomic CRPV DNA fragments that have been cloned in the RNA expression vector Bluescribe M13+ and served to generate the antisense probes are illustrated in Fig. 1. All plasmids were linearized prior to transcription. Depending on the choice of the promoter, the in vitro-synthesized RNAs were either in the same polarity as mRNA (sense orientation) or in the opposite polarity (antisense orientation). In vitro RNA synthesis in the presence of ^{35}S-UTP resulting in probes with a specific activity of 3.5×10^8 cpm/μg was followed by alkaline hydrolysis to reduce the probe length to approximately 200 nt (12). The quality of the transcribed RNAs was tested by polyacrylamide gel electrophoresis. The shortest probe (no. 8, with a complexity of 166 nt) was applied at a concentration of 2.9×10^7 cpm/ml of hybridization mixture, and the concentration of the others was normalized in relation to the probe length.

In situ hybridization. For RNA in situ hybridization, serial 5- to 6-μm-thick sections of biopsy specimens were made with a cryostat microtome (Reichert-Jung), mounted on aminopropylsilan-coated slides, fixed in 4% paraformaldehyde in PBS, and dehydrated through graded ethanols. The dry sections were then acetylated in 0.1 M triethanolamine–0.25% acetic anhydride for 10 min, washed in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and preincubated for 2 h at 42°C covered with solution I, containing 45% formamide, 0.6 M NaCl, 2.5× Denhardt's solution, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.15 mg of tRNA per ml. After removal of the prehybridization solution, individual tissue sections were encircled with rubber cement and incubated at 42°C with different ^{35}S-labelled antisense riboprobes in solution I containing 10% dextran sulfate. After 16 h of hybridization, the strings of rubber cement were removed and the slides were washed in 50% formamide–1× SSC at 55°C and then treated with RNase A (10 μg/ml), subsequently washed twice for 2 h each in 0.1× SSC at 60°C, dehydrated, and finally coated with Kodak NTB-2 emulsion. All slides from one experiment were developed after the same exposure time (10 to 12 days) to allow direct comparison of signals obtained with different probes. Hematoxylin-and-eosine-counterstained sections were evaluated and photographed with a Zeiss Axiophot microscope by using a dark- or bright-field condenser. The background for RNA hybridization was determined by using sense riboprobes and by pretreatment of the tissue with DNase-free RNase before hybridization.

Microdissection of rabbit hair follicles and cell culture. Skin biopsy specimens were obtained from the backs of two New Zealand White rabbits, and hair follicles were isolated under a dissecting microscope. Each biopsy specimen was sliced with a scalpel into small pieces containing few intact follicles. The fat and connective tissue surrounding each follicle were then carefully removed by using fine needles and forceps, and single follicles were isolated. The isolated hair follicles were photographed individually, cut into five fragments, and then re-photographed. At that stage, special care was taken to keep the follicular papilla in place to avoid damaging the matrix cells. Each fragment was then individually transferred to a 35-mm-diameter culture dish containing 1 ml of collagenase-dispase (1 mg/ml; Boehringer Mannheim) and incubated for 30 min at 37°C. Each fragment was next teased with fine needles to separate the dermal sheath from the epithelial core and was then incubated in a solution of trypsin (0.05%) for 2 h. The extent of the dissociation was monitored under a dissecting microscope. When dissociation was complete, the cells were harvested, centrifuged, and then cultivated on lethally irradiated feeder layers of 3T3-J2 cells according to the method of Rheinwald and Green (22). The culture medium, a 3:1 mixture

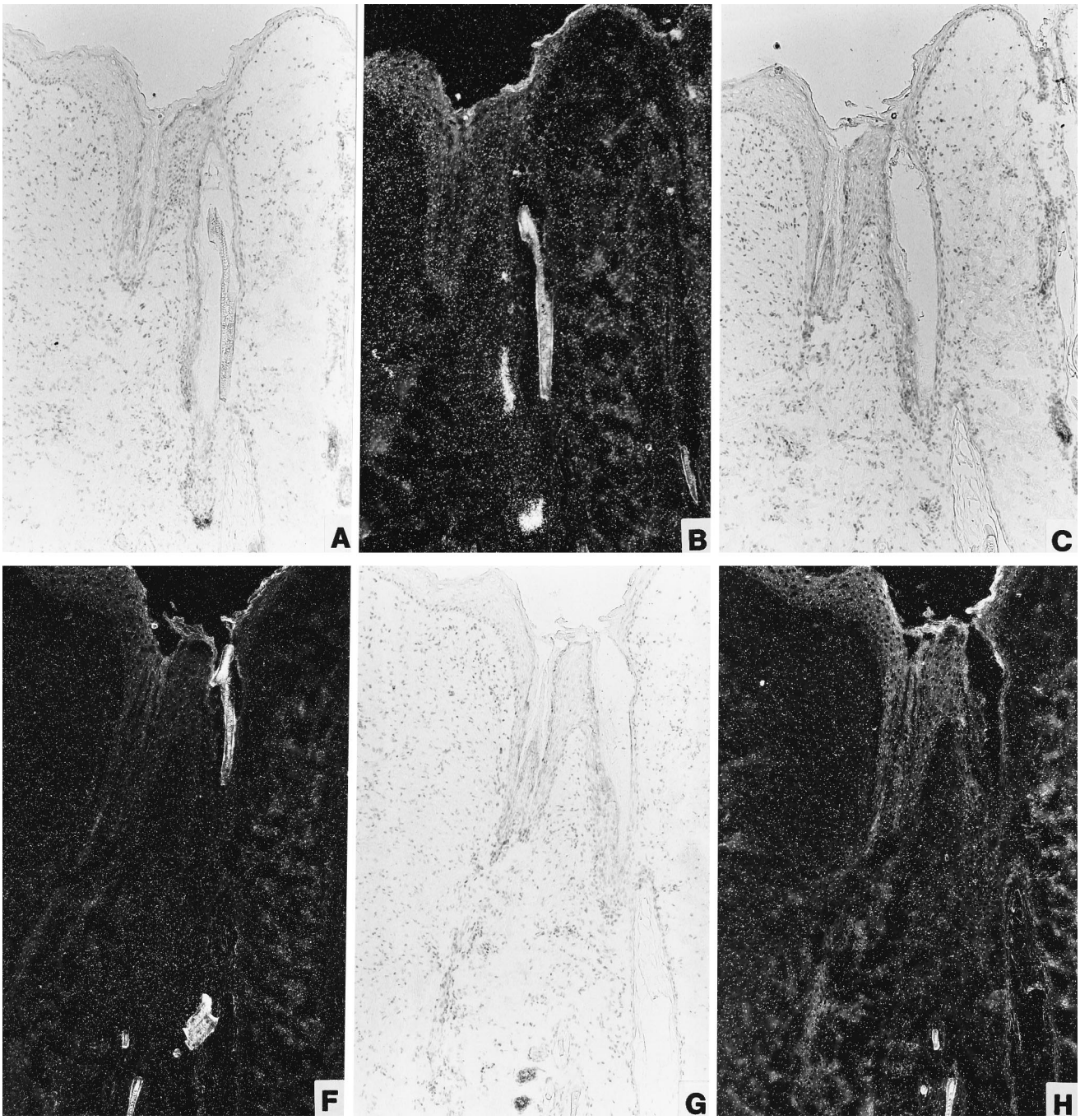


FIG. 2. In situ hybridization of CRPV-infected rabbit skin containing an infected hair follicle with normal morphology. Shown is in situ hybridization of serial tissue sections from a specimen taken 11 days after experimental infection from rabbit 344. Individual adjacent tissue sections after hybridization with riboprobe 2 (B and K), probe 1 (D), probe 3 (F), and probe 4 (H) are shown by dark-field illumination. (A, C, E, G, and J) Corresponding histopathology of the epithelium shown by bright-field illumination and stained with hematoxylin and eosine. The silver grains generated in the film emulsion after exposure to the ^{35}S-labelled probes are visible as white grains under dark-field illumination.

of the Dulbecco-Vogt modification of Eagle's medium and Ham's-F12 medium, was supplemented with 10% fetal bovine serum as described elsewhere (13, 23). Recombinant human epidermal growth factor was added at 10 ng/ml beginning at the first feeding. The cells were fed every 4 days. After 12 days, the cultures were fixed with formalin and stained with 1% rhodamine B and keratinocyte colonies were counted by using a binocular microscope. All experiments were carried out with a single batch of fetal bovine serum (Hyclone Laboratories, Inc.).

RESULTS

New Zealand White rabbits infected with CRPV develop macroscopically detectable papillomas after 3 to 4 weeks. To identify the initial target cells and characterize the pattern of viral gene expression, biopsy specimens were removed before the appearance of papillomas. Serial cryostat sections were

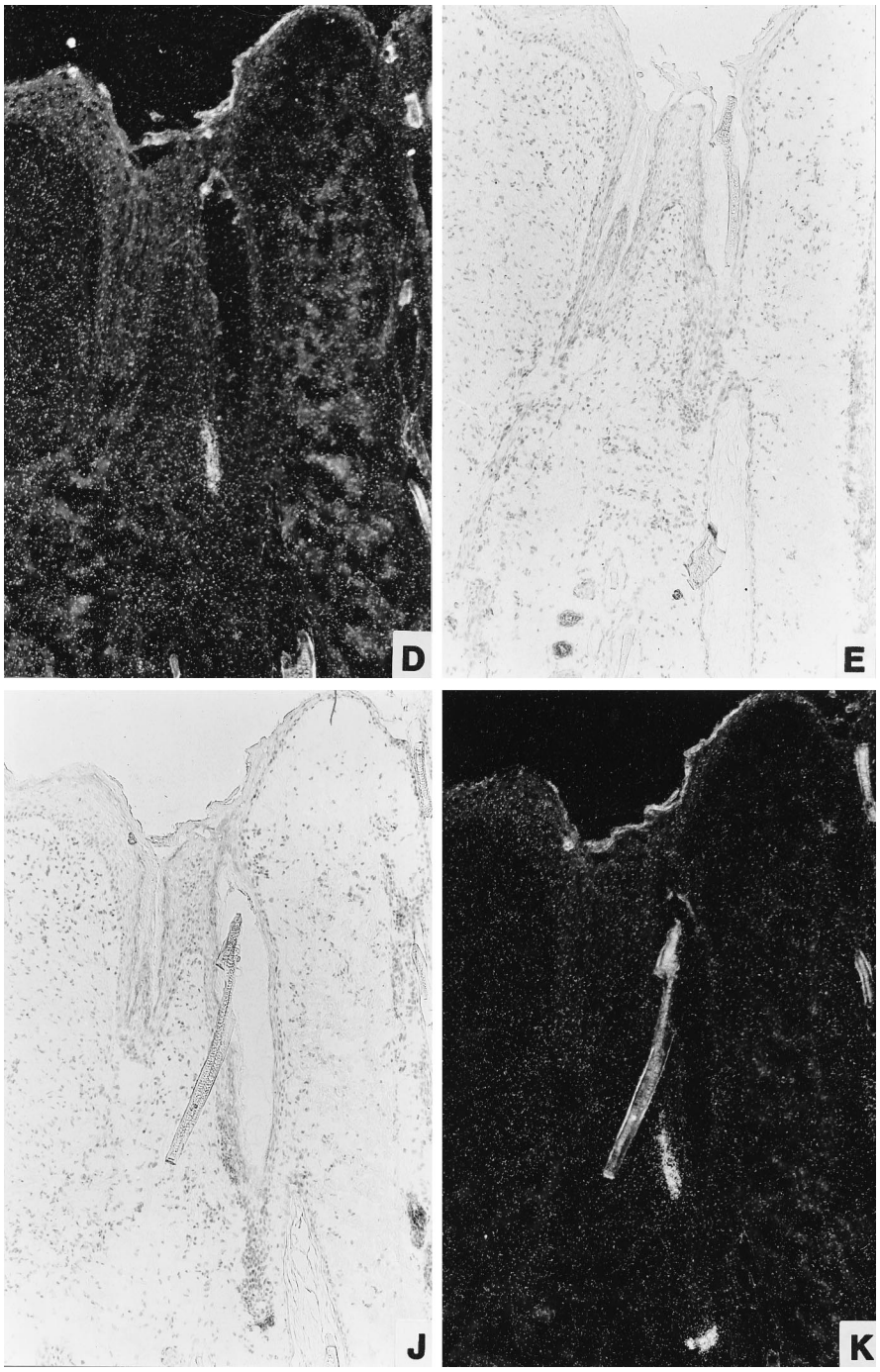


FIG. 2—*Continued*.

prepared and hybridized in situ with CRPV mRNA-specific probes.

In order to facilitate identification of the cells from which papillomas develop, we minimized the destruction of the epithelium by high-pressure injection of CRPV particles into the skin of rabbits rather than applying virus onto scarified areas as is customary. Three New Zealand White rabbits (no. 344, 345, and 346) were injected with 50 μl of CRPV suspension in PBS (corresponding to 50 pg of viral DNA or 6×10^6 viral particles per site) at a total of 20 sites per rabbit or with PBS alone as a control. Two to four skin biopsy specimens were excised from each of the infected rabbits after 3, 11, 21, and 45 days; immediately snap-frozen; and sectioned for further analysis. The frequency of papilloma induction was determined after 3 months from nine sites injected with CRPV particles that were not excised. Frequencies were 77% for rabbits 344 and 346 and 22% for rabbit 345. None of the control sites injected with PBS alone developed tumors. After 21 days, macroscopically detectable lesions could already be observed in 2 rabbits (no. 344 and 345). Histological examination revealed inverted papillomas that were up to 0.5 mm in diameter.

Within 45 days, all three rabbits had developed exophytic

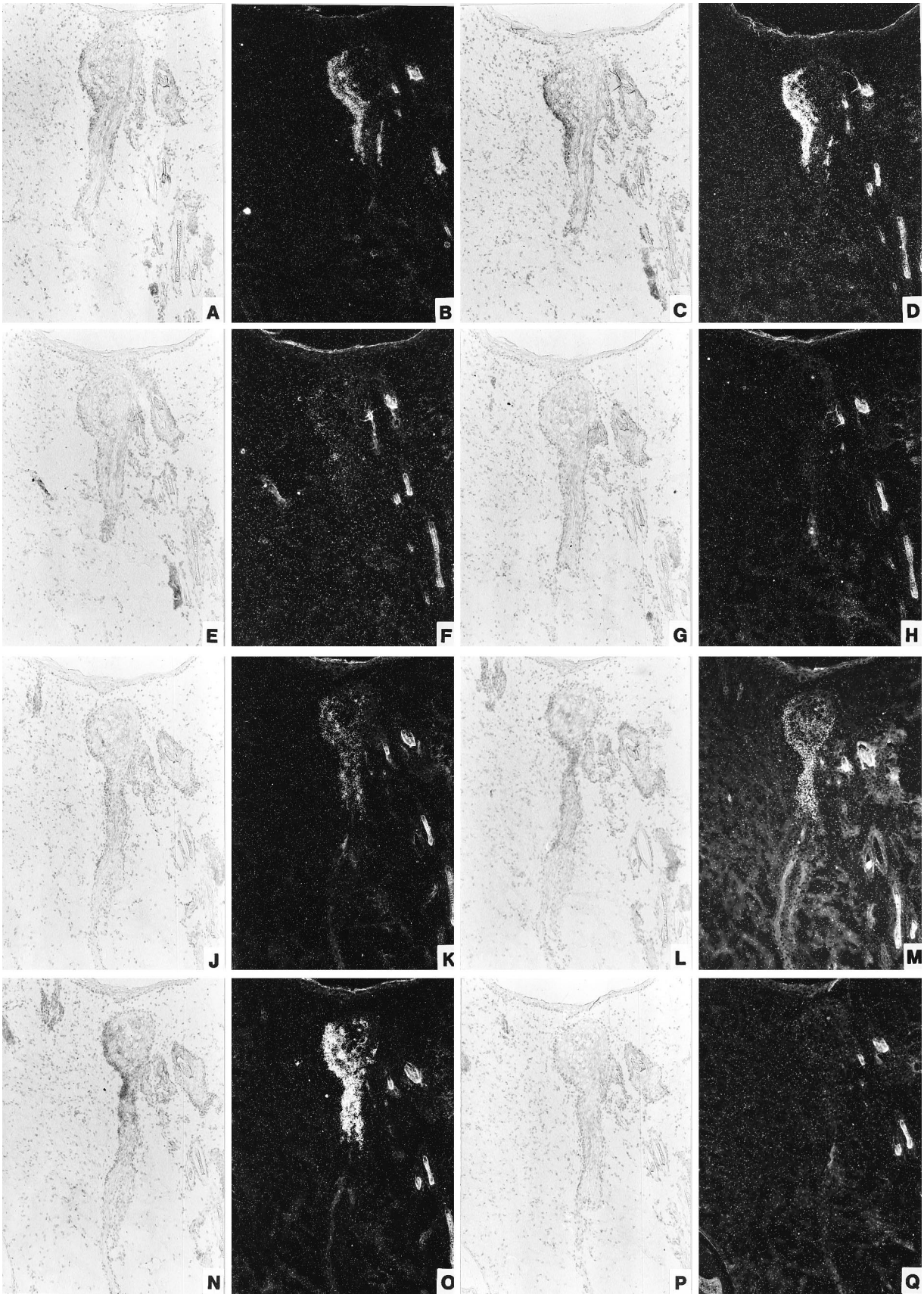


FIG. 3. In situ hybridization of CRPV-infected rabbit skin containing an infected hair follicle exhibiting dysplastic morphological changes. Shown is in situ hybridization of serial tissue sections from a specimen taken 11 days after experimental infection from rabbit 344. Individual adjacent tissue sections after hybridization with probe 1 (B), probe 2 (D and O), probe 3 (F), probe 6 (H), probe 4 (K), probe 5 (M), and probe 7 (Q) are shown by dark-field illumination. (A, C, E, G, J, L, N, and P) Corresponding histopathology of the epithelium shown by bright-field illumination and stained with hematoxylin and eosine.

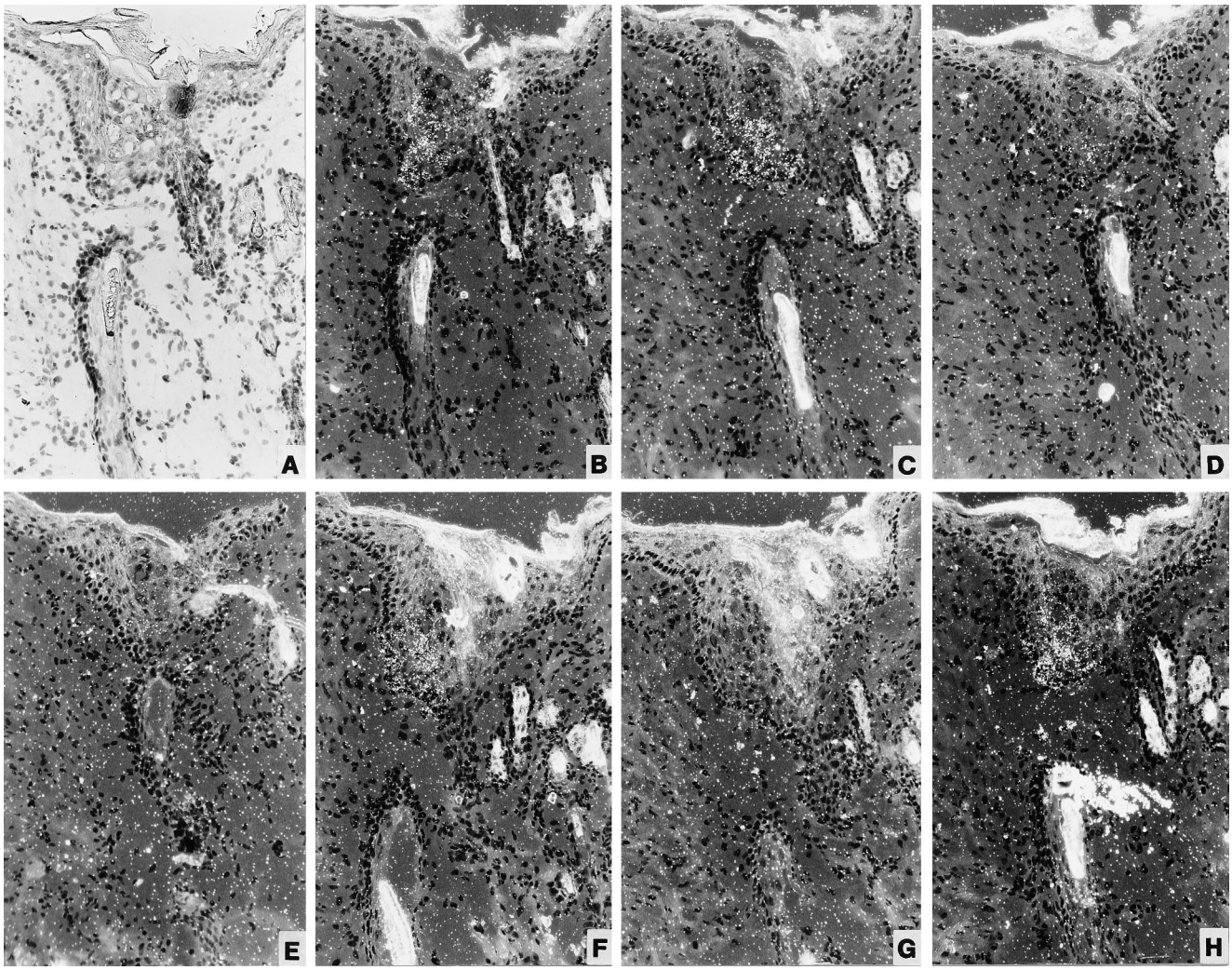
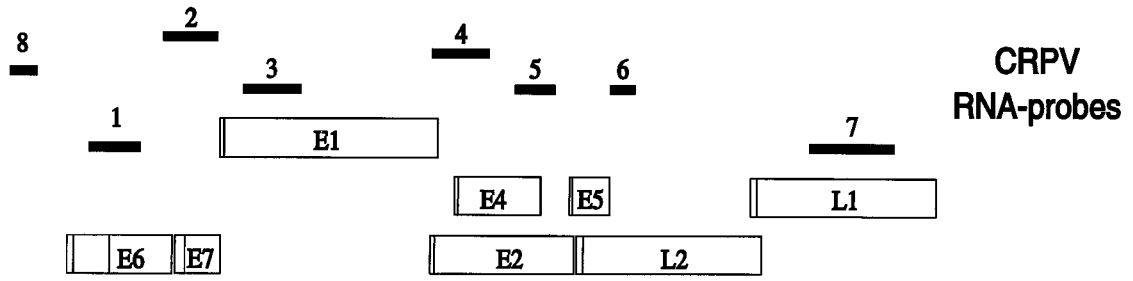


FIG. 4. In situ hybridization of CRPV-infected surface epithelium. Shown is in situ hybridization of serial tissue sections from a specimen taken 11 days after experimental infection from rabbit 345. (A) Histopathology of a hematoxylin-and-eosine-stained section reveals a small region of papillomatous epithelium in close proximity to a hair follicle. Individual adjacent tissue sections hybridized with riboprobes 2 (B and H), 1 (C), 3 (D), 4 (E), and 5 (F) and probe 2 in sense orientation as a negative control (G) are shown by dark-field illumination.

papillomas that were visible with the naked eye. To look for early events of the infection process, biopsy samples were taken at 3 or 11 days after injection and completely sectioned with a cryostat microtome. Serial 5- to 6-μm-thick sections were then postfixed with 4% paraformaldehyde in PBS, and every 10th section was stained with hematoxylin-eosine for histological examination. Microscopic evaluation revealed no obvious morphological or histological alterations of the skin for the first 11 days; indeed, no differences between biopsy specimens from rabbits injected with virus particles and those injected just with PBS were detected. We therefore probed a total of 5,000 serial sections by in situ hybridization with eight CRPV-specific riboprobes to identify cells expressing viral mRNA (Fig. 1). The hybridization specificities of the eight riboprobes for the known mRNA species of CRPV are as follows. Probe 1 is specific for the long E6 and short E6 mRNAs initiated at the promoters P1 and P2 (Fig. 1), respectively. Probe 2 hybridizes with both E6 mRNAs and the E7 mRNA initiated at the promoter P3 and potentially with transcripts encoding the structural genes L1 and L2 (Fig. 1). Late transcripts, however, have not been detected in domestic rabbit tumors (18, 20), except for a small number of terminally differentiated cells in the superficial layers of large exophytic papillomas (37). Since CRPV-infected tissues, in contrast to tissues infected by human genital papillomaviruses, do not contain a major E1ˆ E4 mRNA initiated within the E7 ORF (4, 11, 18, 29, 37), a stronger hybridization signal with probe 2 than with probe 1 indicates the presence of E7 mRNA. Probe 3 identifies potential transcripts covering the E1 ORF. Probe 4 hybridizes to mRNA species with the coding capacity for a full-length E2 protein. Probe 5 is the least specific probe and hybridizes to all early transcripts and potentially to L2 and E4 mRNA. Probe 6 is unique for the L2 mRNA, and probes 7 and 8 hybridize to both major late transcripts. For in situ RNA analysis, nine contiguous biopsy sections were placed together on one slide, individually encircled with rubber cement, and hybridized with the different riboprobes. To evaluate if each of the nine serial sections on the same slide was within the borders of a putative lesion, we usually hybridized the first and the ninth serial sections with riboprobe 2 (Fig. 1) at a concentration of 1.1×10^7 cpm/ml of hybridization mixture. A positive signal with riboprobe 2 in sections 1 and 9 would suggest that



probe #		1	2	3	4	5	6	7	8
N-HF		++	+++	-	-	+++	-	-	-
A-HF		++	+++	+/-	++	+++	-	-	-
SE-PAP 11d		++	+++	+/-	-	+++	-	-	-
I-PAP 21d		+++	++++	+	+	++++	-	-	-
E-PAP 45d	up	+	++	++[b]	++	++	-	+[a]	-
	lo	+++	++++	+	++	++++	-	-	-

FIG. 5. Summary of results of in situ hybridization. The upper part of the figure shows the genetic map and the localization of the mRNA-specific riboprobes 1 to 8 used in this study. The lower part gives the intensities of the in situ hybridization signals detected with the CRPV-specific riboprobes for each individual case (+, weak; ++, moderate; +++, strong; ++++, very strong; +/−, just above the level of background hybridization; −, no signal; [a], signals only in a few single cells; [b], signals only in some areas of the tissue section). up, upper layers of the stratified epithelium; lo, lower layers of the epithelium; N-HF, hair follicles with normal morphology; A-HF, hair follicles with dysplastic changes; SE-PAP 11d, papillomatous outer surface epithelium, 11 days after infection; I-PAP 21d, inverted growing papilloma at day 21; E-PAP 45d, exophytic papilloma at day 45.

all other sections on the slide were likely to be within the borders of the same lesion.

The search for viral gene expression in biopsy samples taken 3 days after infection gave negative results. Also, most sections from day 11 were negative; however, in a number of slides some sections of hair follicles and in one slide a small area of papillomatous epithelium showed positive signals. Examples of such positive sections are shown in Fig. 2, 3, and 4. Some of the positive hair follicles still revealed a normal morphology (Fig. 2; Fig. 5 N-HF) whereas others already exhibited morphological abnormalities (Fig. 3; Fig. 5, A-HF). Moderate to strong signals were detected with probe 1 (Fig. 2D, 3B, and 4C), probe 2 (Fig. 2B and K, 3D and O, and 4B and H), and probe 5 (Fig. 3M and 4F). Weak signals were also detected with the E1-specific probe 3 in a small area of papillomatous surface epithelium (Fig. 4D; Fig. 5, SE-PAP), as well as in morphologically altered hair follicles (Fig. 3F) which also displayed moderate signals after hybridization with the E2-specific probe 4 (Fig. 3K). The strong signals observed in these biopsy specimens with riboprobes 1, 2, and 5 indicate the presence of transcripts encoding E6 and E7, because no signals with the late-gene-specific probe 6, 7, or 8 were detected (Fig. 3H and Q; Fig. 5).

21 days after infection, macroscopically visible papillomas were observed in skin biopsy specimens (Fig. 6; Fig. 5, I-PAP). The tumors were about 0.5 mm in diameter and showed a histology reminiscent of that of an inverted condyloma; they projected into the dermis and grew laterally to underlie the normal epidermis (Fig. 6A and B). Again, high levels of E6 and E7 mRNA were observed after hybridization with probe 2 (Fig. 6C).

The results of the in situ hybridization experiments with lesions at different stages show that in positive hair follicles with normal morphology there was evidence only for transcripts encoding E6 and E7, while more advanced lesions in general also contained low levels of E1 and E2 transcripts. Finally, with 45-day exophytic papillomas (Fig. 5, E-PAP) the previously established pattern of transcription in CRPV-induced domestic-rabbit papillomas was observed, with strong expression of E6 and E7 in the basal layers that decreased significantly in the more differentiated layers (37).

The exclusive expression of E6 and E7 transcripts at 11 days in the normal hair follicle was therefore the earliest stage of infection that we could detect. Interestingly, the transcripts encoding the viral oncoproteins E6 and E7 not only were confined to the hair follicle but also were distributed within the follicle in a nonuniform manner, rather being restricted to two distinct locations, the bulb and the intermediate part of the follicle (Fig. 2B and H). The lack of signal in the bulb region of the hair follicle shown in Fig. 2 after hybridization with the E6-specific probe 1 (Fig. 2D) is related to the fact that the hybridized biopsy section did not contain a full-length longitudinal section through the hair follicle of interest (the bulb is not visible). The bipartite localization of the CRPV E6- and E7-specific signals (Fig. 2B and H) in positive hair follicles was highly reminiscent of the distribution of stem cells in human and rat hair follicles as previously described (13, 23). Unfortunately, no specific marker for keratinocyte stem cells is available and they can be identified only by their capability of initiating a progressively growing colony in culture. To investigate this correlation, pelage hair follicles in the growth phase of the hair cycle were microdissected from pelage biopsy sam-

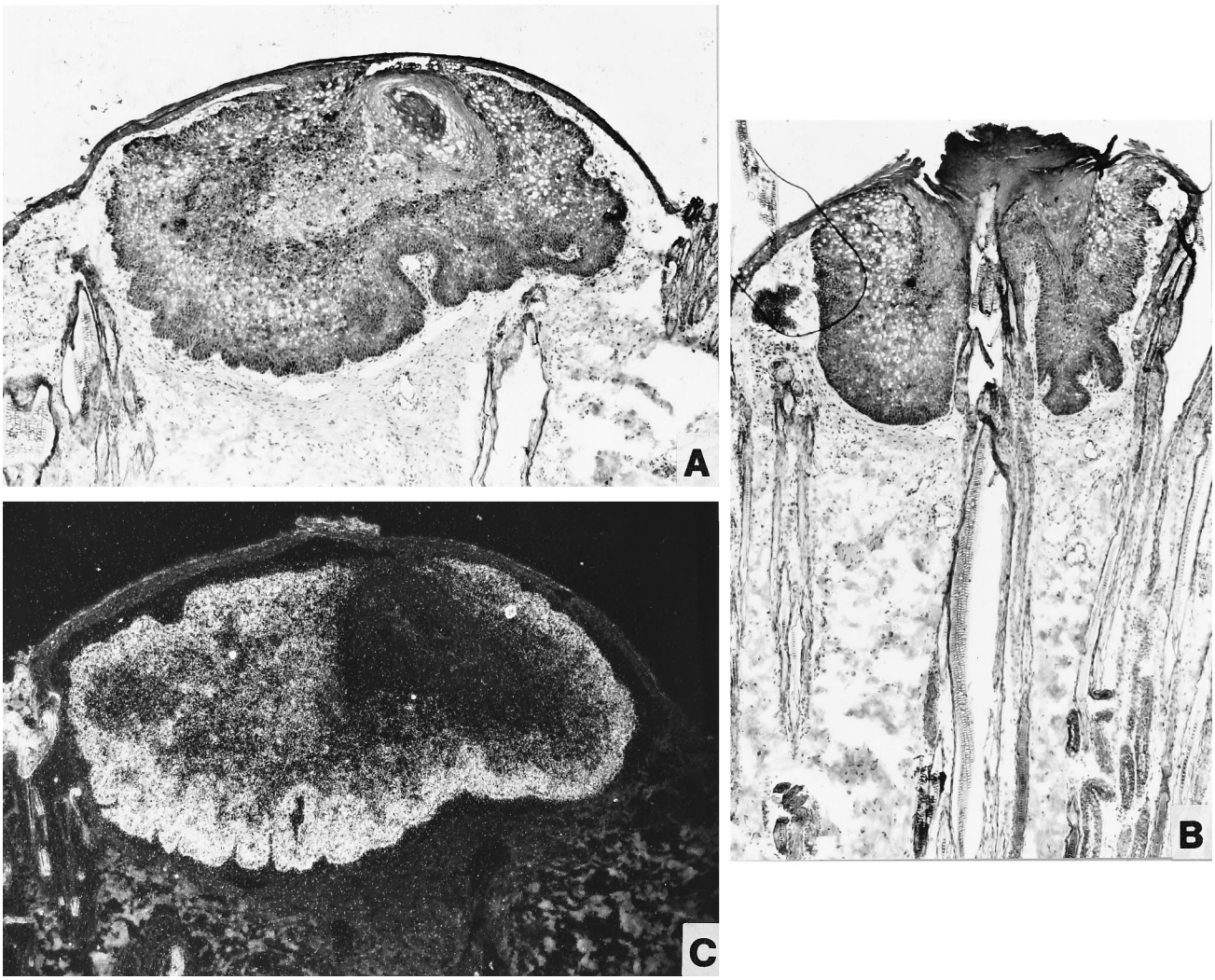


FIG. 6. Histological appearance of CRPV-induced tumors. (A and B) Histology of papillomas excised 21 days after experimental infection of rabbits (no. 344 and 345). The sections stained with hematoxylin and eosine reveal inverted papillomas with a diameter of 0.5 mm located in close proximity to hair follicles. (C) In situ hybridization of a tissue section of the papilloma shown in panel A with riboprobe 2 demonstrates the presence of large amounts of transcripts with a coding potential for CRPV E6 and E7.

ples obtained from two New Zealand White rabbits. The very high density of hair in rabbit pelage renders the microdissection of the follicles extremely difficult, and special care was taken to remove any adjacent vellus hair. Only a few intact hair follicles could be dissected from a skin biopsy sample. Each microdissected hair follicle was photographed (Fig. 7, left) and further cut into five fragments, termed P_1 to P_5. The bulb-containing fragment was called P_1, the lower-intermediate fragment was called P_2, the middle-intermediate fragment was called P_3, the upper-intermediate fragment was called P_4, and the sebaceous-gland-containing fragment was called P_5. The fragments were then photographed (Fig. 7, right) and dissociated enzymatically in order to obtain a single-cell suspension. To assay the colony-forming ability of the cells isolated from each fragment, they were cultivated in individual tissue culture dishes containing a feeder layer of irradiated 3T3 cells, as previously described (13, 23). Cultures were fixed after 12 days and stained with rhodamine B (Fig. 8). Keratinocyte colonies were then easily identified and scored under a binocular microscope.

Keratinocyte colony-forming cells (K-CFCs) were found mainly in the lower fragment (P_1), containing the hair bulb, and in the upper-intermediate fragment (P_4) (Fig. 8). Few K-CFCs were also present in fragments P_3 and P_5, indicating that the K-CFC-containing area is larger than P_4, and it is likely that the K-CFCs isolated from P_3 and from P_5 are located close to the P_4 fragment boundaries. No colonies were obtained from fragment P_2. These results demonstrate that K-CFCs are segregated in rabbit hair follicles as in other species (13, 23). The segregation of the K-CFCs in the bulb (fragment P_1) corresponds to one of the two specific sites of expression of CRPV E6 and E7 transcripts in the hair follicle (Fig. 2). To determine whether the other region containing K-CFCs (fragment P_4) colocalizes with the second site of viral gene expression situated in the intermediate part of the hair follicle, we measured the size of fragments P_4 and P_5. This allowed us to define the layout of the K-CFC-containing region with respect to the epidermis. The K-CFCs were segregated in a region of the follicle (corresponding to fragment P_4) located between 0.67 and 1.39 mm from the epidermis for follicle A

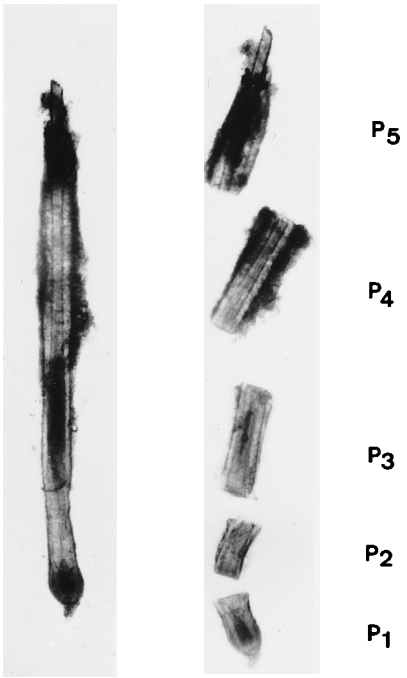


FIG. 7. Morphological aspect of a microdissected rabbit pelage hair follicle. (Left) Pelage guard hair follicle microdissected from a skin biopsy sample taken from the back of a 16-week-old New Zealand White female rabbit. The total length of this anagen follicle (i.e., in the growth phase of the hair cycle) is 2.67 mm. (Right) Same follicle after it was cut into five fragments: P_1, bulb-containing fragment, 0.34 mm; P_2, lower-intermediate fragment, 0.31 mm; P_3, middle-intermediate fragment, 0.63 mm; P_4, upper-intermediate fragment, 0.72 mm; P_5, sebaceous-gland-containing fragment, 0.67 mm.

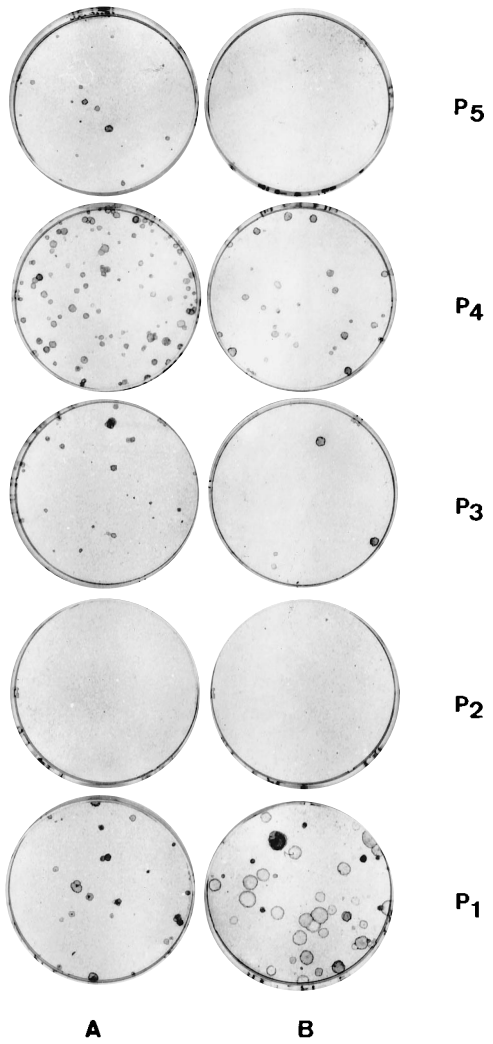


FIG. 8. Segregation of K-CFCs in rabbit pelage hair follicles. Keratinocytes were isolated from fragments P_1 to P_5 of two follicles. (A) Follicle shown in Fig. 7; (B) pelage guard hair follicle (total length, 1.91 mm) microdissected from a skin biopsy sample obtained from the back of a 20-week-old New Zealand White male rabbit. Cells were cultured for 12 days on tissue culture dishes containing a feeder layer of irradiated 3T3 cells (13, 22, 23). Cultures were then fixed with formalin and stained with rhodamine B. The two follicles displayed the same pattern of distribution of the colonies, which were segregated in two regions. First, numerous K-CFCs were found in the bulb-containing fragment, P_1 (A, 48; B, 95), whereas no colonies were obtained in the P_2 fragment. Second, fragment P_4 displayed a fair number of K-CFCs (A, 200; B, 67). Few colonies were also present in the adjacent fragments P_3 (A, 36; B, 11) and P_5 (A, 52; B, 3).

and 0.51 and 0.81 mm for follicle B (Fig. 8). On the other hand, the cells displaying CRPV E6 and E7 signals in the intermediate part of hair follicles were observed at a distance of 0.60 and 0.84 mm from the epidermis, as determined by measurements on photographs. This demonstrates that the cells containing CRPV E6 and E7 transcripts and the K-CFCs are located in the same two regions of the follicle, the bulb and a part of the outer epithelial sheath which is situated at a similar distance from the surface of the epidermis.

DISCUSSION

We have used a method of CRPV infection of rabbit skin which minimizes destruction of the epithelium. This has permitted us to identify cells in two different regions of hair follicles as the cells from which papillomas can develop. Microdissection of hair follicles established that the same fragments also contained K-CFCs with properties of stem cells.

We detected the earliest evidence of viral infection by the presence of transcripts encoding the viral E6 and E7 oncogenes in normal hair follicles of biopsy samples excised 11 days after infection. The particular localization of cells expressing E6 and E7 transcripts within these hair follicles correlated with the location of K-CFCs. This suggests that hair follicle stem cells are initial target cells for CRPV. The lack of unambiguous markers of epidermal stem cells renders their identification in situ rather difficult. However, they can be identified and studied by clonal analysis in culture. They were precisely located by this means to two defined areas (13, 23), in the area of the hair bulb (10) and in an intermediate part of the follicle corresponding to the bulge in the rat vibrissal follicle (13) or underneath the insertion of the arrector pili muscle in the human scalp follicle (23).

As shown in Fig. 3, the infection of hair follicle stem cells can clearly cause epithelial abnormalities with a histological picture of PV-induced changes in stratifying epithelium. This suggests that the K-CFCs in hair follicles which were identified by clonal analysis are competent to initiate the development of a papilloma after infection with CRPV. We certainly cannot exclude the possibility that stem cells located exclusively in the stratifying surface epithelium may be also directly targeted by CRPV, although this seems to represent a rather rare event.

Importantly, some of the colony-forming cells in hair follicles in humans have been demonstrated to be stem cells (23). Hair follicle stem cells may be pluripotent cells, which have

been suggested to be involved not only in hair follicle regeneration but also in the reconstitution of wounded surface epithelium (16, 30). Some evidence from a number of studies dealing with tumor induction in animal systems suggests that the bulge region of the pelage hair follicle in mice contains the precursor cells from which the majority of skin cancers in mice develop (16). It is an intriguing coincidence that cells from a similar region of the hair follicle suspected for a long time to be involved in skin cancer formation have now been found to be target cells for a high-risk PV.

The finding that only a certain subset of keratinocytes within the epithelium reveals viral gene expression points to a highly specific mechanism controlling permissivity. The observation that only cells expressing viral transcripts were also found to be weakly positive by in situ hybridization for the detection of viral DNA (data not shown) leaves open the question of whether these cells carry a specific receptor molecule for PV or whether they provide a unique transcription machinery for PV gene expression. Very little is known about the nature of the PV receptor and mechanisms involved in virus entry into host cells (17). Recently, binding of bovine PV1 virions or HPV11 virus-like particles to the surface of human cultured keratinocytes and to a number of cell lines of epithelial origin or of fibroblastic origin has been reported (17, 24). These results show that PV can bind to a large variety of cells and suggest the ubiquitous presence of PV receptor molecules. They also suggest that viral gene expression is likely to be regulated by the transcription machinery provided solely by permissive host cells, e.g., those from pluristratified epithelia. However, it should be strongly emphasized that these results were obtained with cultured primary cells or cell lines whose properties differ greatly from those of in vivo cells. This is particularly true for keratinocytes, whose programs of proliferation and differentiation differ significantly in vivo and in vitro (21, 33). Additional support for a unique transcription specificity for PV in permissive host cells comes from studies with transgenic mice in which the cell type specificity of the long control region of HPV18 (5) and HPV11 was investigated by using β-galactosidase as the indicator gene. HPV18 transgenic mice revealed β-galactosidase expression in many different epithelia. In the skin, however, no expression was detected in the dermis or epidermis, except in hair follicles, which were stained in the outer root sheath near the opening of the sebaceous gland (5). HPV11 transgenic mice, however, revealed an exclusive staining of cells within hair follicles (9a). Our study may also have implications for the large group of PV affecting the mucosal epithelium. Although there are no hair follicles in these epithelia, cells with features of stem cells are present in discrete areas (16, 30) and the overall process of PV infection might be quite analogous. It has been proposed in earlier works that wounding of the skin is essential for CRPV-induced papilloma formation in rabbits and that the target cells are keratinocytes migrating out of the hair follicle to reepithelialize the wound (8). Wound healing is likely to result in the migration and proliferation of K-CFCs, increasing their competence for viral transcription and thus their ability to escape from normal cell cycle control. Using the high-pressure injection technique to infect rabbits with CRPV, we found no evidence that an extensive injury of the epithelium is absolutely required for papilloma development. We observed nodular lesions of up to 0.5 mm in diameter in the skin of two rabbits as early as 21 days after infection (Fig. 6). This is a time period for papilloma development similar to that observed when scarified skin is infected. However, it is quite possible that wound healing-associated migration of the target cells increases significantly the number of cells competent to express high levels of E6 and E7.

So far, the majority of human carcinogenic PV are involved in the development of anogenital carcinomas devoid of hair follicles. However, single cells with characteristics of stem cells are believed to be located at discrete intervals in the basal layer of the pluristratified epithelium of the cervix (16, 30), and the observed preference of certain PV types for different types of epithelium may simply reflect differences in the transcriptional milieus of different stem cells. Thus, the observations made here for CRPV might indeed be relevant to other PV, including low- and high-cancer-risk types infecting the mucous epithelium.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA 1815 awarded by the National Cancer Institute to F.O.W.; by grants from the Association pour la Recherche contre le Cancer (ARC) and the Institut de la Sante et de la Recherche Medicale (INSERM) to Y.B.; and by Wilhelm Sander-Stiftung grant 89.042.3 to T.I. A.R. was supported by a postdoctoral fellowship from La Societe de Secours des Amis des Sciences.

REFERENCES

1. **Amella, C. A., L. A. Lofgren, A. M. Ronn, M. Nouri, M. J. Shikowitz, and B. M. Steinberg.** 1994. Latent infection induced with cottontail rabbit papillomavirus. Am. J. Pathol. **144:**1167–1171.
2. **Barbosa, M. S., and F. O. Wettstein.** 1988. Identification and characterization of the CRPV E7 protein expressed in COS-7 cells. Virology **165:**143–150.
3. **Boehm, S., S. P. Wilczynski, H. Pfister, and T. Iftner.** 1993. The predominant mRNA class in HPV16-infected genital neoplasias does not encode the E6 or the E7 protein. Int. J. Cancer **55:**791–798.
4. **Chow, L. T., M. Nasseri, S. M. Wolinsky, and T. R. Broker.** 1987. Human papillomavirus type 6 and 11 mRNAs from genital condylomata accuminata. J. Virol. **61:**2581–2588.
5. **Cid, A., P. Auewarakul, A. Garcia-Carranca, R. Ovseiovich, H. Gaissert, and L. Gissmann.** 1993. Cell-type-specific activity of the human papillomavirus type 18 upstream regulatory region in transgenic mice and its modulation by tetradecanoyl phorbol acetate and glucocorticoids. J. Virol. **67:**6742–6752.
6. **Danos, O., E. Georges, G. Orth, and M. Yaniv.** 1985. Fine structure of the cottontail rabbit papillomavirus mRNAs expressed in the transplantable VX2 carcinoma. J. Virol. **53:**735–741.
7. **Ferenczy, A., M. Mitao, N. Nagai, S. J. Silverstein, and C. P. Crum.** 1985. Latent papillomavirus and recurring genital warts. N. Engl. J. Med. **313:**784–788.
8. **Friedewald, W. F.** 1944. Certain conditions determining enhanced infection with the rabbit papilloma virus. J. Exp. Med. **80:**65–82.
9. **Giri, I., O. Danos, and M. Yaniv.** 1985. Genomic structure of the cottontail rabbit (Shope) papillomavirus. Proc. Natl. Acad. Sci. USA **82:**1580–1584.

9a. **Gissmann, L.** Personal communication.

10. **Hardy, M.** 1992. The secret life of the hair follicle. Trends Genet. **86:**41–78.
11. **Hummel, M., J. B. Hudson, and L. A. Laimins.** 1992. Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. J. Virol. **66:**6070–6080.
12. **Iftner, T., M. Oft, S. Boehm, S. P. Wilczynski, and H. Pfister.** 1992. Transcription of the E6 and E7 genes of human papillomavirus type 6 in anogenital condylomata is restricted to undifferentiated cell layers of the epithelium. J. Virol. **66:**4639–4646.
13. **Kobayashi, K., A. Rochat, and Y. Barrandon.** 1993. Segregation of keratinocyte colony-forming cells in the bulge of the rat vibrissa. Proc. Natl. Acad. Sci. USA **90:**7391–7395.
14. **Lin, Y.-L., L. A. Borenstein, R. Selvakumar, R. Ahmed, and F. O. Wettstein.** 1993. Progression from papilloma to carcinoma is accompanied by changes in antibody response to papillomavirus proteins. J. Virol. **67:**382–389.
15. **Meyers, C., J.-B. Harry, Y.-L. Lin, and F. O. Wettstein.** 1992. Identification of three transforming proteins encoded by cottontail rabbit papillomavirus. J. Virol. **66:**1655–1664.
16. **Miller, S. J., and R. M. Lavker.** 1993. Hair follicles, stem cells, and skin cancer. J. Invest. Dermatol. **100:**288S–294S.
17. **Müller, M., L. Gissmann, R. J. Christiano, X.-Y. Sun, I. H. Frazer, A. B. Jenson, A. Alouso, H. Zentgraf, and J. Zhou.** 1995. Papillomavirus capsid binding and uptake by cells from different tissues and species. J. Virol. **69:**948–954.
18. **Nasseri, M., and F. O. Wettstein.** 1984. Differences exist between viral transcripts in cottontail rabbit papillomavirus-induced benign and malignant

tumors as well as non-virus-producing and virus-producing tumors. J. Virol. **51:**706–712.
19. **Pfister, H.** 1992. Human papillomaviruses and skin cancer. Semin. Cancer Biol. **3:**263–271.
20. **Phelps, W. C., S. L. Leary, and A. J. Faras.** 1985. Shope papillomavirus transcription in benign and malignant rabbit tumors. Virology **146:**120–129.
21. **Potten, C. S., and R. J. Morris.** 1988. Epithelial stem cells *in vitro*. J. Cell Sci. Suppl. **10:**45–62.
22. **Rheinwald, J. G., and H. Green.** 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell **6:**331–344.
23. **Rochat, A., K. Kobayashi, and Y. Barrandon.** 1994. Location of stem cells of human hair follicles by clonal analysis. Cell **76:**1063–1073.
24. **Roden, R. B. S., R. Kirnbauer, A. B. Jenson, D. R. Lowy, and J. T. Schiller.** 1994. Interaction of papillomaviruses with the cell surface. J. Virol. **68:**7260–7266.
25. **Scheffner, M., H. Romanczuk, K. Münger, J. M. Huibregtse, J. A. Mietz, and P. M. Howley.** 1994. Functions of human papillomavirus proteins, p. 83–100. *In* H. zur Hausen (ed.), Human pathogenic papillomaviruses. Springer Press, New York.
26. **Schmitt, A., J.-B. Harry, B. Rapp, F. O. Wettstein, and T. Iftner.** 1994. Comparison of the properties of the E6 and E7 genes of low- and high-risk cutaneous papillomaviruses reveals strongly transforming and high Rb-binding activity for the E7 protein of the low-risk human papillomavirus type 1. J. Virol. **68:**7051–7059.
27. **Shope, R. E., and E. W. Hurst.** 1933. Infectious papillomatosis of rabbits. J. Exp. Med. **68:**607–623.
28. **Siegsmund, M., K. Wayss, and E. J. Amtmann.** 1991. Activation of latent papillomavirus genomes by chronic mechanical irritation. J. Gen. Virol. **72:**2787–2789.
29. **Smotkin, D., H. Prokoph, and F. O. Wettstein.** 1989. Oncogenic and non-oncogenic human papillomaviruses generate the E6 and E7 mRNAs by different mechanisms. J. Virol. **63:**1441–1447.
30. **Stanley, M. A.** 1994. Virus-keratinocyte interactions in the infectious cycle, p. 116–145. *In* P. L. Stern and M. Stanley (ed.), Human papillomaviruses and cervical cancer. Biology and Immunology. Oxford University Press, Oxford.
31. **Steinberg, B. M., W. C. Topp, P. S. Schneider, and A. L. Abramson.** 1983. Laryngeal papillomavirus infection during clinical remission. N. Engl. J. Med. **308:**1261–1264.
32. **Syverton, J. T.** 1952. The pathogenesis of the rabbit papilloma-to-carcinoma sequence. Ann. N. Y. Acad. Sci. **54:**1126–1140.
33. **Watt, F.** 1988. Epidermal stem cells in culture. J. Cell Sci. Suppl. **10:**85–94.
34. **Wettstein, F. O., M. S. Barbosa, and M. Nasseri.** 1987. Identification of the major cottontail rabbit papillomavirus late RNA cap site and mapping and quantitation of an E2 and minor E6 coding mRNA in papillomas and carcinomas. Virology **159:**321–328.
35. **Wettstein, F. O.** 1987. Papillomaviruses and carcinogenic progression, I. Cottontail rabbit (Shope) papillomavirus, p. 167–186. *In* N. P. Salzman and P. M. Howley (ed.), The Papovaviridae, vol. 2. Plenum Publishing Corp., New York.
36. **Wettstein, F. O.** 1990. State of viral DNA and gene expression in benign vs. malignant tumors, p. 155–174. *In* H. Pfister (ed.), Papillomaviruses and human cancer. CRC Press, Boca Raton, Fla.
37. **Zeltner, R., L. A. Borenstein, F. O. Wettstein, and T. Iftner.** 1994. Changes in RNA expression pattern during the malignant progression of cottontail rabbit papillomavirus-induced tumors in rabbits. J. Virol. **68:**3620–3630.
38. **zur Hausen, H.** 1994. Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types, p. 131–156. *In* H. zur Hausen (ed.), Human pathogenic papillomaviruses. Springer Verlag, New York.