Differential Regulation of Human Papillomavirus Type 6 and 11 Early Promoters in Cultured Cells Derived from Laryngeal Papillomas

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Cells cultured from laryngeal papillomas contain episomal human papillomavirus type 6 or type 11 (HPV-6/11) DNA. We developed a sensitive RNase protection assay to simultaneously measure expression from the HPV E6, E7, and E1 promoters (P1, P2, and P3, respectively) in this manipulable culture system and found that P1, P2, and P3 transcript abundances could be independently modulated by culture medium composition and culture substrate. In undifferentiated cells grown in a low-calcium, serum-free medium, P1 transcripts commonly predominated over those from P2, P3 transcripts were often undetectable, and high concentrations of retinoic acid were able to selectively decrease P2 transcript abundance. When cultures were allowed to stratify and differentiate by growth on a collagen gel at the air-liquid interface, total HPV RNA increased up to sixfold because of selective increases in abundances of P1 and P3 transcripts. High-calcium submerged cultures also showed easily detectable P3 transcripts, and isolated suprabasal cells contained almost exclusively these transcripts. Growth arrest alone was not sufficient to induce P3 transcripts. Thus, in contrast to the HPV-6/11 E6 and E7 promoters, the E1 promoter was utilized primarily in a differentiation-specific manner. We also show that increased HPV gene dosage will not necessarily bring about increased HPV transcript abundance, suggesting that other viral and cellular factors are responsible for regulation of total transcript levels as well as specific promoter usage.

The closely related human papillomaviruses type 6 and type 11 (HPV-6/11) are the etiologic agents of anogenital condylomas (8, 14) and laryngeal (15, 25) and conjunctival (23) papillomas. Histologically, these tumors consist of well-vascularized connective tissue cores covered by a hyperplastic stratified squamous epithelium. Viral genomes, consisting of approximately 7,900 bp of double-stranded DNA, are present in the epithelial cells as covalently closed circular episomes. In situ hybridization analysis of genital condylomas showed that both viral DNA and total viral RNA increase in amount in the upper, more-differentiated layers of the epithelium (34). Viral transcription and subsequent synthesis of viral early proteins are required for maintenance of both the viral DNA and the disease phenotype. For example, HPV E1 and E2 are required for viral DNA replication (3), and the E2 proteins also regulate viral transcription (2, 11, 13). HPV E5 exhibits transforming activity (1). E6 can transactivate transcription and form a complex with the tumor suppressor protein p53 (7), while E7 can complex with the tumor suppressor Rb (26).

HPV transcripts encoding these viral proteins are made from only one of the viral DNA strands. They arise from several different promoters and overlap, and the primary transcripts can undergo alternative splicing, sometimes giving rise to polycistronic messages (2, 6, 30–32). Electron microscopic R-loop mapping showed that HPV-6/11 early transcripts can initiate at a promoter (P1) at the 5' end of the E6 open reading frame (ORF) and at a promoter (P3) just upstream of the E1 ORF (6). S1 nuclease and primer extension analyses identified an additional promoter (P2) near the middle of E6 (32). Tran-

scripts initiating from P1 have E6 as the first ORF, while E7 is the first translatable ORF in P2 transcripts. The major early transcript in HPV-6/11-induced lesions initiates at P3 and encodes an E1⁺E4 fusion protein (6, 27). Among the other transcripts initiating at P3 is an unspliced E1 message (6).

In an attempt to understand the regulation of these different promoters, in situ hybridization analysis was previously performed with anogenital condylomas (18, 36). It is clear from these studies that the E1⁺E4 transcript, initiating from P3, is most abundant in the more-differentiated layers of the epithelium. However, the distribution of P1 and P2 transcripts, encoding E6 and E7, respectively, remains controversial. In vitro transfection studies using reporter constructs containing HPV regulatory sequences and the E6 promoter have yielded detailed information about the regulation of this promoter (2, 11, 13); however, whether similar regulation operates on the intact viral genome during a natural infection cannot be known for certain. Studies similar to those used to investigate P1 regulation have not been reported for P2 and P3.

Given the importance of viral transcription and synthesis of viral proteins in the pathogenesis of HPV-induced tumors, we have chosen to study the factors controlling the abundance of transcripts initiating from the promoters P1, P2, and P3 using cultured cells derived from laryngeal papillomas. We have developed a sensitive RNase protection assay which allows the abundances of transcripts initiating from these three early promoters to be determined simultaneously from a single RNA sample. Although we have previously reported that cells cultured from laryngeal papillomas contain replicating HPV-6 or HPV-11 episomes (9), this is the first detailed report describing the viral transcription that occurs in these cells. Using the RNase protection assay and this manipulable cell culture system, we have determined that P1, P2, and P3 transcript

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abundances can be independently regulated by modulation of culture medium composition and culture substrate. Taking advantage of the fact that cultures derived from different papilloma biopsy specimens have different average HPV DNA copy numbers (9), we have also discovered that HPV DNA amount and HPV RNA amount are not strongly correlated, suggesting that HPV transcript abundance is not controlled by HPV gene dosage alone. In addition, we have used RNase protection to determine P1, P2, and P3 transcript abundances in samples of laryngeal papillomas from patients so that in vivo and in vitro transcription patterns could be compared, and we have independently confirmed the existence of the P2 promoter which was first described by Smotkin and coworkers as being active in HPV-6/11-containing genital condylomas (32).

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MATERIALS AND METHODS

Cell culture media. F12 medium consisted of Ham's F12 supplemented with 10 μ g of hydrocortisone per ml, 10% fetal bovine serum or Fetal Clone II (HyClone), 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. Low-calcium, serum-free medium contained Keratinocyte Basal Medium (Clonetics) supplemented with 0.15 mM calcium, 1 ng of epidermal growth factor per ml, 5 μ g of insulin per ml, 0.5 μ g of hydrocortisone per ml, 2 μ g of transferrin per ml, 11 μ g of bovine pituitary extract (Clonetics) per ml, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, and 10^{-9} M all-trans retinoic acid (unless otherwise stated). High-calcium, serum-free medium had an identical formulation except that it contained 1 mM calcium.

Cell culture methods. Laryngeal papilloma biopsy specimens were obtained from patients with recurrent laryngeal papillomatosis whose lesions were known to contain HPV-6 or HPV-11 DNA. Primary explant cultures were initiated essentially as described previously (33). Briefly, specimens were minced and embedded in collagen gels containing 1.5 mg of type I collagen per ml in F12 medium. After 2 weeks, cells were harvested from the explant cultures with collagenase and seeded in serum-free medium (see above) in submerged or raft cultures.

For submerged cultures, cells were seeded onto tissue culture-treated plasticware at a density of 1×10^4 to 5×10^4 cells per cm^2 . DNA and RNA isolations were performed 3 to 8 days after seeding, usually before the cultures had attained confluence. Raft cultures, in which cells are grown on a fibroblast-containing collagen gel, were prepared essentially as described previously (24). For these cultures, the laryngeal papilloma cells were seeded and maintained in high-calcium, serum-free medium.

RNA isolation. For most experiments, total RNA was isolated from cells by using TriReagent (Molecular Research Center) (5). For isolation of total RNA from laryngeal papilloma tissues, biopsy specimens were frozen in liquid nitrogen and disrupted by vibration with a Braun Micro-Dismembrator II, and RNA was isolated by the guanidinium-cesium chloride method of Chirgwin et al. (4). In our early experiments, we isolated mRNA, rather than total RNA, using the Quick Prep mRNA Purification Kit (Pharmacia). Prior to slot blot or RNase protection analysis, RNA samples were treated with RNase-free DNase I to eliminate any contaminating DNA.

In vitro transcription. HPV-6 sequences from nucleotides (nt) 37 to 846 and HPV-11 sequences from nt 37 to 837 were amplified by PCR and cloned into the *Sma*I site of pGEM-4Z (Promega). Radiolabelled antisense RNA probes were synthesized from these templates by using the Ambion MAXIScript *in vitro* Transcription Kit. Transcription was performed in Ambion's Transcription Buffer in a 20- μ l reaction mixture containing 1 μ g of *Eco*RI-digested template DNA, 0.2 μ mol of dithiothreitol, 10 nmol of ATP, 10 nmol of GTP, 10 nmol of CTP, 154 pmol of unlabelled UTP, 62.5 pmol of [α - 32 P]UTP (800 Ci/mmol), 40 U of RNase inhibitor, and 10 U of T7 RNA polymerase. Transcription was carried out for 1 h at 37°C, and the resulting RNA probe had a specific activity of 3×10^8 to 4×10^8 cpm/ μ g. Template DNA was digested with RNase-free DNase I. The probe was separated from residual template DNA and prematurely terminated transcripts by using a 5% acrylamide-8 M urea gel and was eluted from the gel by incubation in 0.5 M ammonium acetate-1 mM EDTA-0.2% sodium dodecyl sulfate (SDS) at 37°C for 2 h. Probes were used in the RNase protection assay immediately after elution.

Unlabelled sense transcripts (used as controls in the RNase protection assays) were also synthesized as described above, except that templates were linearized with *Bam*HI, 10 nmol of unlabelled UTP was used, the [α - 32 P]UTP was omitted, and SP6 RNA polymerase was used instead of T7.

RNase protection assay. RNase protection assays were performed with the aid of Boehringer Mannheim's RNase Protection Kit. A 2-fmol amount of an HPV-6 or HPV-11 antisense RNA probe was coprecipitated with each RNA sample.

Precipitates were resuspended in 40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 6.4)-400 mM NaCl-1 mM EDTA-80% formamide and heated to 95°C for 5 min, and hybridization was performed overnight at 45°C. Usually, 3 to 8 μ g of total cellular RNA or 1 μ g of tissue RNA was used for each hybridization reaction, and the total amount of RNA in each reaction mixture was brought to 10 μ g with yeast RNA. When two or more samples were to be directly compared, equal amounts of RNA were used for the mixtures. Two control reaction mixtures contained 10 μ g of yeast RNA alone, and one contained 10 μ g of yeast RNA combined with 45 pg of HPV-6 or HPV-11 unlabelled sense transcript. The next day, 12 volumes of RNase digestion buffer (10 mM Tris [pH 7.5], 5 mM EDTA, 300 mM sodium acetate) were added to each reaction mixture and digestion was performed by incubation with 0.07 U of RNase T₁ per μ l at 30°C for 30 min. (RNase A was not used because it resulted in degradation of hybrids.) One of the yeast RNA reaction mixtures was not treated with RNase to confirm that the probe remained intact in the absence of deliberate RNase T₁ digestion. Protected fragments were purified by proteinase K digestion, phenol extraction, and ethanol precipitation with yeast tRNA as the carrier. Protected fragments were separated on 5% acrylamide-8 M urea gels. The entire reaction mixture was loaded on the gel, except for the sense transcript and the undigested yeast RNA reaction mixtures, of which only a small portion was used. Molecular weight markers were end-labelled *Sau*3A fragments of pUC19. Autoradiograms were prepared by using intensifying screens and Kodak X-Omat AR film. Exposures were done at -70°C, and film was usually preflashed by using a Sensitize preflash unit (Amersham) so that small amounts of radioactivity would not be underrepresented on the autoradiogram.

Removal of suprabasal cells from cultures. Cells were seeded in the high-calcium, serum-free medium already described. When cultures appeared confluent and multilayered, they were incubated for 1 day in a calcium-free medium consisting of calcium-free minimum essential medium, nonessential amino acids, and 13% Chelex 100-treated fetal bovine serum. After 1 day, many of the suprabasal cells had been shed into the medium and were used for RNA analysis. RNA was also prepared from the cells that were still attached to the culture dish, which were a mixture of involucrin-positive and involucrin-negative cells (data not shown).

Determination of BrdU labelling index. Laryngeal papilloma cells were seeded onto glass coverslips in low-calcium, serum-free medium, and bromodeoxyuridine (BrdU) incorporation was measured 2 and 21 days after seeding. Cultures were incubated for 24 h with 3 μ g of BrdU per ml, and cells that incorporated BrdU were detected with an anti-BrdU antibody (Becton Dickinson) and an immunoperoxidase detection system (Vector).

Determination of average HPV DNA copy number. Cultured cells were lysed in 0.3 M sodium acetate (pH 7.5)-5 mM EDTA-0.5% SDS. Proteins were removed with phenol, and DNA was precipitated with ethanol. Contaminating RNA was removed by digestion with RNase A. DNA samples (usually 0.1 to 1 μ g) were slot blotted onto GeneScreen Plus membrane as recommended by the manufacturer, along with standards consisting of known quantities of HPV-6 or HPV-11 DNA (depending on whether the cell samples to be analyzed contained HPV-6 or -11 DNA) and standards consisting of known quantities of human DNA. Blots were first probed with cloned HPV-6 or -11 DNA gel purified from vector sequences and labelled with [α - 32 P]dCTP by random priming. After stringent washing (at 22°C below melting temperature), HPV signals on the resulting autoradiograms were quantified by densitometry. Comparison with HPV standards allowed the quantity of HPV DNA in each sample to be determined. The HPV probe was removed by boiling the blot in 10 mM Tris (pH 8)-1 mM EDTA-1% SDS. Blots were reprobed with a 32 P-labelled human β -actin cDNA (16). Autoradiograms were quantified, and comparison with human DNA standards allowed the amount of cellular DNA in each sample to be determined. Average HPV DNA copy numbers for each culture could then be calculated (e.g., 1.5 pg of HPV DNA per μ g of cellular DNA equals one copy of HPV DNA per cell). Larynx is a nonkeratinizing epithelium, with only minimal DNA degradation in terminally differentiated cells; therefore, we would expect little apoptotic degradation in our cultures.

Quantitation of total HPV RNA. RNA samples (1 to 4 μ g) were slot blotted onto GeneScreen Plus membrane as recommended by the manufacturer, along with standards consisting of known quantities of HPV-6 or HPV-11 DNA and known quantities of a 196-bp *Pst*I fragment of the 36B4 cDNA (22). (36B4 is a housekeeping gene encoding the acidic ribosomal phosphoprotein P0 [21], a protein whose transcript abundance has been shown to be unchanged by a variety of in vitro manipulations [20, 39].) The amount of HPV RNA in each sample was determined in a manner identical to that described above for the quantitation of HPV DNA. Blots were then stripped and reprobed with a 32 P-labelled fragment of 36B4. As we had previously determined that a signal of 0.6 pg of 36B4 corresponds to 1 μ g of total RNA, the total picograms of HPV RNA per microgram of total cell RNA for each culture could be calculated from these measurements.

RESULTS

RNase protection analysis of HPV transcripts in laryngeal papillomas. Before investigating HPV transcription in cell cultures derived from laryngeal papillomas, we studied HPV-6/11

FIG. 1. (A) Schematic explanation of the RNase protection assay. The upper part shows the ORFs and genome organization of HPV-6/11. In the lower part, sample transcripts initiating at the three HPV early promoters P1, P2, and P3 are shown, along with the probe used in the RNase protection assay and the approximate sizes of the probe fragments protected by each class of transcript. The transcripts are shown unspliced, because alternate splicing may occur within each class. The probe used was 5' to all previously described splice donor sites. (B and C) P1, P2, and P3 transcripts in laryngeal papillomas. RNase protection analysis using the antisense HPV-6 (B) or HPV-11 (C) probe was performed with RNA from laryngeal papillomas (Pap), yeast RNA (Ye), or the in vitro-synthesized HPV-6 (B) or HPV-11 (C) sense transcript (Sen). The full-length probe can be seen in the yeast RNA reaction mixture that was not digested with RNase (Ye, -RNase). Numbers to the right indicate the positions of the size markers (M). Fragments protected by P1, P2, and P3 transcripts are indicated on the left.

transcription by RNase protection using RNA extracted directly from laryngeal papilloma tissues. With an antisense probe from nt 37 to 846 of HPV-6 or nt 37 to 837 of HPV-11, transcripts initiating at the E6, E7, and E1 promoters (P1, P2, and P3, respectively) can easily be distinguished, because they will protect different-sized fragments of the radiolabelled probe. This is explained diagrammatically in Fig. 1A. The probe used was 5' to all previously described splice donor sites.

The results obtained with two different laryngeal papillomas, one containing HPV-6 and one containing HPV-11, are shown in Fig. 1B and C. In both, P1 transcripts are represented by the band of approximately 750 nt, P2 transcripts are represented by the doublet of 580 nt, and P3 transcripts by the set of bands ranging in size from 111 to 160 nt. HPV-6/11 P1 transcripts in genital condylomas have been shown previously to initiate at approximately nt 90 (32). Our results with laryngeal papillomas agree with this earlier work. HPV-6 P2 transcripts in a genital condyloma were previously shown, by S1 nuclease analysis, to have a major start site at nt 270 and two minor start sites at nt 238 and nt 260 (32). P2 transcripts appear as a doublet in Fig. 1, indicating that HPV-6 and HPV-11 P2 transcripts in laryngeal papillomas can have at least two different 5' ends. Others have reported that HPV-6/11 P3 transcripts have heterogeneous start sites in laryngeal papillomas (38) and in genital condylomas (32). For the HPV-6-containing papilloma, the protected fragments of 111 to 160 nt correspond to transcripts having 5' ends at nt 686 to 735. (Because the HPV-11 probe was 9 nt shorter than the HPV-6 probe, the protected fragments of this size in the HPV-11-containing papilloma correspond to transcripts having 5' ends at nt 677 to 726.)

Note that in the RNase protection assay, we included a control hybridization reaction in which the RNA sample was an in vitro-synthesized sense transcript that protected all of the HPV sequences in the radiolabelled probe. This control confirmed that the P2 and P3 protected fragments were not artifacts resulting from partial degradation of the larger hybrid, as only one large protected fragment was observed in this control reaction.

The antisense RNA probes used in the RNase protection assay were synthesized in the presence of [α - 32 P]UTP and were uniformly labelled along their length. The signals from each protected fragment can be used to determine the relative abundances of HPV transcripts initiating from P1, P2, and P3, provided that the signals are corrected for size. If, for example, P1 and P3 signals were to appear equal, this would indicate that there were five times more P3 transcripts than P1 transcripts, because fragments protected by P1 transcripts are about five times longer than those protected by P3 transcripts. Densitometric analysis showed P1/P2/P3 ratios of 1:2.5:5.5 (Fig. 1B) and 1:1.7:31 (Fig. 1C). Clearly, in these two papillomas HPV-6/11 P3 transcripts were the most abundant and P2 transcripts were more abundant than P1 transcripts.

RNase protection analysis of cells cultured from laryngeal papillomas. We next used our RNase protection assay to measure the abundances of P1, P2, and P3 transcripts in cells cultured from laryngeal papillomas. This analysis was first performed with cells cultured in a low-calcium, serum-free medium, conditions which minimize differentiation and stratification. Representative results are shown in Fig. 2. In the two low-calcium cultures shown, HPV transcripts initiating at P1 and P2 were detected but transcripts initiating at P3 were not. In these cultures and in nearly all of the low-calcium cultures examined in this way, P1 transcript abundance appeared greater than or equal to P2 transcript abundance. P3 transcripts were always the least abundant and were sometimes undetectable even on very long exposures.

In situ hybridization analysis of genital condylomas showed that the total amount of HPV RNA is greatest in the upper, more-differentiated layers of lesions (34). We asked whether changing culture conditions to favor stratification and differentiation would increase the amount of viral RNA present in cells derived from laryngeal papillomas. We have shown previously that when these cells are cultured on fibroblast-containing collagen gels at the air-liquid interface (raft cultures),

FIG. 2. P1 and P2 transcripts in low-calcium cultures. RNase protection analysis with the antisense HPV-6 probe was performed with RNA from low-calcium cultures derived from different laryngeal papillomas (Low), yeast RNA (Ye), or the in vitro-synthesized HPV-6 sense transcript (Sen). Note that a small amount of undigested probe was sometimes observed with yeast RNA alone (Ye). Numbers to the right indicate the positions of size markers. Fragments protected by P1 and P2 transcripts are indicated on the left. P3 transcripts were not observed, even on a much longer exposure. Ye, -RNase, no RNase digestion.

the cultures stratify and the suprabasal cells stain positive for involucrin, a marker of squamous differentiation (29). Using RNA slot blot analysis, we compared the amounts of HPV RNA present in low-calcium submerged cultures and raft cultures (Fig. 3A and B). Total HPV RNA increased up to sixfold in raft cultures in comparison with low-calcium submerged cultures. (Note that for each of the comparisons shown in Fig. 3, the submerged and raft cultures were both derived from the same papilloma biopsy specimen.) RNase protection analysis showed that P1 and P3 transcripts both increased in raft cultures while P2 transcripts remained unchanged (Fig. 3C). This suggests that the HPV-6/11 E6 and E7 promoters (P1 and P2, respectively) can be independently regulated. In contrast to the situation with low-calcium submerged cultures, P3 transcripts (initiating from the E1 promoter) were detectable in all raft cultures examined by RNase protection, with P3 transcripts often being predominant or the second most abundant transcripts. (Relative abundances of P1 and P3 transcripts in submerged cultures versus raft cultures were not quantified in these experiments, since P3 transcripts could not be accurately measured in low-calcium submerged cultures.)

High-calcium submerged cultures of laryngeal papilloma cells show stratification and differentiation that is intermediate between that seen in low-calcium cultures and raft cultures. Because submerged cultures are more amenable to certain types of analyses than are raft cultures, we next examined HPV transcript abundance in high-calcium submerged cultures using the RNase protection assay. A representative example is shown in the first lane of Fig. 4A, where it can be seen that significant amounts of P3 transcripts were detected in this culture, as they were in most of the high-calcium cultures studied. When low- and high-calcium submerged cultures derived from a single laryngeal papilloma were compared, P3 transcript abundance was found to be increased in high-cal-

FIG. 3. Effect of differentiation on HPV transcript abundance. (A) Slot blot analysis of RNA from cells derived from different laryngeal papillomas (specimens 1, 2, and 3) and cultured in low-calcium medium (L) or on rafts (R). The left column shows hybridization with an HPV probe. The right column shows the same slots rehybridized with a 36B4 probe after removal of the HPV probe. (B) Graphic representation of the results in panel A. Signals were read by densitometry and compared with standards to calculate absolute HPV RNA amounts. (C) Cells derived from the same laryngeal papilloma were cultured in low-calcium medium or on a raft. RNase protection analysis with the antisense HPV-6 probe was performed by using RNA from the low-calcium culture (Low), RNA from the raft culture (Raft), yeast RNA (Ye), or the in vitro-synthesized HPV-6 sense transcript (Sen). Ye, -RNase, no RNase digestion. Numbers to the right indicate the positions of size markers. Fragments protected by P1, P2, and P3 transcripts are marked on the left. The lower portion shows a 14-day exposure, while the upper portion shows a 1-day exposure.

FIG. 4. (A) HPV transcripts in high-calcium submerged culture and isolated suprabasal cells. Cells derived from a laryngeal papilloma were grown in high-calcium submerged culture and then transiently treated with calcium-free medium to remove some of the suprabasal cells. RNase protection analysis with the antisense HPV-6 probe was done with RNA from isolated suprabasal cells (Supra), RNA from the remaining attached cells (Att), RNA from an untreated high-calcium culture derived from a different laryngeal papilloma (High), yeast RNA (Ye), and the in vitro-synthesized HPV-6 sense transcript (Sen). Ye, -RNase, no RNase digestion. Numbers to the right indicate the positions of size markers. Fragments protected by P1, P2, and P3 transcripts are marked on the left. In all cases, a uniform exposure was used for the entire lane. (B) HPV transcripts in subconfluent and confluent low-calcium cultures. Cells derived from a single laryngeal papilloma were seeded in duplicate low-calcium cultures, and RNA was isolated before and after confluence was reached. RNase protection analysis with the HPV-6 probe was performed with RNA from a subconfluent culture (Subcf) and a confluent culture (Cf). The subconfluent and confluent cultures had BrdU labelling indices of 42 and 7%, respectively. Fragments protected by P1 and P2 transcripts are marked on the left. A very long exposure revealed low but equivalent levels of P3 transcripts in the two cultures (data not shown). Controls were as described for panel A.

cium cultures while the abundances of P1 and P2 transcripts were not significantly changed (data not shown).

In order to measure the HPV transcripts present in the upper, differentiated layer of a high-calcium culture, we incubated such a culture transiently in a calcium-free medium. Under these conditions, some of the suprabasal cells (approximately one-third of the total cells) detach from the rest of the culture, so a population consisting only of differentiated suprabasal cells can be obtained (19, 28). When this suprabasal cell population was analyzed by RNase protection, P3 transcripts were found to be the most abundant, P1 transcripts were detectable but far less abundant, and P2 transcripts were not visible (Fig. 4A, second lane). In contrast, the remaining attached cells contained easily detectable transcripts from all three promoters (Fig. 4A, third lane). The lower abundance of HPV RNA relative to cellular RNA in the suprabasal cells could reflect RNA degradation in these terminally differentiated cells. However, we have no reason to believe that these HPV transcripts, which have identical 3' ends and use the same polyadenylation signal, would show differential stability.

The suprabasal cells described above, in which P3 transcripts predominated, not only were differentiated but also were non-proliferating. We therefore asked whether low-calcium cultures of laryngeal papilloma cells would be induced to synthesize P3 transcripts in abundance if they were growth arrested

by confluence. RNase protection analysis was used to compare HPV transcript abundances in subconfluent and confluent cultures of cells derived from the same laryngeal papilloma biopsy specimen. As shown in Fig. 4B, abundant P3 transcripts were not induced in the confluent culture. A very long exposure showed extremely low but equivalent levels of P3 transcripts in the subconfluent and confluent cultures (data not shown).

Although the number of cells that can be obtained from primary explant cultures of laryngeal papillomas is somewhat limited (ranging from 1×10^6 to 5×10^6 cells), the sensitivity of our RNase protection assay allows useful data to be obtained from as few as 10^5 cells. Therefore, cells cultured from a single laryngeal papilloma biopsy specimen can be grown under different culture conditions and the effects on HPV transcript abundance can be monitored by this assay. We previously reported that the treatment of laryngeal papilloma cell cultures with increasing concentrations of retinoic acid resulted in decreased HPV-6/11 DNA copy number (29). Using RNase protection to study the effects of retinoic acid on the abundances of P1, P2, and P3 transcripts, we found that increasing concentrations of retinoic acid resulted in a decrease in the abundance of P2 transcripts while P1 transcript abundance was less affected (Fig. 5). Again, this illustrates differential regulation of P1 and P2 transcript abundances.

HPV DNA and HPV RNA amounts in cells cultured from laryngeal papillomas. As mentioned earlier, the upper layers of genital condylomas show increased amounts of both HPV DNA and HPV RNA. We have previously reported that cell cultures derived from laryngeal papillomas can have average HPV DNA copy numbers ranging from less than 1 to greater than 50 HPV episomes per cell (9). Taking advantage of this intrinsic difference, we performed DNA and RNA slot blot

FIG. 5. Effect of high concentrations of retinoic acid on P2 transcript abundance. Cells derived from a single laryngeal papilloma were cultured in low-calcium medium containing 1 nM, 0.01 μ M, 0.1 μ M, or 1 μ M all-trans retinoic acid. RNase protection analysis with the HPV-6 probe was performed with RNA from each culture as indicated. Numbers to the right indicate the positions of size markers. Fragments protected by P1 and P2 transcripts are marked on the left. Controls were as described in the legend for Fig. 4.

FIG. 6. HPV DNA and RNA amounts in laryngeal papilloma cell cultures. Cells derived from 14 different laryngeal papillomas were cultured in low-calcium or high-calcium submerged cultures or on rafts. HPV DNA and RNA slot blot analyses were performed on each culture as detailed in Materials and Methods. (The number of points exceeds 14 because in some cases, cells from a single papilloma were used for more than one culture condition.)

analyses of several different cultures, including the matched sets shown in Fig. 3B, to determine whether the total HPV RNA amount would also vary from culture to culture and whether the total HPV RNA amount would correlate with HPV DNA copy number. The HPV RNA amount did vary from 10 to 170 fg of HPV RNA per μ g of cell RNA. However, as shown in Fig. 6, we did not find a clear relationship between HPV DNA and HPV RNA amounts. This suggests that increased HPV gene dosage is not sufficient to bring about increased HPV transcription and that other cellular and viral factors must regulate total transcript levels as well as specific promoter usage. This is consistent with our analysis of HPV RNA increases in raft cultures (Fig. 3B), since only experiment 3 showed a slight increase in HPV DNA copy number for raft cultures.

DISCUSSION

The goal of this work was to begin to understand the regulation of HPV-6/11 gene expression. We developed an RNase protection assay to simultaneously measure expression from the E6, E7, and E1 promoters (P1, P2, and P3, respectively) in a manipulable system consisting of cultured cells derived from laryngeal papillomas and found that the abundances of transcripts initiating from these three promoters can be modulated differentially.

HPV transcription in respiratory papillomas has been studied previously by Northern (RNA) blot analysis (10, 37), and S1 nuclease and primer extension analyses have been used to map the predominant transcript initiating at P3 and encoding E1⁺E4 (38). However, as the simultaneous measurement of P1, P2, and P3 transcript abundances has not been performed with laryngeal papillomas, we chose this as our starting point so that *in vivo* and *in vitro* HPV-6/11 transcription patterns could later be compared. *In vivo*, P3 transcripts were the most abundant while P2 transcripts were two- to three-fold more abundant than P1 transcripts. When Smotkin et al. used S1 nuclease analysis to measure HPV transcripts in genital condylomas, they also found that P3 transcripts were the most abundant and P1 transcripts were the least abundant (32). Interestingly, however, their data indicate that P2 transcripts are at least 10-fold more abundant than P1 transcripts in genital lesions, while we observed only a two- to three-fold difference in laryngeal le-

sions. Although these results suggest differential transcriptional regulation in the two body sites, this would need to be confirmed by studying more lesions from both sites.

Regulation of the HPV-6/11 E6 promoter (P1) has been extensively studied through use of reporter constructs in *in vitro* transfection assays (2, 11, 13). These studies have highlighted the importance of the viral upstream regulatory region, host transcription factors (such as Sp1), and viral E2 proteins (including full-length E2, E2-C, and E1M⁺E2C) in the regulation of the E6 promoter. However, attempts to localize E6 transcripts by *in situ* hybridization in genital condylomas have yielded conflicting results. In one study, E6 transcripts were localized primarily to basal cells, with occasional weak signals observed in suprabasal cells of some lesions (18). In contrast, an earlier study detected E6 transcripts exclusively in suprabasal cells (36). We found that E6 transcripts were easily detectable in undifferentiated basal cell-like cells grown in low-calcium cultures (where differentiation and stratification are minimized), that proliferation did not alter P1 transcript levels, and that high-calcium submerged cultures did not show increased E6 expression. Rather, stripped suprabasal cells from high-calcium cultures contained far fewer E6 transcripts than did the attached cells, suggesting that the most-differentiated cells contain fewer E6 transcripts. It should be noted, however, that when cells cultured from a single lesion were grown either under low-calcium conditions or in a raft culture (where stratification and differentiation are maximized), E6 transcripts were reproducibly increased in the raft culture. We cannot know if this reflects increased E6 expression in the basal cells of the raft culture compared with the basal cell-like cells in the low-calcium culture, increased E6 expression in the more-differentiated cells of the raft culture (as was observed by Stoler et al. for genital condylomas [36]), or a combination of the two. Despite this uncertainty, our studies of submerged cultures clearly demonstrate that the P1 promoter is active in basal cell-like cells.

Although E6 promoter regulation has been studied in detail *in vitro*, no such studies have been reported for the E7 promoter (P2). The splice sites and ORFs of P2 transcripts have not been determined either. However, Chow et al. (6), in their legend to Fig. 1, state that "additional molecules" of an HPV-11 P1 transcript, later found to be spliced from nt 847 to 2622, "appeared to have their 5' ends shortened to variable extent." We think it may be possible that these molecules were actually P2 transcripts. *In situ* hybridization analysis of genital condylomas has yielded confusing results concerning the localization of P2 transcripts. Iftner et al. found them in the two to three lowest layers (18), while Stoler et al. found them only in suprabasal cells (36). Although P2 transcripts were more abundant than P1 transcripts in laryngeal papillomas, this was generally not the case in cells cultured from these lesions, regardless of the culture conditions employed. Occasionally, P2 transcripts were observed to exceed P1 transcripts in a low-calcium culture (for example, Fig. 3C). More commonly, P1 transcripts were 2- to 10-fold more abundant than P2 transcripts in low-calcium cultures. We cannot, as yet, explain this variation, but we do know that different proliferation rates cannot explain the different P1/P2 ratios observed, nor can different HPV DNA copy numbers (data not shown). Growth of cells in high-calcium submerged cultures or on rafts did not increase P2 transcript abundance, suggesting that this promoter is not upregulated by squamous differentiation under our culture conditions.

In the course of our work, we found conditions under which P1 and P2 were differentially regulated. P1 transcript abundance was increased in raft cultures in comparison with the

abundance in low-calcium cultures, while P2 transcript abundance was unaltered. High concentrations of retinoic acid dramatically decreased P2 transcript abundance, with much smaller effects on P1 transcript abundance. In addition, we have preliminary evidence to suggest that serum may also diminish P2 transcript levels, but not those of P1 or P3 (data not shown).

Transcripts initiating at promoter P3 include the transcript that is the predominant RNA in lesions, encoding an E1⁺E4 fusion protein, as well as the much more rare unspliced E1 message (6, 27). In situ hybridization analysis of genital condylomas, and explant cultures of them, has clearly shown that the abundance of the HPV 11 E1⁺E4 transcript increases in the upper, nonproliferating, differentiated cells (12, 18, 36), and abundance of the E1⁺E4 transcript was substantially increased when cells containing episomal HPV-31b were allowed to differentiate on rafts (17). In our low-calcium cultures, P3 transcripts were least abundant and were sometimes undetectable even on long exposures. Growth arrest did not induce P3 transcripts in low-calcium cultures. However, P3 transcripts increased in raft cultures compared with levels in low-calcium cultures, some high-calcium submerged cultures showed easily detectable levels of P3 transcripts, and P3 transcripts predominated in isolated suprabasal cells. Taken together, these results indicate that the P3 promoter is utilized in a differentiation-specific manner in vitro and in vivo. This is in contrast to the situation with the HPV-6/11 E6 and E7 promoters, which do not appear to be differentiation dependent in vitro.

Finally, in situ hybridization analysis of genital condylomas shows that HPV DNA and HPV RNA both dramatically increase in abundance in the upper cell layers (34). It has been hypothesized that the increase in HPV transcription is due, in part, to increased HPV gene dosage (35). To test this hypothesis in vitro, we measured HPV DNA and HPV RNA abundances in cultures of laryngeal papilloma cells derived from many different lesions. There was no clear relationship between HPV DNA and total HPV RNA amounts, indicating that increased gene dosage alone is not sufficient to increase total HPV transcription in vitro and may not be sufficient in vivo.

In conclusion, we have shown that RNase protection can be used to study HPV early promoter use in vivo and in vitro. At the present time, our culture systems permit only partial recapitulation of in vivo differentiation and HPV gene expression. Our work does demonstrate that cells cultured from HPV-6/11-induced lesions support HPV transcription and can be used to better understand HPV gene expression. The promoters P1, P2, and P3 clearly are differentially regulated, suggesting that it is important for the viral life cycle that there be independent regulation of the levels of E6, E7, and E1⁺E4, and perhaps the other viral proteins as well. It should be noted that R-loop mapping of the HPV transcripts in genital condylomas identified another HPV promoter near the 5' end of the E1 ORF (6). A transcript that could potentially encode the transcriptional repressor E2-C initiates at this promoter (31). It will be of interest to use RNase protection to study the abundance of this transcript in laryngeal papillomas and in cells derived from these lesions and cultured under various conditions. In this way, further information concerning the differential regulation of the HPV-6/11 early promoters will be gained.

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