Transcription of the E6 and E7 Genes of Human Papillomavirus Type 6 in Anogenital Condylomata Is Restricted to Undifferentiated Cell Layers of the Epithelium

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The E6 and E7 genes of human genital papillomaviruses (HPVs) appear to transform cells by different mechanisms. They seem to act synergistically but are not equally important when tested under diverse experimental conditions. We were therefore tempted to investigate the E6- and E7-specific transcription pattern in HPV6-infected condylomas separately, by in situ hybridization. Recent studies have identified three promoters within the E6-E7 region of HPV6 and HPV11 by applying S1, exonuclease VII, and cDNA analyses. On the basis of these data, we cloned subgenomic fragments of HPV6 into plasmid pBS to obtain riboprobes that differentiated between transcripts starting upstream of the E6 and E7 open reading frames, respectively. These different species of mRNAs were analyzed in serial thin sections of eight HPV6-positive anogenital condylomas. The E6 probe (nucleotides 7862 to 241) led to weak signals within the basal layer. In three cases, rather strong signals were confined to a few basal cells. The E7 probe (nucleotides 242 to 534) gave rise to a more pronounced labeling of all cells within the two to three lowest epidermal layers. In situ hybridization with a riboprobe for human *c-fos* revealed an expression pattern similar to that observed with the E7 probe. In contrast to the preferential expression of the transforming E6 and E7 genes in the lower epithelium, the major transcriptional activity of the virus was detected in the middle and upper third by probes colinear with the 3' moiety of the early region.

The oncogenic potential of human papillomaviruses (HPVs) is reflected in vitro by their ability to immortalize keratinocytes and to change their differentiation behavior (17). Genetic experiments identified the E6 and E7 genes of HPV16 as both necessary and sufficient for this activity (9, 11). However, under certain experimental conditions, E6 or E7 was able to immortalize human primary epithelial cells by itself (1, 8). It was recently suggested that genital HPVs induce proliferation, at least in part, through interaction of the viral oncoproteins E6 and E7 with the tumor suppressor proteins p53 and Rb, respectively (7, 12, 23).

The E6 and E7 genes were shown to be retained and transcribed in most cervical carcinomas (24), which may indicate that they contribute to the proliferative potential of the cancer cells. The activity of these genes in benign HPV-induced tumors is not yet clearly elucidated. Northern (RNA) blot studies of HPV16 RNA revealed no differences in the amount of E6 and E7 transcripts in premalignant versus malignant lesions (19). In situ hybridization with HPV6-, HPV11-, and HPV33-infected condylomas showed a differentiation-linked gene expression, however, and it was surprising to note that E6 and E7 transcripts were almost undetectable in the proliferating lower layers (2, 21). The separate expression of E6 and E7 was not assessed because of uncertainties concerning the structure of the gene-specific transcripts.

Recently, two promoters were identified within the E6 open reading frame (ORF) of HPV6 and HPV11 (20). Initiation at promoter P2 (Fig. 1) leads to a transcript with E7 as the first translatable ORF. Transcripts derived from P1 (Fig.

1) could encode both E6 and E7, but reinitiation of translation at the E7 AUG is highly unlikely (10, 14); therefore, this mRNA is probably E6 specific. On the basis of these data, we designed probes for in situ hybridization which specifically detect the E6 and E7 transcript of HPV6. Care was taken to avoid any overlap with the major E1-E4 mRNA class, which initiates around nucleotide (nt) 700 in HPV6 or between nt 674 and 714 in HPV11 (Fig. 1) (4, 20).

MATERIALS AND METHODS

Biological specimens and HPV typing. Specimens from randomly selected patients of different sex and age (Table 1) were snap-frozen immediately after surgery and stored at -70°C until processed. High-molecular-weight DNA was extracted from the frozen tumor tissue. The polymerase chain reaction for HPV classification was performed as described previously (3). Eight condylomas from different sites of the anogenital region (Table 1) that were proven to contain HPV6 DNA were chosen for in situ hybridization analysis. Normal ectocervical mucosa was obtained from a patient with a history of breast cancer and was proven to be HPV negative.

Construction of specific probes. For the synthesis of specific RNA probes, subgenomic restriction fragments (Fig. 1) of HPV6b (6) were cloned into the *Sma*I site of the vector pBS+ (Stratagene) or pGEM1 (Promega). The identity and orientation of each fragment were confirmed by restriction enzyme analysis. The construct to generate antisense riboprobes for the *c-fos* gene is available from Amersham.

Depending on the choice of the promoter, the in vitro-synthesized RNAs were either in the same polarity as mRNA (sense) or in the opposite polarity (antisense). To

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FIG. 1. Genetic and transcriptional map of the early region of HPV6 and localization of the mRNA-specific probes 1 to 4 used in this study. The ORFs (shaded boxes) and the nucleotide positions of the probe boundaries (at the top) correspond to the published sequence (18). Vertical lines within the ORFs represent the first translational start codon. The major transcripts of the early region (a to c) are depicted at the bottom as lines with different thicknesses corresponding to their relative abundances determined in previous investigations (13, 20). The promoters are indicated as P1 to P3, and the major splice acceptor is indicated as SA.

generate antisense probes, the pBS+ -based RNA expression vectors were each linearized with *Hind*III cutting in the multiple cloning site distal from the T3 bacteriophage promoter used for transcription. The HPV6 DNA fragment cloned in pGEM1 to synthesize probe 4 (Fig. 1) spanned nt 2792 to 4029. After cleavage with *Ava*II at nt 3491, the appropriate antisense RNA was transcribed from the T7 promoter. In vitro RNA synthesis in the presence of [35 S]UTP, resulting in probes with a specific activity of 3×10^8 cpm/ μ g, was followed by alkaline hydrolysis to reduce the probe length to approximately 200 nt (5). The quality of the transcribed RNAs was tested by agarose gel electrophoresis. The shortest probe (probe 1, with a probe complexity of 143 nt) was applied at a concentration of 6×10^7 cpm per ml of hybridization mixture (0.15 μ g/ml/kb of probe complexity), and the others were normalized in relation to the probe length.

RNA in situ hybridization. Serial cryostat sections of biopsy material, mounted on aminopropylsilane-coated slides, were fixed in 4% paraformaldehyde in phosphate-buffered saline and dehydrated through graded ethanol. The dry sections were then acetylated in 0.1 M triethanolamine-0.25% acetic anhydride for 10 min, washed in $0.2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and preincubated for 2 h at 45°C covered by solution I (50%

formamide, 0.6 M NaCl, $2.5 \times$ Denhardt's solution, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.1% sodium dodecyl sulfate, 0.15 mg of tRNA per ml). After removal of this prehybridization solution, the tissue sections were incubated for 16 h at 45°C with 35 S-labeled antisense riboprobes in solution I containing 10% dextran sulfate. The incubated slides were washed in 50% formamide- $1 \times$ SSC at 50°C, treated with RNase A (5 μ g/ml), washed for 1 h each in $0.2 \times$ SSC at 50°C and $0.1 \times$ SSC at 60°C, dehydrated, and finally coated with Kodak NTB-2 emulsion. All slides from one specimen were developed after the same exposure time (10 to 14 days) to allow direct comparison of signals obtained with different probes. Hematoxylin-and-eosin-counterstained sections were observed and photographed with a Reichart & Jung Polyvar microscope by usage of a dark- or bright-field condenser. The specific detection of RNA by in situ hybridization was established by using sense riboprobes, which gave only background signals, or by pretreatment of the tissue with DNase-free RNase before hybridization. The intactness of the RNA in the tissue sections could be demonstrated by hybridization with a probe specific for an epithelial cell adhesion molecule (E-cadherin [16]).

RESULTS

To obtain riboprobes specific for different mRNA classes of HPV6, we cloned subgenomic viral DNA fragments into the RNA expression vector pBS+ (Stratagene) or pGEM1 (Promega). Probe 1 is specific for E6 mRNAs starting at promoter P1 (Fig. 1), whereas probe 2 recognizes all mRNAs starting at promoter P2 or upstream of it. The net signal resulting from the E7 mRNA starting at P2 can be obtained by subtracting signals generated by probe 1 from signals generated by probe 2. Probe 3 hybridizes only to mRNA species with the coding capacity for a full-length E2 protein (Fig. 1). Antisense probe 4 is complementary to all known mRNA species of HPV6 except for the L2 mRNA.

With the help of these probes, we analyzed the pattern of HPV6 RNA expression in adjacent serial sections of eight condylomata acuminata from distinct sites of the anogenital region, which were proven by polymerase chain reaction and in situ hybridization to contain HPV6 DNA (data not shown). Each of the four different riboprobes resulted in highly characteristic signal patterns (Fig. 2), which were consistent for all condylomas tested.

Five condylomas hybridized with the E6 probe (probe 1) revealed homogeneously distributed weak signals in the cytoplasm of basal cells (Fig. 3A and B), whereas three other condylomas exhibited only a few heavily labeled cell nuclei within the basal layer (Fig. 2B and 3C). In some cases, weak signals were also discernible in suprabasal cells.

The E7 probe (probe 2) gave rise to a rather uniform cytoplasmic labeling of all cells within the two to three lowest layers of the stratum germinativum (Fig. 4A, C, and E). No signals could be observed in higher layers, with the exception that in one condyloma, labeling extended throughout the lower third (Fig. 2C). The signals in Fig. 2C that seem to appear in the upper third of the epidermis are located in undifferentiated basal cells around a crosswise-cut papilla, as can be seen by comparison with the bright-field histology micrograph (Fig. 2A). When adjacent sections of all condylomas were analyzed for transcription of the *c-fos* gene by hybridization with an antisense probe, the distribution of signals was similar to that observed with the E7 probe (Fig.

TABLE 1. Clinical data

Case no.	Localization of lesion	Patient data		Pretreatment
		Age (yr)	Sex ^a	
1	Genital	2	F	None
2	Vagina	22	F	None
3	Perianal	22	M	Unknown
4	Perianal	31	M	None
5	Vulva	16	F	None
6	Perianal	28	F	None
7	Genital	2	M	None
8	Penis	41	M	None

^a F, female; M, male.

FIG. 2. In situ hybridization analysis of an anal condyloma whose histopathology is shown by light microscopy after hematoxylin-and-eosin staining (A). Adjacent tissue sections were hybridized to HPV6-specific probes 1 (B), 2 (C), 3 (D), and 4 (E) (Fig. 1) and with a human *c-fos* antisense riboprobe (F). 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 (B to F). Black arrowheads indicate the locations of two crosswise-cut papillae.

2F and 4). Investigation of normal HPV DNA-negative ectocervical mucosa revealed an identical *c-fos* expression pattern, whereas the HPV6 E7 probe did not hybridize (data not shown).

Hybridization with the E2 probe yielded signals dispersed through the lower and middle parts of the epithelium, with some strong signals in the nuclei of koilocytes (Fig. 2D and 5A). The major transcriptional activity of the virus was detected with probe 4 (Fig. 1) in the middle and upper third

of the epithelium. The strongest signals were seen in koilocyte clusters with a perinuclear cytoplasmic distribution (Fig. 2E and 5B). As there were no E6- or E7-specific signals detectable in these differentiated areas, this massive label probably represents the highly abundant E1-E4 transcripts, which are initiated downstream from the 3' end of the E7 probe (4), and late transcripts having a short exon from the E4 region (15). Hybridization with probe 4 also led to a signal within the lower third of the epithelium, which was compa-

FIG. 3. In situ hybridization of a genital and two perianal condylomata with the E6-specific riboprobe 1 demonstrated by light microscopy. Shown are details of the basal layers in dark-field (A) and bright-field (B and C) illumination.

able to that obtained with the E7 probe in both localization and signal strength (Fig. 2E). This finding was not unexpected, because E7 mRNAs are known to contain a second exon largely colinear with probe 4 (20). The spatial separation of the E7-specific mRNA (detected by probes 2 and 4) and the E1-E4 mRNA (detected by probe 4 only) becomes particularly obvious in Fig. 6, in which the strong signals generated by probe 4 appeared to be unusually focal.

DISCUSSION

The infectious cycles of all DNA viruses appear to be sequentially organized. Early proteins of papovaviruses and adenoviruses drive the host cell into the S phase to allow viral DNA replication and then the expression of structural proteins. This activity of the early proteins may lead to an oncogenic transformation of abortively infected cells.

In the case of papillomaviruses, the multiplication cycle is particularly complicated because of its strong dependence on the differentiation state of the host cells. It starts with a latent infection of the basal keratinocyte, proceeds with ongoing cell maturation, and ends with the production of virus particles in the uppermost layers of the epithelium. This process is reflected by a cell differentiation-dependent gene expression, which could be demonstrated by morphology-based mRNA detection in genital condylomas (2, 21).

With each of four early-gene-specific probes, we observed a characteristic differentiation-dependent transcription pattern that was consistent in eight condylomas from four distinct sites of the anogenital region. The majority of transcripts of the early region appeared in the middle and upper third of the epithelium. In accordance with previous *in situ* hybridization studies with subgenomic probes (2, 21), the bulk of the signal in this area most likely represents the E1-E4 mRNA, which was identified previously by Northern blot hybridization as the most abundant transcript (13). A minor percentage of the signal in the midstratum seems to be due to mRNA with a coding potential for a full-length E2 protein.

Previous *in situ* hybridization studies with a probe colinear with the growth-promoting E6 and E7 genes surprisingly revealed an expression pattern similar to that of the E1-E4 mRNA, with almost no E6 and E7 transcripts detectable in the mitotically active basal cell layer (2, 21). In contrast, we found E6- and E7-specific mRNAs strictly confined to the lowest layers of the epithelium. These differences are partially due to the fact that the earlier *in situ* studies applied ORF-specific probes, whereas transcript-specific probes were used in this study. The ORF-specific probe for the detection of HPV6 or HPV11 E6 and E7 mRNA (nt 7694 to 811 [21]) contains approximately 100 nt that hybridize with the 5' end of the E1-E4 messages, which were shown to initiate between nt 674 and 714 (Fig. 1, P3 [4, 20]). It will therefore primarily detect the highly abundant E1-E4 mRNA transcripts, which are 50 to 100 times more prevalent than the E6- and E7-specific transcripts in basal cells. Using such an ORF-specific probe for the analysis of our specimens, we observed prominent signals in the upper part of the epithelium (data not shown). They were similar in location to those

FIG. 4. Correlation of HPV6 E7 and *c-fos* expression in two perianal condylomas and one genital condyloma. In situ hybridization was carried out with riboprobe 2 in antisense (A, C, and E) or sense (G) orientation, a riboprobe for human *c-fos* (B, D, and F), and a riboprobe for the epithelial cell-specific adhesion molecule E-cadherin (H). Details of bright-field photographs of the lower strata are shown as insets in panels A, C, and E.

FIG. 5. In situ hybridization of a genital condyloma with riboprobes 3 (A) and 4 (B) as demonstrated by bright-field illumination. Shown are details of the midstratum of the epithelium.

obtained with probe 4 although lower in intensity, as one would expect from the different lengths of the 5' and 3' exons of the E1-E4 mRNA. It is not quite clear why previous studies failed to detect major signals in the lowest layers of the epithelium, which were detected here both with probe 2 (Fig. 2C, 4, and 6) and the E6 and E7 ORF-specific probe mentioned above (data not shown) as well as with probe 4 (Fig. 2E and 6B), which recognizes the second exon of E6 and E7 transcripts (Fig. 1 [4, 22]). This discrepancy could be due to different sensitivities obtained with frozen versus paraffin-embedded material and ^{35}S - versus ^3H -labeled riboprobes. The exclusive expression of the transforming E6 and E7 genes in the basal cell layers is fully in line with their expected role early in the viral life cycle.

The different signal intensities observed after hybridization with either probe 1 or probe 2 point to the existence of two classes of mRNAs from the E6-E7 region. The majority of the transcripts do not contain sequences between nt 7862 and 241 because they are not detectable with probe 1. These RNAs contain E7 as the first translatable ORF and are likely to initiate at nt 270, which was identified as a major cap site of mRNAs from HPV6-infected condylomas (20). The transcripts hybridizing to probe 1, which was designed to detect E6-specific mRNAs, were much less abundant. The relative signal intensities obtained with probes 1 and 2 correspond to the amounts of mRNAs initiated at promoters P1 and P2, respectively (20).

A relatively sharp borderline above which no E7-specific

FIG. 6. In situ hybridization of an anal condyloma with riboprobe 4. The same section is shown in light-field (A) and dark-field (B) illumination. The result of hybridization with riboprobe 2 is visualized by dark-field optics (C).

transcripts could be observed was a consistent feature of all condylomas. That line also formed the boundary of the area showing *c-fos* expression. This coincidence does not reflect an influence of E7 on the transcription of *c-fos*, because the *c-fos* expression pattern did not differ between condylomatous and HPV-negative, normal mucosa. However, one might speculate that only *c-fos*-expressing cells provide the conditions allowing transcription from the E7 promoter. The lack of E6 and E7 expression in the more differentiated cells of the condylomas points to a very effective control of the activity of promoters P1 and P2 (Fig. 1). Synthesis of the E6 mRNA seems to be even more restricted than that of the E7 mRNA. Previous studies noted that the total amounts of E6 and E7 mRNAs from HPV-infected condylomas differ by 1 order of magnitude (20). Our data provide the additional information that this difference is due not only to a lower abundance of E6 mRNA per cell but also to an expression strictly confined to undifferentiated cells of the basal layer. This highly restricted synthesis of the E6 mRNA indicates that the E6 protein acts very early during the multiplication cycle of the virus.

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