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Synthesis of Viral DNA and Late Capsid Protein L1 in Parabasal Spinous Cell Layers of Naturally Occurring Benign Warts Infected with Human Papillomavirus Type 1

Kiyofumi Egawa,*[,1] Angelika Iftner,† John Doorbar,‡ Yumi Honda,§ and Thomas Iftner †

*Department of Dermatology and §Department of Pathology, Kumamoto University School of Medicine, Kumamoto, Japan; †Sektion Experimentelle Virologie, Universitätsklinikum Tuebingen, Calwer Strasse 7/6, 72076 Tuebingen, Germany; and ‡National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

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We investigated human papillomavirus type 1 (HPV1)-specific transcription, viral DNA replication, and viral protein expression in naturally occurring benign tumors by *in situ* hybridization, 5-bromodeoxyuridine (BrdU) incorporation, and immunohistochemistry and obtained results different from other HPV-infected benign tumors characterized so far. Moderate amounts of transcripts with a putative coding potential for E6/E7, E1, and E2 were demonstrated from the first subrabasal cell layer throughout the stratum spinosum and granulosum. In addition very large amounts of E4 and L1 transcripts were present in the same epithelial layers. This finding was substantiated by the demonstration of L1 and E4 protein already in the bottom-most spinous cell layer. Furthermore massive amplification of the viral DNA as measured by BrdU incorporation and different methods of *in situ* hybridization took place in the lowest 5 to 10 suprabasal cell layers. These findings are in contrast to the assumption that late gene expression and viral DNA synthesis are restricted to the more differentiated cell layers of the epithelium and point to differences in the regulation of the vegetative life cycle between different papillomavirus types.

Key Words: HPV-1; life cycle; transcripts; proteins; DNA replication.

INTRODUCTION

Human papillomaviruses (HPVs) are nonenveloped double-stranded DNA viruses, which are able to induce benign or malignant tumors of both the human skin and the mucosa (Galloway and McDougall, 1989; Laimins, 1996; zur Hausen, 1996). At present, more than 77 different types of HPV have been characterized (de Villiers, 1997). Despite differences in the oncogenic potential and tissue tropism all HPVs share a common genomic organization and are dependent on the differentiation of the infected epithelium for the completion of their vegetative life cycle (Laimins, 1996; zur Hausen, 1996). The viral genome is divided into an early region that encodes the genes E1 to E8 required for viral DNA replication, gene regulation, and cellular transformation, a late region that codes for the capsid proteins L1 and L2, and a regulatory region that contains the origin of DNA replication and many of the control elements for transcription and replication (Laimins, 1996; Thomas *et al.,* 1998). Recent studies revealed that the L1 protein has the capacity to self-assemble into virus-like particles (VLPs) when expressed in different eukaryotic systems (Hagensee *et al.,* 1993; Kirnbauer *et al.,* 1993). The L2 protein is capable of binding to DNA and is required to assemble infectious BPV1 efficiently (Roden *et al.,* 1996; Zhou *et al.,* 1993). It has been speculated that the L2 protein is involved in viral DNA encapsidation (Zhou *et al.,* 1993) and in capsid stabilization at low pH (Liu *et al.,* 1997). In accordance with such a regulatory function the molar ratio of the L2 to the L1 protein in virus particles is approximately 1:30 (Schiller and Roden, 1996). Although viral E4 proteins were originally classified on the basis of their distribution in benign tumors as early proteins, recent studies suggest that they are late polypeptides expressed during and after the onset of vegetative viral DNA replication in the parabasal layers of infected skin (Doorbar *et al.,* 1997; Rogel-Gaillard *et al.,* 1992). Our current understanding of the biology of HPVs is based primarily on *in vitro* experiments performed with molecularly cloned viral DNA, on the biochemical analysis of viral gene products, and on *in situ* hybridization studies applied to infected biopsy material. The latter experiments revealed that vegetative viral DNA replication, expression of viral structural genes, and viral capsid assembly were found to be restricted to keratinocytes committed to terminal differentiation (Böhm *et al.,* 1993; Dürst *et al.,* 1992; Higgins *et al.,* 1992; Iftner *et al.,* 1992, 1994; Zeltner *et al.,* 1994). In addition experimental *in vivo* studies with HPVs in animal model systems could not be performed because of the strict species specificity of HPVs and results

[1] To whom correspondence and reprint requests should be addressed at Department of Dermatology, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860, Japan. Fax: +81(0)96-373-5235. E-mail: kuroibo@kaiju.medic.kumamoto-u.ac.jp.

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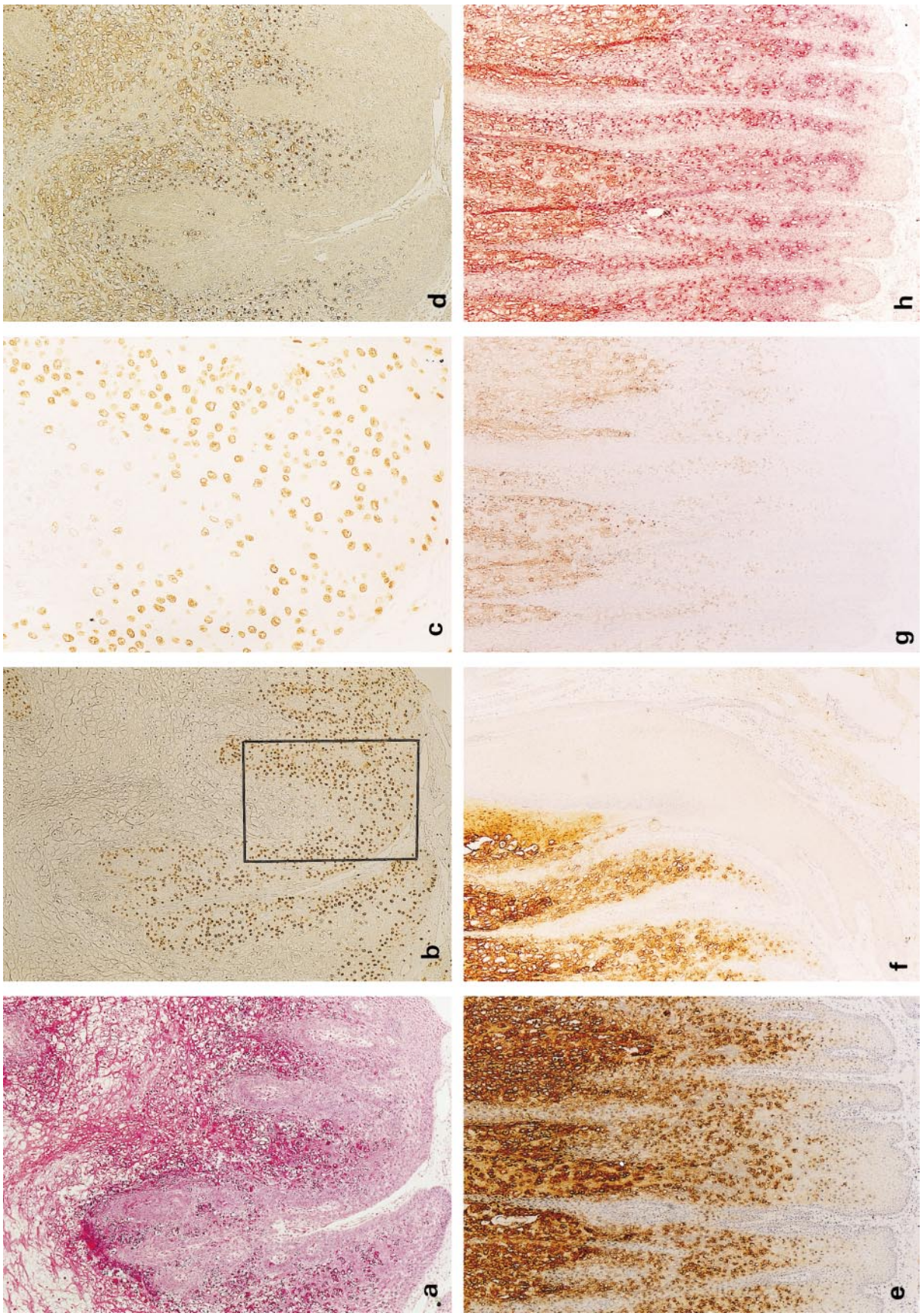
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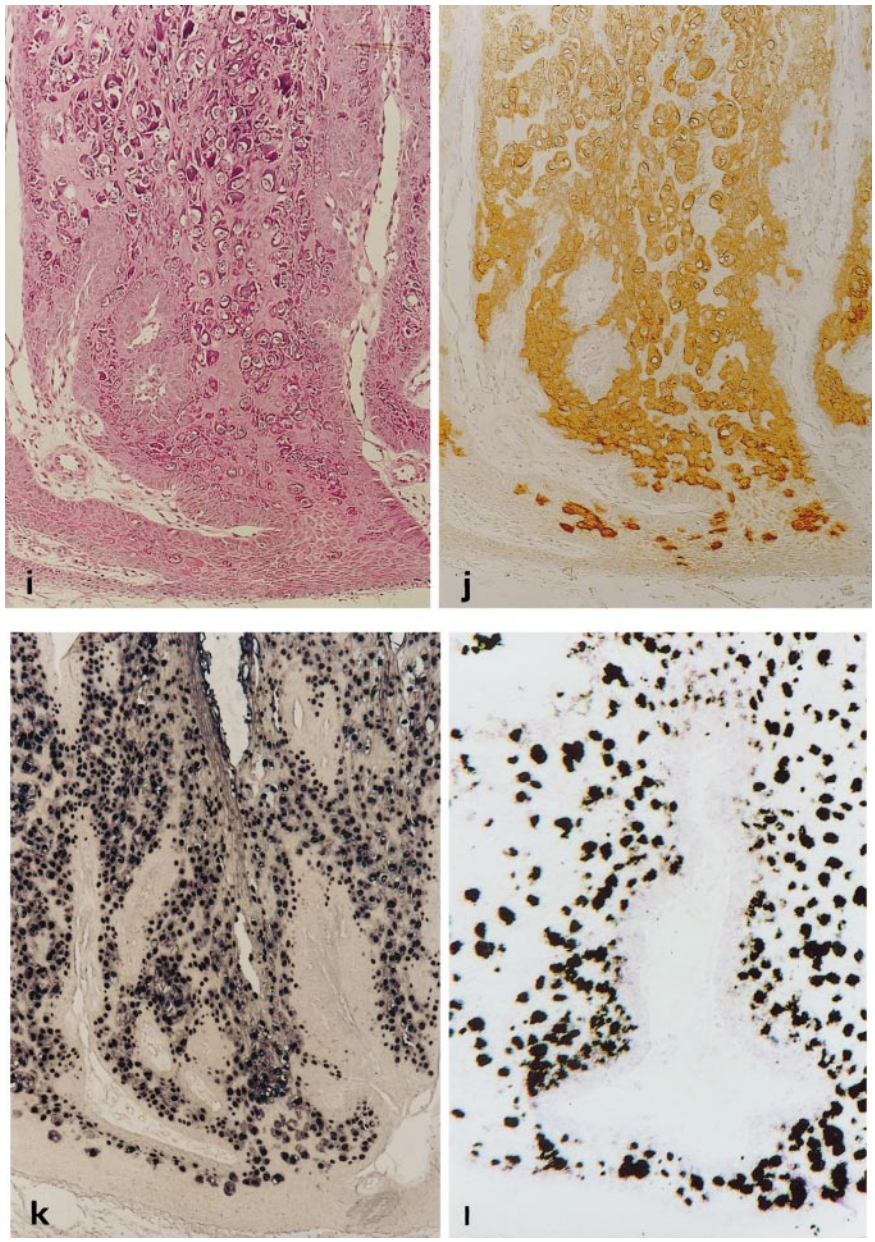


FIG. 1. Immunohistochemical detection of HPV1 proteins and *in situ* hybridization for HPV1 DNA. Hematoxylin–eosin staining of ethanol-fixed (a) and formalin-fixed (i) sections of a HPV1-infected plantar wart. Detection of BrdU incorporation in a tissue section at lower (b) and higher (c) magnification identifies cells with replicating DNA. (d) Immunohistochemical detection of both L1 and L2 capsid proteins in a formalin-fixed tissue section. (e) L1 capsid protein in a frozen unfixed section of infected wart tissue. (f) L1 capsid protein in a frozen unfixed section of infected wart tissue with adjacent uninfected epidermis demonstrating the specificity of the antibody staining. (g) L2 capsid protein in a frozen unfixed section. (h) L1 (red) together with L2 (brown) capsid protein in a frozen unfixed section of infected wart tissue. (j) E4 proteins are detected in all suprabasal cell layers having a distribution similar to that of the intracytoplasmic inclusion bodies seen in (j) and the HPV1 DNA as detected by *in situ* hybridization using a biotinylated probe (k) or radioactive probe 2 in sense orientation (l; Fig. 4) on a denatured proteinase-treated tissue section.

obtained in these systems with animal HPV types are not easily transferable to the human system. However, recent advances in organotypic (raft) and semisolid medium culture techniques allowed the investigation of vegetative viral DNA replication and expression of viral structural genes as well as viral progeny production for a limited number of "high-risk" human papillomavirus types, but not for HPV types that are unable to immortalize primary keratinocytes as in the case of HPV1 (Frattini *et al.,* 1996; Laimins, 1996; Ruesch *et al.,* 1998; Schmitt *et al.,* 1994).

In this study we investigated the life cycle of HPV1 in ordinary plantar warts, which is an extremely prevalent disease present in roughly 30% of all schoolchildren at

the age of 10 to 11 years in Germany. When analyzed by PCR and subsequent sequencing of the amplification products 31% of 130 plantar warts contained HPV1 in addition to HPV27 (17%), HPV57 (9%), and HPV2 (8%; Iftner, 1999). By applying *in situ* hybridization, 5-bromodeoxyuridine (BrdU) labeling, and immunohistochemistry to HPV1-infected plantar warts we correlated the distribution of HPV1-specific transcripts and of viral DNA with the specific detection of the viral proteins L1, L2, and E4. Our results reveal a difference between the pattern of viral DNA amplification, viral RNA expression, and protein expression found in HPV1-infected benign tumors compared to the patterns previously observed for other HPV types (Böhm *et al.,* 1993; Dürst *et al.,* 1992; Higgins *et al.,* 1992; Iftner *et al.,* 1992, 1994). While in the single basal cell layer of HPV1 warts no viral DNA or RNA was detectable, transcripts with a putative coding potential for E6/E7, E1, E2, E4, and L1 were demonstrated already in the first suprabasal cell layer and throughout most of the stratum spinosum and granulosum. In contrast, transcripts with a coding potential for L2 seemed to be expressed preferentially in higher differentiated layers of the epithelium. In accordance with these data the late capsid protein L1 was identified by immunohistochemistry already in suprabasal layers while the L2 protein was preferentially detected in the upper epidermal cell layers. Interestingly the results of DNA–DNA *in situ* hybridization and BrdU incorporation suggest that gross synthesis of the viral DNA is performed in the lowest suprabasal cell layer of the epidermis coincident with the presence of transcripts with a coding potential for E1 and E2 and with the presence of the E4 protein.

RESULTS

Microscopic examination of sections of all 16 HPV1-infected plantar warts revealed common histological characteristics, which were compatible with features previously described in HPV1-associated cutaneous tumors (Figs. 1a and 1i). The epidermis showed clear signs of acanthosis, hyperkeratosis, parakeratosis, and papillomatosis. Intracytoplasmic vacuolization developed in parallel with the differentiation of the epithelial keratinocytes. Eosinophilic granular intracytoplasmic inclusion bodies were present throughout most suprabasal layers and were a specific feature seen in all samples (Fig. 1i; Egawa *et al.,* 1993, 1994; Egawa, 1994; Jablonska *et al.,* 1985). Increased numbers of mitotic figures were seen in the basal and the first suprabasal cell layers in contrast to adjacent normal skin. When small pieces (less than 1 mm^3) of freshly taken wart tissue were homogeneously labeled with BrdU (Fig. 2b) and afterward embedded in paraffin we observed in 5-μm sections of the tissue that cells labeled with BrdU were found not only in the basal cell layer but also in the adjacent 5 to 10 suprabasal layers. Such labeled cells were surprisingly not detected in the upper spinous and granular cell layers of the HPV1-infected acanthotic epithelium although the penetration of the tissue sample with BrdU was equal for all epithelial cell layers (Figs. 1b and 2b). In general, incorporation of BrdU as a result of cellular DNA replication is not distinguishable from BrdU incorporation resulting from viral DNA replication. However, the strong positive staining with BrdU in spinous cell layers, especially in cells that were morphologically well differentiated (Figs. 1b and 1c), was never observed in noninfected normal epithelium (Fig. 3b). This staining therefore seems to be due to the presence of actively replicating viral DNA. In addition amplified viral DNA was identified within the nuclei of epidermal keratinocytes by nonradioactive DNA–DNA *in situ* hybridization (Fig. 1k). Very strong positive signals were present in the epithelium as low as in the first suprabasal layer and were distributed throughout the whole thickness of the epithelium. The intensity of the signals was almost even between the upper and the lower epidermal cell layers, suggesting the presence of similar copy numbers of viral DNAs in all suprabasal layers. Even in very short-exposure DNA *in situ* hybridizations of HPV1-infected warts (3 days) we detected uniformly strong signals in all suprabasal epithelial layers representative of viral DNA presence. Together with the results from the BrdU labeling experiment this finding suggests that the viral DNA is not further up-replicated in the upper third of the epithelium. By applying radioactive DNA *in situ* hybridization using riboprobe 1 (data not shown) or 2 (Fig. 1l) in sense orientation on denatured proteinase-treated frozen wart sections, it could be confirmed that indeed a large proportion of the cells in the first suprabasal cell layer already contained a high copy number of viral DNA.

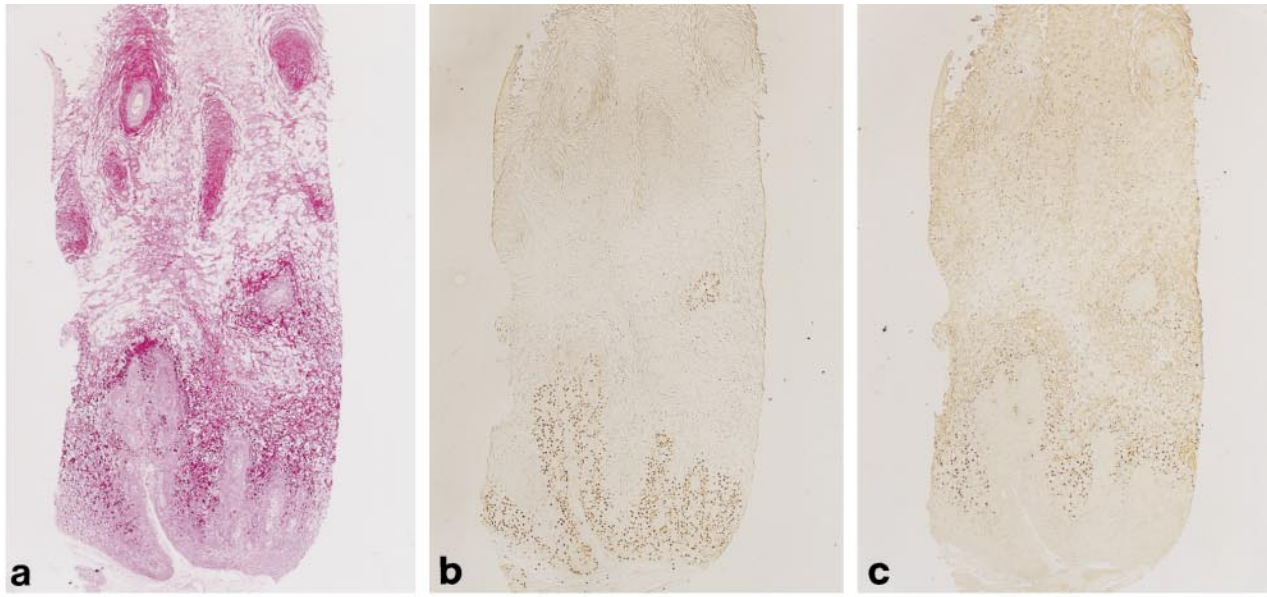
Based on the already known transcription pattern for HPV1-specific mRNAs (Chow *et al.,* 1987; Palermo-Dilts *et al.,* 1990), we constructed seven RNA expression plasmids to generate antisense riboprobes, which should allow the detection of several mRNA classes in HPV1-infected tissue. The hybridization specificities of the different probes for the known mRNA species are as follows: Probe 1 is specific for a transcript encoding the E6 and E7 proteins as first translatable gene products. Probe 2 recognizes the same transcript and additionally recognizes any transcript initiating downstream from nt 343 and at the E1^E4 promoter located within the 3′-half of ORF E7 (Fig. 4; Palermo-Dilts *et al.,* 1990). Probe 3 identifies putative transcripts encoding the E1 gene product, but does not hybridize to mRNAs that were processed by cleavage at the major splice donor site at the end of ORF E7 (Palermo-Dilts *et al.,* 1990). Probe 4 hybridizes to mRNA species with a coding capacity for a full-length E2 protein. Probe 5 hybridizes to all early and late transcripts. Probe 6 identifies the L2 mRNA and a potential, but so far unknown, E5 transcript, and probe

7 hybridizes to both major late transcripts encoding L1 or L2. With the help of these probes we investigated eight plantar warts from Germany, which were found by PCR/direct sequencing and *in situ* hybridization to contain HPV1 DNA and obtained consistent results for all of them. In all biopsies investigated we detected moderate amounts of transcripts with a possible coding potential for E6/7, E1, and E2 and large amounts of transcripts encoding E1^E4 and L1 already in the first suprabasal cell layer (Fig. 5). Only transcripts detected with probe 6 identifying the L2 mRNA seemed to be expressed preferentially in higher differentiated layers of the epithelium (Fig. 5G). Because in HPV1 only one early promoter immediately upstream of ORF E6 (Palermo-Dilts *et al.,* 1990) has been identified so far, the much stronger signal obtained with probe 2 in comparison with probe 1 (Figs. 4, 5A, and 5B) is likely to be derived from the E1^E4 promoter in the 3′-half of ORF E7. In addition to probe 1, probe 3 (Figs. 4 and 5C), probe 4 (Figs. 4 and 5D), and probe 6 (Fig. 5G) resulted in faint to moderate signals within the epithelium. Therefore the strong specific signals obtained with riboprobes 2 (Fig. 5B), 5 (Fig. 5E), and 7 (Fig. 5F) most probably correspond to transcripts with a coding potential for E1^E4 and L1, respectively. In the case of L1 this conclusion is further supported by comparing the signal intensities obtained after hybridization with probe 6 or with probe 7 (Figs. 5F and 5G). First, the difference in signal intensity and second, the difference in location of the signals after hybridization with probe 6 or 7 provide support for the presence of L1 transcripts in suprabasal cells. These results could be further confirmed by using another L1-specific antisense probe (data not shown). This clearly shows that the expression pattern of the transcripts for the capsid proteins L1 and L2 is highly divergent in the case of HPV1-infected warts. Whereas L1-specific transcripts were highly abundant already in suprabasal cells and less prominent in higher differentiated cells, transcripts encoding L2 were preferentially expressed within the upper part of the epithelium.

When fixed tissue sections (ethanol or formalin) were investigated with immunohistochemistry using both anti-L1 and -L2 antibodies, cells with positive signals were detected in higher cell layers adjacent to cells positive for incorporation of BrdU (Figs. 1b, 1d, 2b, and 2c). This positive signal for the capsid proteins persisted into the upper spinous, granular, and horny layers (Fig. 2c). In contrast to this finding we surprisingly noticed that using frozen sections a positive signal for the L1 capsid protein was detected already in the lowest spinous cell layers and persisted throughout all the upper epidermal cell layers (Figs. 1e and 1f). L1 protein-specific signals were never seen in the adjacent epidermis not infected by HPV1 (Fig. 1f, right half). Interestingly, abundant L1-specific mRNA was detected in lower epidermal layers (Fig. 5F) than the L1 protein, which may be indicative of putative translational regulation of the L1 protein. Even in frozen sections, however, a detectable signal for the L2 capsid protein was preferentially seen in higher differentiated epidermal cell layers (Fig. 1g) as shown already in ethanol- (Fig. 1d) or formalin-fixed sections. This divergent distribution of L1 versus L2 protein observed within the epithelium may just reflect differences in the level of protein expression together with a different threshold of detection for each protein dependent on the affinities of the different antibodies used, although the results of the RNA–RNA *in situ* hybridization using L2-specific probe 6 clearly argue against this possibility. In addition double-immunoenzymatic staining of the same section with antibodies against L1 and L2 (Fig. 1h) revealed again a different distribution of both proteins. When using anti-E4 antibodies positive staining was seen mainly in cells having intracytoplasmic inclusion bodies (Figs. 1i and 1j). The intensity of E4-positive staining persisted from lower spinous cell layers throughout the upper epidermal cell layers and correlated with the presence of viral DNA.

DISCUSSION

It is generally assumed that papillomavirus DNA amplification takes place in higher differentiated keratinocytes, which is usually accompanied by the expression of the viral structural proteins L1 and L2. This is believed to be the major obstacle for the development of a simple productive tissue culture model to propagate papillomaviruses. However, a few already existing data are not completely in line with this widespread assumption. For example, previous findings obtained with the CRPV animal model system demonstrated gross viral DNA amplification in undifferentiated dysplastic epithelium being adjacent to well-differentiated papillomatous tissue that did not support viral DNA synthesis (Zeltner *et al.,* 1994) and an increase of L1 antibodies in the serum of rabbits with progressive malignant disease (Lin *et al.,* 1993). This L1-mediated immune response in rabbits correlated with detectable L1-specific transcripts of CRPV in cell layers forming the outer border of invasive squamous epithelial carcinomas (Zeltner *et al.,* 1994). Second, using a mouse model it was shown that a dedifferentiated tumor cell line containing full-length HPV16 DNA plus EJ*ras* surprisingly expressed sufficient levels of L1 to serve as a target for L1-VLP-specific cytotoxic T cells (De Bruijn *et al.,* 1998). And third, it was described that papillomavirus DNA amplification correlated with E4 protein expression, but not with known cellular differentiation markers such as nuclear degeneration and filaggrin or loricrin expression (Doorbar *et al.,* 1997; Jareborg and Burnett, 1991). Taken together with our earlier reports showing HPV6/16 DNA amplification in cancer tissue (Böhm *et al.,* 1993; Oft *et al.,* 1993) the necessity for a fully differentiated epithelial



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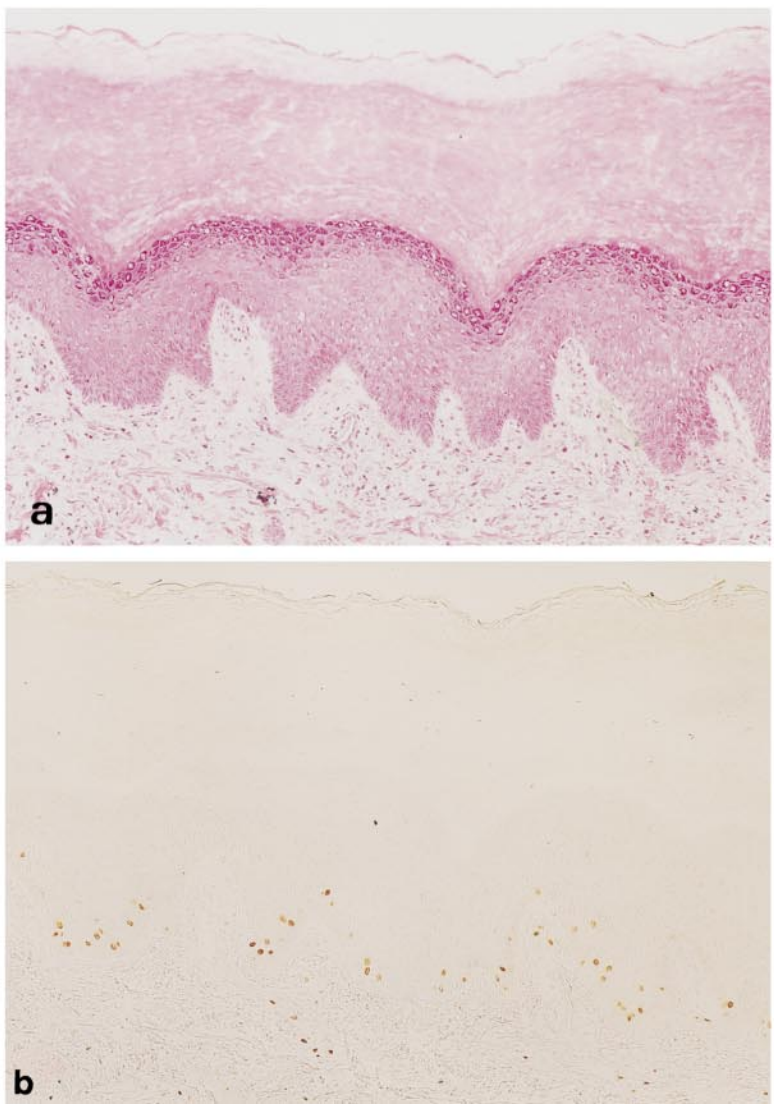

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cell to support amplification of viral DNA does not seem to be absolute.

In this study we observed in all 16 naturally HPV1-infected benign tumors a clear difference from the well-believed dependence of DNA amplification and late gene expression on a progressed differentiation status of the infected keratinocyte. When BrdU incorporation was used as a measurement for DNA synthesis we found a considerably increased number of labeled cells in the basal cell layer within the lesions compared to the adjacent normal skin. This suggests an increased proliferation of basal cells by infection with HPV1. However, BrdU was also incorporated in 5 to 10 layers of almost all suprabasal cells. Since BrdU could be incorporated as a result of viral and cellular DNA replication, a part of the incorporated BrdU, especially that seen in the cells that were morphologically well differentiated, could be due to viral rather than to cellular DNA amplification. This is further supported by the results of two different approaches to demonstrate viral DNA by *in situ* hybridization, which clearly showed the presence of large amounts of viral DNA from the bottom-most parabasal cell layer up to the corneal layers. These findings are in contrast to older results obtained in HPV-induced verruca vulgaris using tritiated thymidine, where viral DNA amplification was found to take place only in the upper differentiated epidermal cell layers (Rashad, 1969) and also in contrast to previous results of *in situ* hybridization studies with HPV6- or HPV16-infected benign tumors (Böhm *et al.,* 1993; Dürst *et al.,* 1992; Higgins *et al.,* 1992; Iftner *et al.,* 1992, 1994). However, very early findings by Grußendorf and zur Hausen (1979) using c-RNA probes for *in situ* hybridization experiments with denatured sections of HPV1-infected warts suggested the presence of viral DNA and transcripts already in the first or second suprabasal cell layer (Grußendorf and zur Hausen, 1979) and also in bovine papillomavirus type 1 (BPV1)-infected fibropapilloma viral DNA amplification was found to begin in the spinous cell layer (Barksdale and Baker, 1993). One might speculate that regulation of vegetative DNA replication is less tightly controlled in the case of HPV1 than in genital HPV types. In addition, even the pattern of late gene expression in HPV1-infected warts was shown here to be highly divergent from other HPV types (Böhm *et al.,* 1993; Dürst *et al.,* 1992; Higgins *et al.,* 1992; Iftner *et al.,* 1992, 1994) and also from BPV1-infected tissues (Baker and Howley, 1987). Transcripts with a coding potential for L1 were observed starting from the first suprabasal cell layer and extended throughout the epithelium. This finding again is in contrast to the current model that late gene expression is restricted only to higher differentiated cell layers. Interestingly transcripts encoding HPV1-L2 were detected preferentially in the upper epidermal cell layers of the infected plantar warts. This unusual late gene expression pattern of HPV1 was further confirmed using immunohistochemistry to demonstrate L1 capsid protein in the bottom-most spinous cell layers and by the detection of L2 protein in higher differentiated layers in tissue sections. Interestingly we noticed differences in the results between frozen and fixed tissue in the detectability of L1 protein. These differences may suggest that ethanol or formalin fixation could affect the reactivity of epitopes on the capsid proteins. In accordance with previous studies the HPV1 E4 gene product was detected in the cytoplasm of keratinocytes in association with intracytoplasmic inclusion bodies (Breitburd *et al.,* 1987; Doorbar *et al.,* 1988). Positive cells containing E4 protein could be found in the first suprabasal layer and persisted throughout all epithelial cell layers. This distribution of cells expressing E4 was very similar to that of cells in which viral DNA amplification was strong enough to be detected by *in situ* hybridization.

To summarize these findings we included our data in a hypothetical model of the life cycle of HPV1 in plantar warts. Within the epidermis a continuous renewal takes place through mitotic division of basal cells. Daughter cells of dividing basal cells move upward from the basal cell layer to form layers of progressively more differentiated cells comprising the "epidermal flow." Combining the results of the *in situ* hybridization, which detects all viral DNA, with the results obtained from the BrdU incorporation experiment, which detects only synthesis of DNA, it is reasonable for us to assume that the viral DNA is amplified already in the lowest parabasal cell layer and is afterward carried by the epidermal flow into the upper part of the skin. DNA replication in the lower epithelial layers is accompanied by the presence of transcripts with a coding potential for the gene products of E1 and E2, known to be involved in viral replication. The unusual early expression of the late protein L1, which we detected already in suprabasal cell layers, may

FIG. 2. BrdU staining of an HPV1-infected plantar wart and detection of late proteins. (a) Hematoxylin–eosin staining of HPV1-infected plantar wart tissue at low magnification. (b) BrdU incorporation is detected in 5 to 10 suprabasal cell layers in the lower part of the wart tissue and in a cross-sected papilla in the upper right part, demonstrating homogeneous BrdU incorporation into the whole block of the tissue. (c) Immunohistochemical detection of both L1 and L2 capsid protein in a formalin-fixed tissue section demonstrates the presence of late proteins in adjacent higher epithelial cell layers positive for BrdU staining.

FIG. 3. BrdU staining of normal plantar skin. (a) Hematoxylin–eosin staining of normal plantar skin. (b) BrdU incorporation is detected only in a few cells in the basal and first suprabasal cell layers of the epithelium.

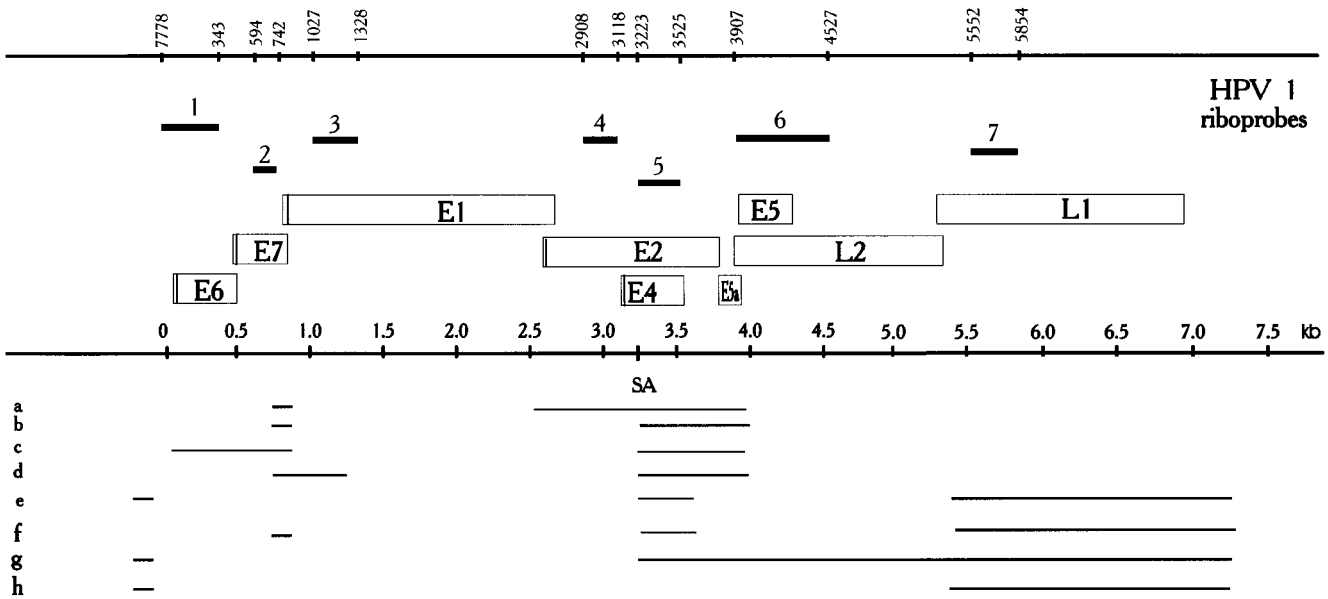


FIG. 4. Genomic organization of HPV1 and location of the riboprobes used for *in situ* hybridization. Genetic and transcriptional map of HPV1 and localization of mRNA-specific probes 1–7. The ORFs (open boxes) and the nucleotide positions of the probe boundaries (top) correspond to the published sequence of HPV1 (Danos *et al.,* 1982). Vertical lines within the ORFs represent the translational start codon. The lower part of the figure summarizes already known transcript structures (a–h) derived from RT-PCR or R-loop mapping methods (Chow *et al.,* 1987; Palermo-Dilts *et al.,* 1990).

shift the balance between the protein levels of L1 and L2 in infected cells to be able to achieve a putative final ratio of 30:1 (L1:L2) in analogy to HPV16 within the mature viral capsid (Roden *et al.,* 1996; Schiller and Roden, 1996). Although the function of the L2 protein is not fully understood, there is some evidence to suggest that it is a DNA-binding protein (Roden *et al.,* 1996; Zhou *et al.,* 1993) that may be involved in viral DNA encapsidation (Zhou *et al.,* 1993). The observed distribution of L2 protein in HPV1-infected warts would be compatible with a putative role of L2 as a trigger molecule for the final assembly of the mature virions.

In summary this report demonstrates viral DNA amplification and late protein expression in the first suprabasal cell layers of naturally occurring tumors infected with HPV1. The results obtained suggest major differences in the level of control of the switch from early to late replication cycles between individual papillomavirus types.

MATERIALS AND METHODS

Eight plantar warts induced by HPV1 (from Japan) were employed for BrdU incorporation, nonradioactive DNA *in situ* hybridization, and immunohistochemistry and an additional eight plantar warts (from Germany) were further investigated with a radioactive *in situ* hybridization protocol for the presence of viral transcripts and DNA. All lesions were located on the soles of children ranging in age from 7 to 13 years and showed typical clinical features for such infection, i.e., dome-shaped tumors with a central depression (myrmecia). These warts were of 3 to 6 months duration. All warts had no previous history of treatment. The lesions were excised under local anesthesia and cut into several small pieces, one of which was fixed in 10% formalin and embedded in paraffin by routine processing for light microscopic examination, immunohistochemistry, and nonradioactive biotin-based DNA–DNA *in situ* hybridization, and one of which was employed for cell kinetics study using BrdU (Gratzner, 1982), while the others were frozen and stored at −80°C for further analyses.

BrdU incorporation and detection

Small pieces less than 1 mm^3, which were taken from each biopsy specimen, were placed in a glass vial containing nucleoside- and nucleotide-free RPMI medium 1640 (Gibco) with 10% heat-inactivated fetal calf serum and 400 μM BrdU (Sigma). The vial was tightly sealed with a Teflon rubber cap after the air was replaced with 95% O_2 and 5% CO_2 under 3 atm of pressure and incubated in a shaking water bath for 1 h at 37°C. The labeled tissues were fixed in 70% ethanol overnight at 4°C and then embedded in paraffin. For detection of incorporated BrdU, 5-μm-thick sections were cut, deparaffinized, and rehydrated. DNA was denaturated by incubation in 2 N HCl for 30 min at room temperature and neutralized by 0.1 M $Na_2B_4O_7$ for 15 min at room temperature. The incorporated BrdU was visualized immunohis-

tochemically using an anti-BrdU monoclonal antibody (Becton-Dickinson, San Jose, CA).

Immunohistochemistry

Immunohistochemical staining was performed with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) on serial sections of ethanol-fixed, formalin-fixed, and/or frozen specimens to visualize the incorporated BrdU and to detect HPV 1-encoded (L1, L2, and E4) proteins. Briefly, 4-μm cryostat sections were air-dried and fixed in acetone at −20°C for 20 min. After being washed in phosphate-buffered saline (PBS), the sections were treated with 3% hydrogen peroxide solution to reduce endogenous peroxidase activity. The slides were incubated with the respective primary antibodies overnight at 4°C, washed in PBS, and treated with biotinylated anti-mouse, anti-rabbit, or anti-rat antibodies. After 60 min of incubation with avidin–biotin–peroxidase complex (ABC, Vector Laboratories), the sections were washed in PBS, developed in 0.05% diaminobenzidine diluted in PBS with 0.01% hydrogen peroxide, dehydrated, and mounted. The same staining procedure was employed on ethanol-fixed (for the above-mentioned BrdU incorporation study) and formalin-fixed materials, after deparaffinization and rehydration. Nonimmunized mouse, rabbit, and rat sera (Cappel Division, Organon Technika Co., West Chester, PA) were used as negative controls. Antibodies against HPV1 L1, L2, and E4 proteins were developed by Doorbar and co-workers (Doorbar and Gallimore, 1987; Doorbar *et al.,* 1988).

Double-immunoenzymatic staining was performed to clarify the correlation between the L1 and the L2 protein distribution. In brief, a sequential immunostaining procedure was carried out as follows. First, immunoperoxidase staining against L2 protein using the ABC method (Hsu *et al.,* 1981) was performed with a Vectastain ABC kit (Vector Laboratories). After pictures were taken of the distribution of the L2 protein within the tissue, the respective slide was put into xylene to remove the cover glass and subsequently the section was again rehydrated. The immunoalkaline phosphatase antialkaline phosphatase (APAAP) method (Cordell *et al.,* 1984) was then applied for L1 protein with an APAAP kit (Zymed Laboratories, San Francisco, CA). As substrate solution for the alkaline phosphatase reaction, naphthol-AS-phosphatase (Sigma Chemical Co., St. Louis, MO) and fast red TR (Sigma) were dissolved in dimethylformamide and diluted with 0.1 M Tris buffer, pH 8.2. To inhibit endogenous tissue enzyme activity, 0.25 mM levamizole (Sigma) was added to the incubation medium.

DNA–DNA *in situ* hybridization with biotinylated probes

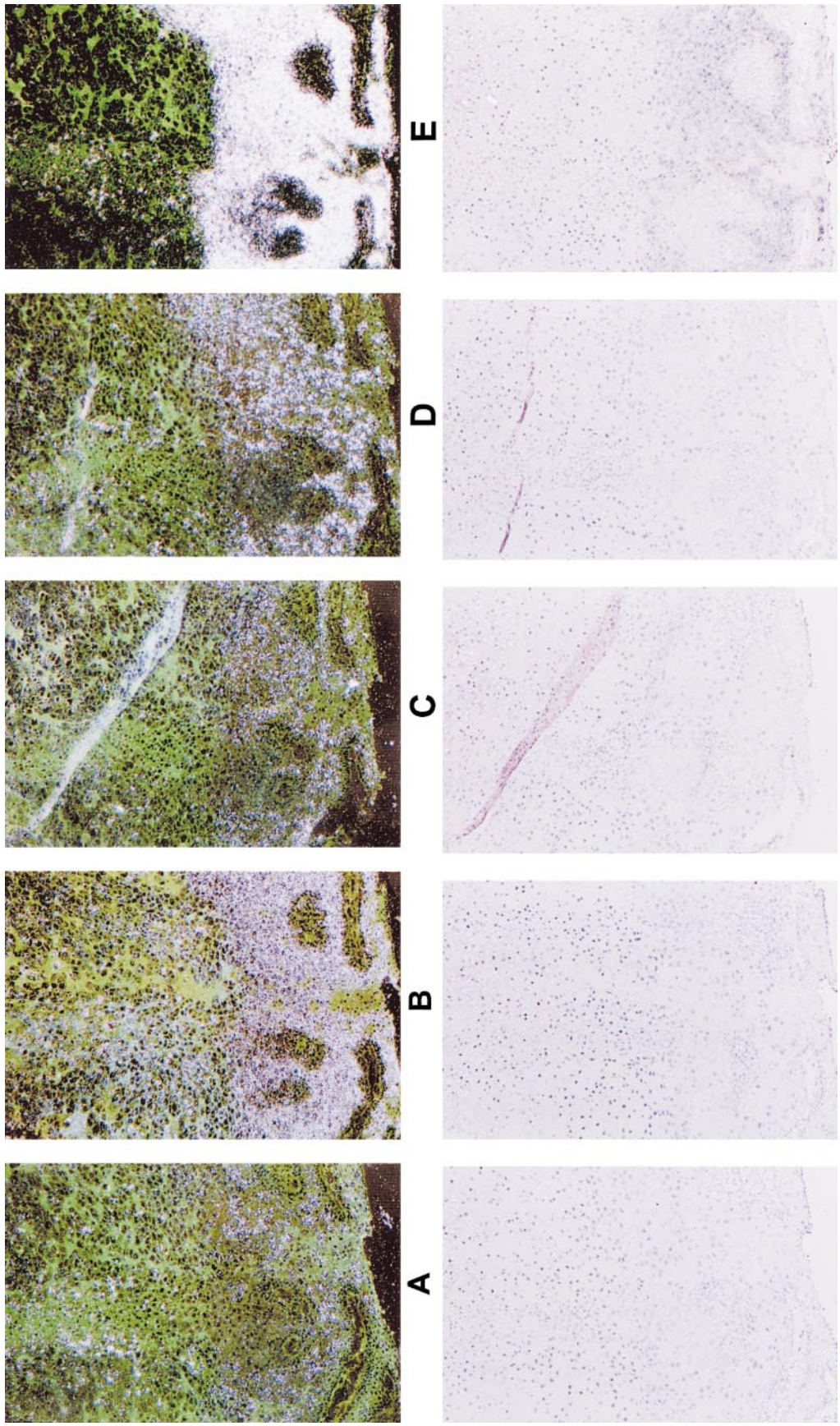
HPV DNA was detected in formalin-fixed, paraffin-embedded tissue sections by using a DNA *in situ* hybridization method described previously (Egawa *et al.,* 1993). Hybridization was performed at 37°C for 16 h using the biotinylated HPV1 DNA complete genome (a gift from the Japanese Cancer Research Resources Bank). The hybridization mixture consisted of 10% dextran sulfate, 2× SSC, 400 μg/ml sheared herring sperm DNA (Sigma), 50% formamide, and 2 μg/ml of biotinylated probe DNA. The biotinylated probe was detected by the streptavidin-biotinylated polyalkaline phosphatase detection system (DNA detection system; BRL, Gaithersburg, MD). Pheochromes used were nitroblue tetrazorium and 5-bromo-4-chloro-3-indolylphosphate.

Radioactive labeled RNA probes used for *in situ* hybridization

The method to generate riboprobes and the conditions for DNA hybridization with sense strand riboprobe 2 have been described previously (Schmitt *et al.,* 1996). The map positions of the seven small subgenomic HPV1 DNA fragments that have been cloned in the RNA expression vector Bluescribe M13+ (Vector Cloning System) and that served to generate the antisense probes are illustrated in Fig. 4. All plasmids were linearized prior to transcription. Depending on the choice of 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 3×10^8 cpm/μg was followed by alkaline hydrolysis to reduce the probe length to approximately 200 nt (Cox *et al.,* 1984). The quality of the transcribed RNAs was tested by polyacrylamide gel electrophoresis. The shortest probe (probe 2 with a complexity of 148 nt) was applied at a concentration of 2.7×10^7 cpm/ml hybridization mixture and the concentration of the others was normalized in relation to the probe length.

RNA–RNA *in situ* hybridization

For RNA *in situ* hybridization, serial 5- to 6-μm sections of biopsies 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, 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% SDS, and 0.15 mg/ml tRNA. After removal of the prehybridization solution, individual tissue sections were encircled with rubber cement and incubated at 42°C with different ^{35}S-labeled antisense riboprobes in solution I, containing 10% dextran sulfate. After 16 h of hybridization the rubber cement strings were removed and the slides were washed in 50% formamide, 1× SSC at 55°C and then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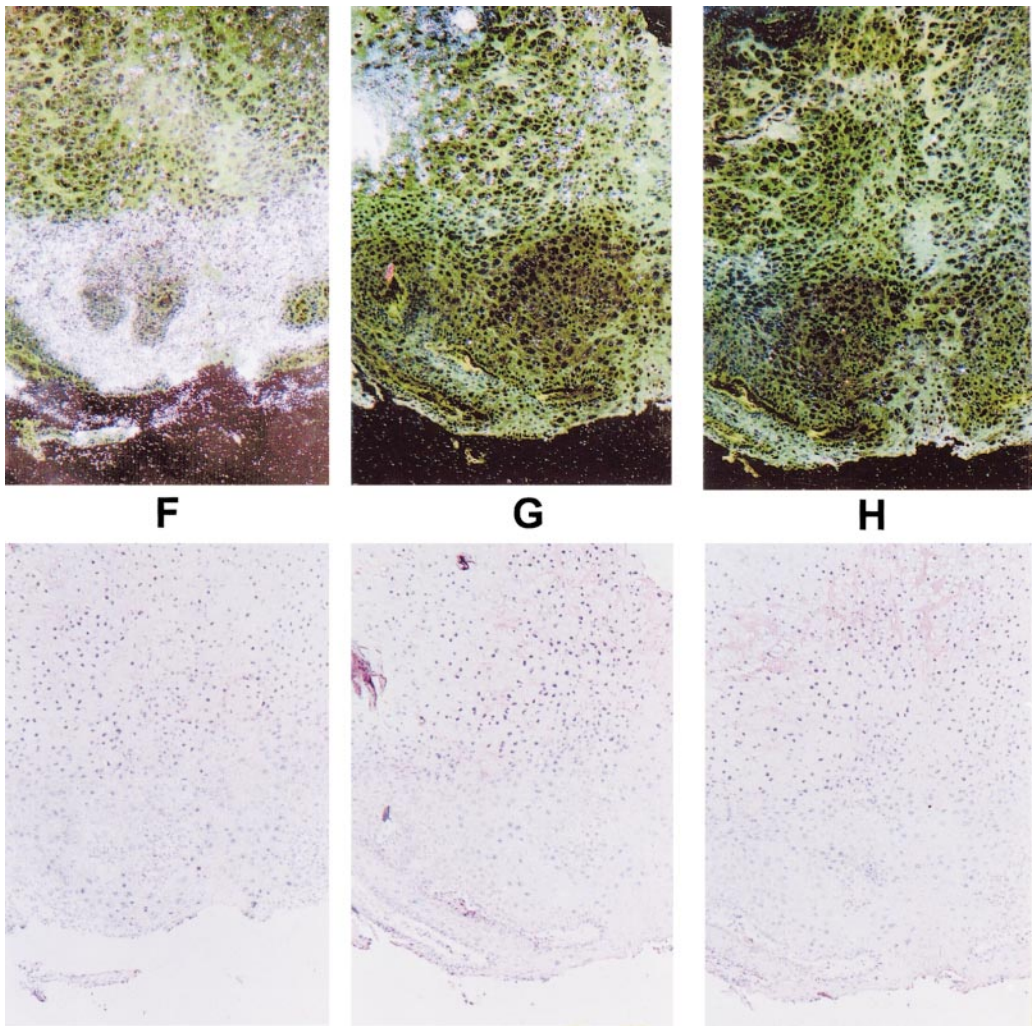

FIG. 5. RNA–RNA *in situ* hybridization of adjacent tissue sections of a HPV1-infected plantar wart. Individual nondenatured sections were hybridized with riboprobes 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 7 (F), and probe 6 (G). The silver grains generated in the film emulsion after exposure to the ^{35}S-labeled probes are seen as white grains under dark-field illumination (top row). Histopathology observed by bright-field microscopy after staining with H & E stain is shown in the lower corresponding pictures. Hybridization of nondenaturated sections with riboprobe 2 in sense orientation served as a negative control (H) and resulted in low background signals.

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 (6–10 days), to allow direct comparison of signals obtained with different probes. Hematoxylin and eosin counterstained sections were evaluated and photographed with a Zeiss Axiophot microscope using a dark-field or bright-field condenser. The background for RNA hybridization was determined using sense riboprobes and by pretreatment of the tissue with DNase-free RNase before hybridization.

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