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Inverse Relationship between Human Papillomavirus (HPV) Type 16 Early Gene Expression and Cell Differentiation in Nude Mouse Epithelial Cysts and Tumors Induced by HPV-Positive Human Cell Lines

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Two human papillomavirus type 16 (HPV 16)-immortalized human keratinocyte cell lines (HPK) were shown to have retained the ability for differentiation after subcutaneous injection into nude mice. These properties were maintained even at late passage. HPK cells gave rise to transiently growing cysts which exhibited an epitheliumlike architecture. Moreover, differentiation-specific markers such as cytokeratin 10, involucrin, and filaggrin were shown to be expressed in an ordered succession. RNA-RNA in situ hybridization revealed heterogeneous and low levels of HPV 16 E6-E7 RNA in the basal layer of the cysts. In contrast, in progressively growing tumors induced by HPK cells containing an activated *ras* oncogene (EJ-*ras*) or in tumors induced by the cervical carcinoma cell line CaSki, high levels of E6-E7-specific RNA could be detected. Irrespective of the growth potential of these cell lines in nude mice, viral transcription was always more evident in the basal layer and in proliferatively active cells rather than in differentiated cells. This contrasts with viral gene expression in HPV 16 positive low-grade cervical dysplasia, in which abundant viral transcriptional activity was mapped to the upper third of the epithelium. It is suggested that the physical state of the viral DNA, i.e., integrated viral DNA in the cell lines as opposed to extrachromosomal DNA in low-grade cervical dysplasia, may influence viral gene regulation.

To date, more than 60 different human papillomavirus (HPV) genotypes are known. A subset of these viruses is found in the urogenital tract. HPV types 6 and 11 (HPV 6 and HPV 11) induce preferentially benign proliferative changes of the epithelium (condylomata acuminata), while others such as HPV types 16, 18, 31, 33, and 35 are associated with flat condyloma, precancerous lesions (squamous intraepithelial neoplasia), and squamous cell carcinoma.

The assumption that certain papillomaviruses play an active role in the development of cervical cancer is based not only on the detection of viral DNA in most premalignant and malignant lesions but also on, among other findings, the ability of viral DNA to alter the growth behavior of human cells in vitro (for a review, see reference 36). Recent studies have shown that cultivated human primary foreskin keratinocytes became immortalized in response to transfection with HPV 16 DNA (11, 25). Similar results were observed for HPV 18 and other cervical cancer-linked HPV types but not for HPV 6 or HPV 11 (24, 29, 33). The immortalizing function could be mapped to the viral genes E6 and E7 (15, 22). The observation that the HPV 16 E7 protein forms a specific complex with the human retinoblastoma suppressor gene product p105-RB (13, 23) may be relevant for the mechanism by which these viruses contribute to cell transformation. Similarly, the E6 protein has been shown to bind p53 (32), another protein suspected to play a role in tumor suppression. None of the aforementioned HPV-immortalized human keratinocyte cell lines were tumorigenic in nude mice. However, the tumorigenic phenotype could be induced in these cells by the cooperating effect of an activated *ras* oncogene (10, 12).

In natural hosts, HPV infection is morphologically characterized by epithelial hyperplasia, with cells exhibiting a number of abnormalities such as nuclear changes (enlargement, binucleation, and degeneration) and cytoplasmic vacuolization (koilocytosis). In cervical intraepithelial neoplasia (CIN), histological grading is based on epithelial criteria such as the proportion of the epithelial thickness occupied by undifferentiated basal layer-like cells and on single-cell criteria such as nuclear abnormalities (e.g., an increased nuclear-cytoplasmic ratio) and the presence of atypical mitotic figures (6, 19, 28). Morphological alterations similar to CIN have recently been described for HPV-immortalized keratinocytes. By using a raft system which permits epithelial cells to stratify and differentiate in vitro, morphological changes which closely resembled those seen in histological sections of low-grade CIN were observed for HPV 16- and HPV 18-transfected primary keratinocytes at low passage. Late passages of the same cells had completely lost the ability to differentiate in the raft system, thus resembling high-grade CIN (16, 18).

In this article, we show that immortalization in vitro of human epidermal keratinocytes is not coupled with a loss of terminal differentiation when the cells are grown under in vivo conditions. Subcutaneous injection of two HPV 16-immortalized keratinocytes cell lines at high passage (>2 years in culture) into nude mice gave rise to encapsulated

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cysts showing an orderly structured and differentiated epidermal tissue. Moreover, in these cysts, as well as in tumors induced by a malignant derivative of one of the immortalized cell lines, HPV early gene expression appears to be inversely related to terminal differentiation, which is marked by expression of cytokeratins 1 and 10 as well as involucrin and filaggrin (9, 21, 27). These observations contrast with the transcriptional activity of HPV 16 in low-grade CIN (8, 31). We suspect that these differences in viral gene expression may in part be attributed to the physical state of viral DNA within the host cell. In CINs the viral DNA predominates in episomal forms, whereas in cell lines established by transfection of HPV DNA the viral genome always persists in an integrated state.

MATERIALS AND METHODS

Cell lines. The HPK IA and HPK II cell lines were established by transfection of human primary foreskin keratinocyte cultures with the entire HPV 16 genome and subsequent selection for unlimited growth in vitro (11). At a later stage, the cell lines were adapted to grow in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum only. They have been kept in culture continuously for over 2 years, being passaged on average once per week (initially at a split ratio of 1:2 and later at a ratio of 1:8). During this time, the HPV 16 DNA integration pattern was not modified and the viral genome remained transcriptionally active. HPK IA cells harbor two to three complete copies of the HPV 16 genome arranged in a head-to-tail fashion, integrated at one chromosomal site. HPK II cells possess about 10 copies of the viral genome, also arranged in a head-to-tail fashion but integrated at two distinct sites within the host DNA. The precise locations of the integration sites within the viral genome of both cell lines have not yet been determined. Southern blot analysis of HPK IA DNA indicates that both virus-cell junctions are within the L1 open reading frame (11). HPK II cells displaying a malignant phenotype were established after transfection with EJ-*ras* (the activated human c-H-*ras*) (10a) and are referred to as HPK II-EJras. CaSki, a cervical cancer-derived cell line containing about 500 copies of HPV 16 DNA integrated at several chromosomal locations (20), was obtained from the American Type Culture Collection.

All cell lines were grown in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum in a 37°C incubator with 5% CO_2.

Cyst and tumor growth in nude mice. For each cell line, 10^7 cells per animal were injected subcutaneously into 6-week-old female nude mice (BALB/c *nu/nu* backcross). For HPK IA and HPK II cells, cystic nodules developed, reaching maximal size 2 to 4 weeks after injection, and then regressed. Cysts which had persisted for months were shown to consist solely of dead keratinized cells. CaSki cells and HPK II-EJras cells gave rise to progressively growing tumors.

Cysts and tumors were excised at different times after injection and frozen in cold (−70°C) 2-methylbutane for subsequent storage in liquid nitrogen.

Indirect immunofluorescence. Serial cryostat sections (5 μm thick) were double stained by indirect immunofluorescence using antibodies directed against various markers for epithelial cell differentiation as first-step reagents and appropriate fluorescein isothiocyanate- or biotin-labeled anti-mouse, anti-guinea pig, or anti-rabbit immunoglobulins as second-step reagents (Fig. 1). Use of the second-step reagents alone did not give rise to any significant staining of the human components of the biopsy specimens, except for dead cornified cells. For double immunofluorescent staining, serial sections were each incubated for 45 min at room temperature with both first antibodies, i.e., panreactive and anti-cytokeratin 10 antibodies or antiinvolucrin and antifilaggrin antibodies. After several washes in phosphate-buffered saline, the sections were incubated with the appropriate second antibodies (see Table 1) for an additional 45 min and washed again. Specifically bound biotinylated anti-mouse immunoglobulins could be detected after a further incubation with Texas red-labeled streptavidin complex followed by washing. The sections were then embedded in aqua mount (BDH Ltd., Poole, England) and photographed (double exposures with appropriate filters) under an Olympus AH2 microscope equipped with epifluorescence optics.

First antibody	Second antibody
Panreactive	Anti-guinea pig FITC
Cytokeratin 10	Anti-mouse biotin
Involucrin	Anti-rabbit FITC
Filaggrin	Anti-mouse biotin

FIG. 1. Indirect double immunofluorescence. Biotin was detected by a third reaction step using Texas red-labeled streptavidin. FITC, Fluorescein isothiocyanate.

The antiserum against human involucrin was kindly provided by Fiona Watt (Kennedy Institute of Rheumatology, London, United Kingdom). A panreactive antiserum directed against epidermal and simple keratins was a gift from Progen, Heidelberg, Federal Republic of Germany. Anti-human cytokeratin 10 (CK 8.60) and antibodies directed against filaggrin were purchased from Renner, Dannstadt, Federal Republic of Germany, and Paesel, Frankfurt, Federal Republic of Germany, respectively. All second antibodies were purchased from Dianova, Hamburg, Federal Republic of Germany.

RNA-RNA in situ hybridization. Serial cryostat sections mounted on 3-aminopropyl-triethoxysilane-coated slides were fixed in 4% paraformaldehyde in 2× SSPE (0.3 M NaCl, 20 mM NaH_2PO_4, 2 mM EDTA) for 10 to 15 min at room temperature, digested with proteinase K (0.5 μg/ml) for 10 min at 37°C, and hybridized with strand-specific RNA probes spanning the URR-E6-E7 open reading frames of HPV 16 (nucleotide positions 7456 to 880) (30) and the human cytokeratin 1 gene (17) as described previously (3). Briefly, radioactively labeled RNA probes were generated in the transcription vector Bluescribe with a solution containing either T3 or T7 RNA polymerase, 100 μCi of [^{32}P]UTP (800 Ci/mmol; Amersham), and 0.25 mM each of the remaining precursors as a cold substrate. This procedure yields an RNA with a specific activity of 10^9 cpm/μg. After DNase treatment, the probes were subjected to limited alkaline hydrolysis. The sense orientation of each probe served as a negative control. Hybridization was performed overnight at 42°C in a solution containing 50% formamide, 2× SSPE, 10% (wt/vol) dextran sulfate, 10 mM Tris (pH 7.5), 1× Denhardt's solution (0.02% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 500 μg of tRNA per ml, 100 μg of herring sperm DNA (sonicated) per ml, 0.1% sodium dodecyl sulfate (SDS), and 10^5 cpm of probe per μl. These conditions approximately equal T_m − 20°C for RNA-RNA in situ hybridization (7). Sections were washed in 50% form-

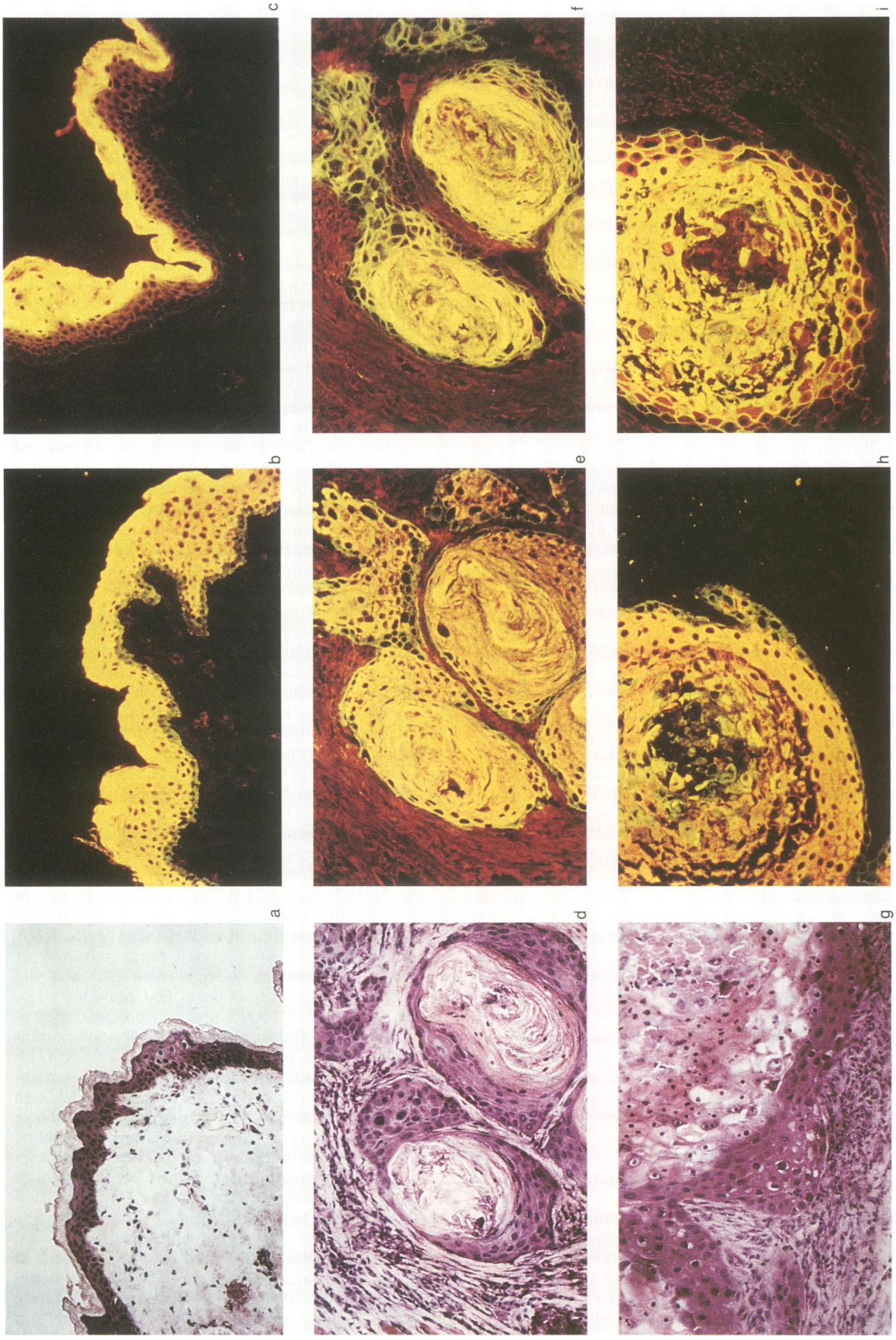


FIG. 2. Hematoxylin- and eosin-stained sections of human native foreskin (a) and of cysts induced in nude mice after subcutaneous injection of HPK IA cells (d) and HPK II cells (g). Serial sections of the foreskin (b and c), the HPK IA cyst (e and f), and the HPK II cyst (h and i) were each stained by indirect double immunofluorescence with (i) a panreactive antibody against epidermal and simple keratins and an antibody against human cytokeratin 10 (b, e, and h) and (ii) antibodies against involucrin and filaggrin (c, f, and i) (Fig. 1). The photomicrographs represent double exposures taken with appropriate filter combinations for fluorescein isothiocyanate (green) and Texas red (red) fluorescent staining. In sections b, e, and h, the basal layer stains green, whereas the suprabasal layer appears yellow. This is because the panreactive antibody stains all epithelial cells (green), whereas the cytokeratin 10 antiserum stains only suprabasal cells (red) and the stain appears yellow in the double exposure. Similarly, in sections c, f, and i, involucrin stains green or yellow and filaggrin stains red or yellow. Squames in the center of cysts and on the surface of the foreskin stain unspecifically. There is also some cross-reactivity between mouse stromal tissue and biotin-labeled anti-mouse antibody. Magnification, ×120.

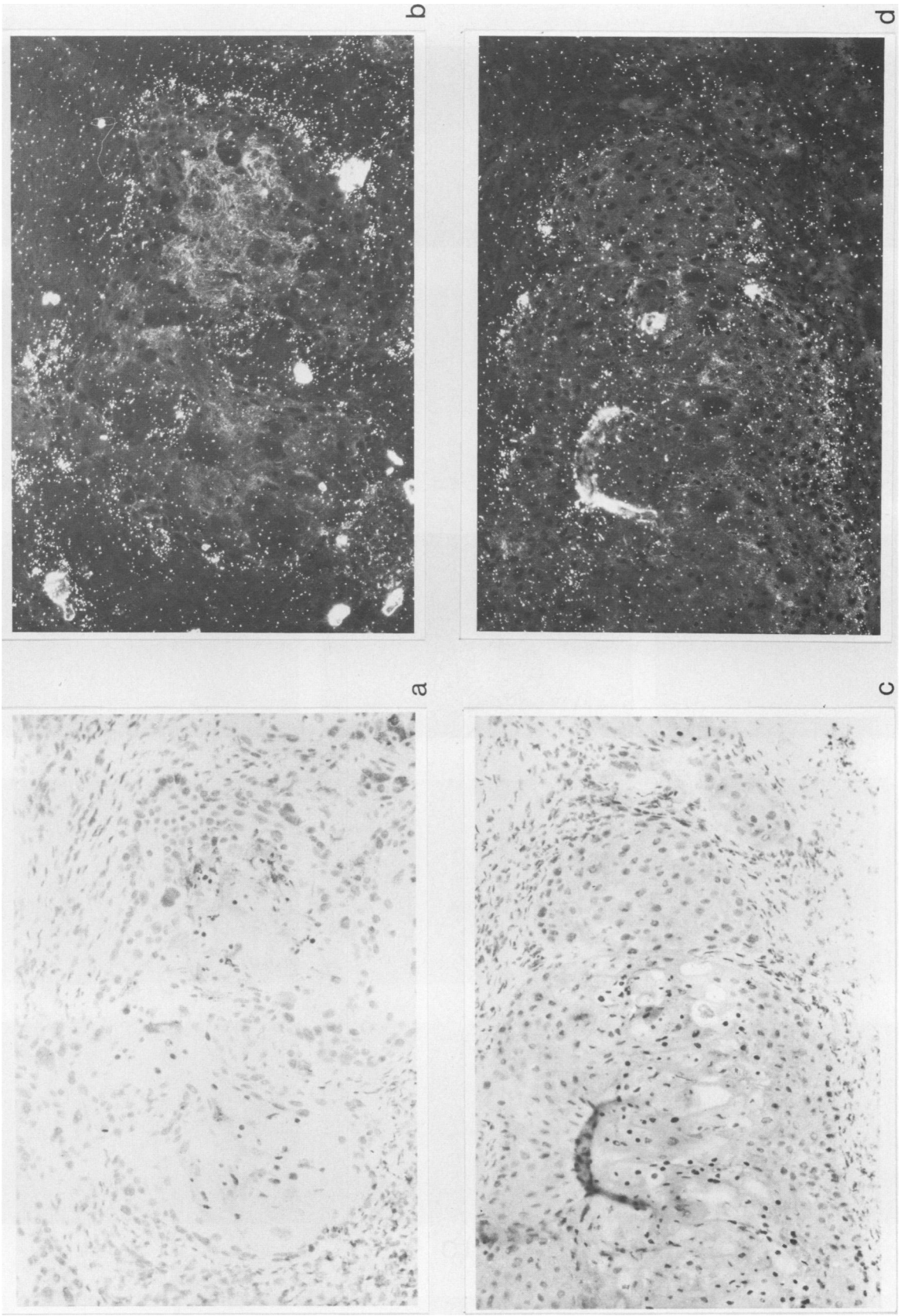
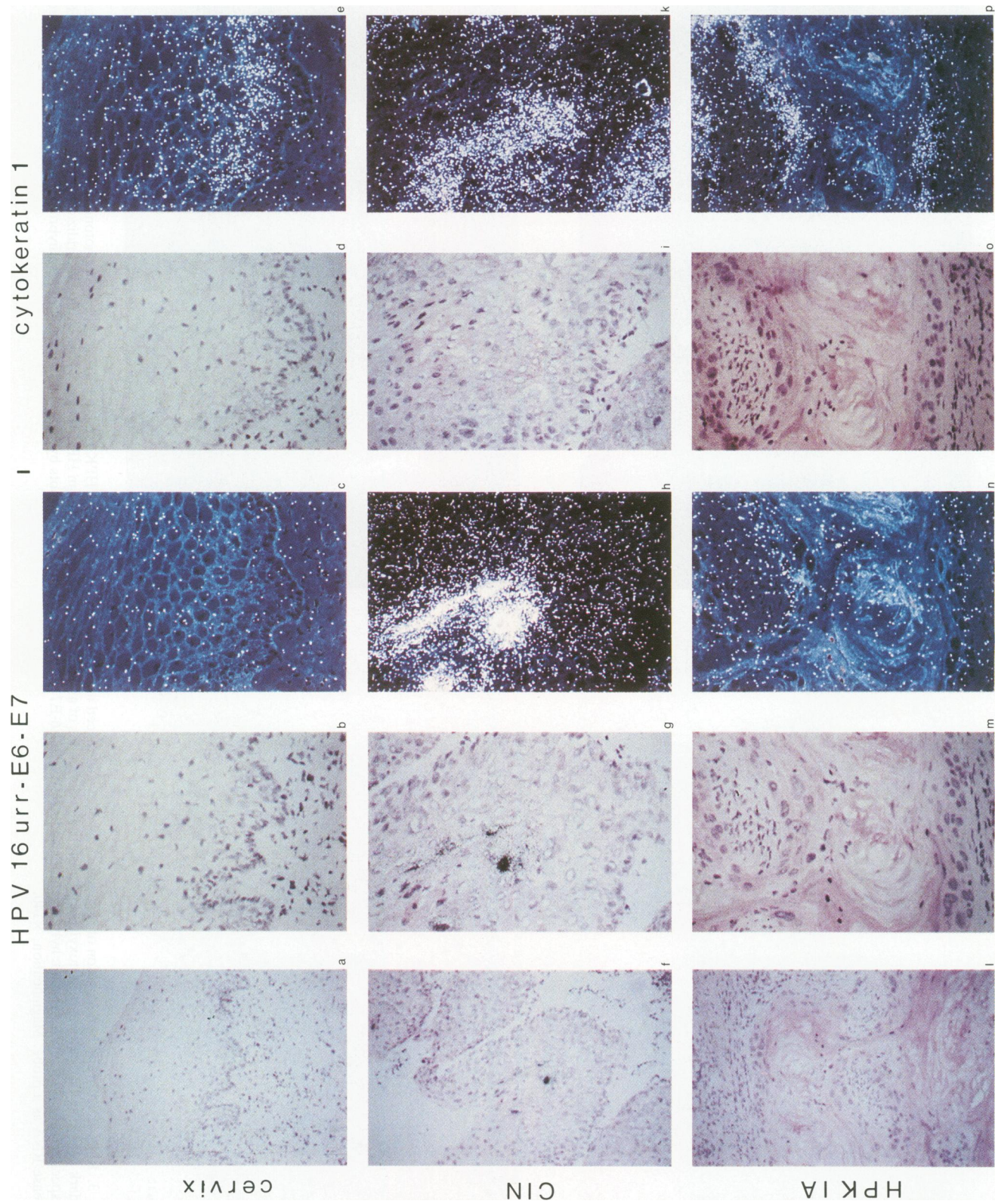


FIG. 3. RNA-RNA in situ hybridization of tissue sections of cysts induced in nude mice after injection of HPK IA and HPK II cells. The photomicrographs show light field (a and c) and dark field (b and d) views of hematoxylin- and eosin-stained sections of an HPK IA cyst (a and b) and an HPK II cyst (c and d) after hybridization with an HPV 16 URR-E6-E7 strand-specific (antisense) probe. Specific hybridization signals (HPV 16 E6-E7 mRNA) are seen as white spots by dark field microscopy. No hybridization signals were obtained by using sense RNA as a probe. Magnification, ×140.


HPV 16 urr-E6-E7
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cervix
CIN
HPK 1A
a
b
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amide–2× SSPE–0.1% SDS for 30 min at 50°C, treated with RNase A (50 μg/ml) for 30 min at 37°C, and subsequently washed in 50% formamide–0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 30 min at the same temperature (T_m − 15°C). Slides were dehydrated in graded alcohols containing 300 mM ammonium acetate, dried, and dipped in Kodak NTB 2 emulsion diluted 1:1 in 600 mM ammonium acetate. After storage for 1 to 14 days, the slides were developed in Kodak D-19 developer, fixed, and counterstained with hematoxylin and eosin. The sections were examined and photographed under a Zeiss Axiophot microscope equipped with rotatable bright field and dark field condensers to reveal tissue histology and to enable good signal visualization, respectively.

RESULTS

Growth and differentiation of HPV 16-immortalized keratinocytes and CaSki cells after subcutaneous injection into nude mice. Despite their apparent unlimited growth potential, HPK cells have retained the ability to reform an orderly structured and differentiated epidermal tissue when injected subcutaneously into nude mice. Cystic structures were formed when suspensions of HPK cells were injected subcutaneously onto the subdermal muscle fascia. Approximately 2 weeks after inoculation the epithelium of these cysts had developed normal tissue architecture, including a stratum granulosum and corneum (Fig. 2; compare panels d and g [cysts] and panel a [normal foreskin]). The living epithelium eventually degenerated, often leaving behind a cyst filled with dead keratinized material. The histologic findings could be substantiated by analysis of differentiation-specific markers. Basal and suprabasal cell compartments were clearly distinguishable (as in the epidermis) by double immunofluorescence using a panreactive antibody specific for all epithelial cells and a polyclonal antibody specific for cytokeratin 10, which is only expressed in the suprabasal layer of the epidermis (21) (Fig. 2; compare panels e and h [cysts] with panel b [normal foreskin]). Moreover, involucrin, a major precursor protein of the cross-linked envelope in the stratum corneum (27) and filaggrin, the major component of keratohyalin granules (9), were detected in the upper living layers of the cysts (Fig. 2; compare panels f and i [cysts] with panel c [normal foreskin]). In addition, human cytokeratin 1 RNA transcription, which in native foreskin and ectocervical tissue is confined to the suprabasal layer, was seen in the corresponding layers in HPK-induced cysts and tumors (see Fig. 4 and 5). HPK cells maintained these in vivo differentiation properties at high passage levels, even though differentiation in vitro (stratification and squame formation) showed a marked decrease at earlier passages (11).

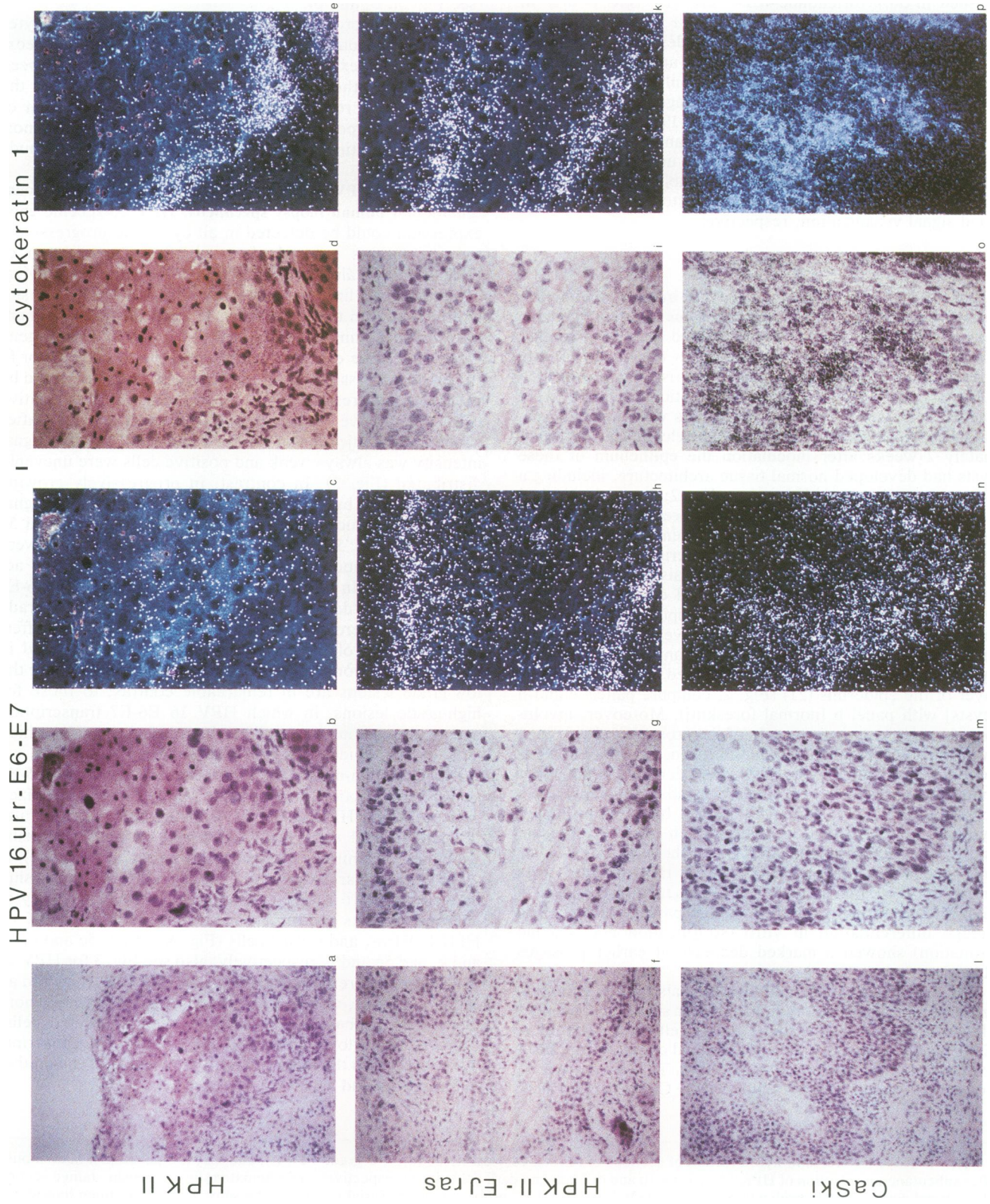
The potential for differentiation was also retained in progressively growing tumors induced after subcutaneous inoculation of EJ-*ras*-transfected HPK II cells into nude mice. Evidence for terminal differentiation and cytokeratin 1 RNA expression is shown in Fig. 5f, i, and k. The same observations were made for tumors induced by CaSki cells, which, in contrast to tumors induced by HeLa and SiHa cells (data not shown), also showed signs of terminal differentiation (see Fig. 5l, o, and p).

The growth properties of normal foreskin keratinocytes after subcutaneous injection into nude mice were not reexamined in our experiments, since they have already been addressed by others (5, 34). Boukamp et al. described the formation of a regular epithelium with the expression of differentiation-specific markers after transplantation of normal human keratinocytes from different body sites onto nude mice (5).

Analysis of HPV 16 E6-E7 gene expression in nude mouse tumors and human biopsy specimens. HPV 16-specific gene expression could be detected in all cysts and progressively growing tumors induced after subcutaneous injection of HPV 16-immortalized cells and an HPV 16-positive cervical carcinoma cell line into nude mice by RNA-RNA in situ hybridization on tissue sections. In each of the experimentally induced tumors, cells positive for viral E6-E7 gene expression were essentially located in the basal layer or in the extended basallike layer. Differences, however, could be observed with respect to the distribution of HPV-positive cells and relative signal intensity. In cysts obtained after subcutaneous injection of HPK IA and HPK II cells, signal intensity was always weak and positive cells were unevenly distributed (Fig. 3). In contrast, in progressively growing tumors induced by HPK II-EJras and CaSki cells, the signal strength was much more intense (compare Fig. 4n and 5c [cysts] with Fig. 5h and n [tumors]). Moreover signals were present throughout the undifferentiated, proliferatively active cell layer. In normal cervical tissue no HPV 16 E6-E7 RNA could be detected (Fig. 4c). However, in low-grade CIN, signals were confined to cells in the terminally differentiated layers of the epithelium, where they occurred in high concentrations (Fig. 4h). The observations made for the low-grade lesions are in remarkable contrast to those for high-grade lesions, in which HPV 16 E6-E7 transcription occurs throughout the proliferating epithelial layer, which fails to show evidence for terminal differentiation (10a).

Differentiation-dependent expression of the viral E6-E7 genes. Serial sections of all biopsy samples were hybridized with probes for HPV 16 URR-E6-E7 and human cytokeratin 1. Cytokeratin 1 is normally expressed in suprabasal cells, which are committed to terminal differentiation (Fig. 4e). It was of particular interest to note an inverse relationship between HPV 16 E6-E7 gene expression and that of cytokeratin 1 in cysts and tumors induced by HPK IA, HPK II, HPK II-EJras, and CaSKi cells (Fig. 4n and p, 5c and e, 5h and k, and 5n and p, respectively; also see Fig. 3 for HPV 16 E6-E7 gene expression in HPK IA and HPK II cysts). In all of these lesions, viral RNA transcription was always more evident in the basallike layer than in differentiated cells. Conversely, in low-grade CIN, HPV 16 E6-E7 transcripts were mapped to the more differentiated layers of the epithelium (Fig. 4h and k).

FIG. 4. RNA-RNA in situ hybridization of sections of normal cervical tissue (a), low-grade CIN (f), and a cyst induced in a nude mouse after subcutaneous injection of HPK IA cells (l). (b and c) Light and dark field views, respectively, of a hematoxylin- and eosin-stained section of a normal cervix after hybridization with the HPV 16 URR-E6-E7 probe. (d and e) Serial section of the same biopsy specimen hybridized with human cytokeratin 1 as a probe. The arrangement of photomicrographs for the other biopsy specimens is the same as that for normal cervical tissue. Specific hybridization signals are seen as white spots by dark field microscopy and, if present in high concentrations, also as black gains by light field microscopy. Magnification, panels a, f, and l ×70; other panels ×140.


HPV 16 urr-E6-E7
cytokeratin 1
HPK II
HPK II-EJras
CaSki

DISCUSSION

Immortalization of human keratinocytes in vitro appears to be a specific property of cervical cancer-linked HPV types such as HPV 16, HPV 18, HPV 31, and HPV 33 but not of HPV 6 and HPV 11, which preferentially induce benign genital lesions (24, 29, 33). None of the HPV-containing cell lines established from primary keratinocytes in different laboratories were tumorigenic in nude mice, but they appear to exhibit a morphology similar to that of CIN when grown in vitro on collagen rafts which permit epithelial differentiation analogous to that seen in vivo (16, 18). Dysplastic differentiation in vivo, characteristic of CIN, was also observed after grafting HPV-immortalized cell lines beneath skin-muscle flaps of nude mice (34). In contrast, the epidermal architecture of the transitory cystic nodules induced by subcutaneous inoculation of two of our HPV 16-immortalized keratinocyte cell lines (HPK IA and HPK II) into nude mice is more reminiscent of normal skin than of CIN. The apparent loss of some differentiation properties in vitro (that is, the loss of both stratification and squame formation when the cells are grown on plastic), which was noticed at an early stage in the passage history of the HPK cell lines, has not influenced the potential for terminal differentiation when these cells are grown under in vivo conditions. Since these two cell lines have retained this ability for differentiation in vivo despite being in culture for over 2 years (>100 passages), it is concluded that immortalization of human keratinocytes with HPV 16 is not necessarily accompanied by major defects in differentiation. There is no obvious explanation for the observed differences in the differentiation potential in vivo of the various HPV-immortalized cell lines (34). Differences in experimental design and conditions as well as factors unrelated to HPV gene expression (such as natural variability among different foreskin keratinocyte cultures or genetic alterations due to passaging) are likely to influence the outcome of each experiment. In contrast to the cell lines examined by Woodworth et al. (34), our cell lines were established on the basis of continuous passage, i.e., immortalization after HPV 16 DNA transfection without the use of any additional dominant selection marker. Moreover, our cultures were continuously grown in a medium with a high calcium content which permits epithelial cell differentiation. It is even more difficult to reconcile our data with those obtained for HPV-immortalized cell lines in the raft system (16, 18). Although the raft system mimics the in vivo situation to some extent, i.e., cultured cells derived from various tissue biopsy specimens can duplicate many of the histologic features observed in either normal epithelium or cervical neoplasia (26), complete normalization of the tissue in the raft system is apparently not achieved, as indicated by irregularities in the spatial organization of differentiation products (1, 2, 14).

By RNA-RNA in situ hybridization, the topographic distribution of HPV 16-specific RNA in HPK cells exhibiting different degrees of growth potential in nude mice could be assessed. In cysts induced by parental HPK cells, HPV 16 E6-E7-specific RNA signals were heterogeneous and were restricted to cells in the basal layer. However, in progressively growing tumors induced by HPK cells containing the activated human EJ-*ras* oncogene, high steady-state levels of viral RNA could be detected throughout the basallike layer. Northern blot analysis of RNA extracted from benign and tumorigenic HPK cells grown in vitro showed no quantitative differences in HPV 16 E6-E7 transcription (data not shown). Thus, HPV 16 gene expression in benign nude mouse tumors, unlike that in cells cultured in vitro, may be governed by the same cellular factors which are thought to negatively regulate HPV 18 E6-E7 gene expression in nontumorigenic HeLa-fibroblast hybrid cell lines (4). Indeed, the very low levels of HPV 16 E6-E7-specific RNA signals in the basal lining of two low-grade CINs which were analyzed at the same time support a postulated tight cellular control on viral gene expression in actively proliferating cells (4, 35). The mechanism by which the activated Ras protein interferes with the postulated modulation of viral gene expression in vivo is not known.

The essence of these results is the observed retention of the differentiation potential of HPV 16-immortalized keratinocytes when analyzed in vivo (nude mouse system) and the inverse relationship between HPV 16 E6-E7 transcription and epithelial cell differentiation. In low-grade CIN, HPV 16 E6-E7 gene expression is typically confined to the upper layer of the epithelium and is not or only rarely detected in the basal layer (8, 31). In our model system, two HPV 16-immortalized keratinocyte cell lines, although capable of terminal differentiation in vivo, are unable to express these viral genes in the appropriate (with reference to naturally occurring lesions) epidermal compartment. Instead, limited viral transcriptional activity takes place in the basallike layer only. The same inverse relationship between HPV gene expression and differentiation was also observed for progressively growing tumors induced by EJ-*ras*-transfected cells (HPK II-EJras) and tumors induced by the cervical carcinoma cell line CaSki.

We suspect that the inability to express HPV genes in differentiated cells may be due to the physical state of the viral DNA within the host cell. In contrast to low-grade CIN, the examined cell lines harbor exclusively integrated HPV 16 DNA. Because the viral DNA in cell lines is an integral part of the chromatin, its expression may be regulated differently from that of episomal DNA. Alternatively, HPV DNA replication which leads to a high number of DNA templates also available for transcription should be considered as a possible explanation for the high level of viral RNA in the upper layer of low-grade lesions. In contrast to naturally occurring lesions, in which HPV DNA is known to replicate in the stratum granulosum, none of the nude mouse tumors induced by the HPV 16-containing cell lines can support extrachromosomal viral DNA replication. We are presently screening for well-differentiated HPV 16-positive cervical carcinomas which contain only integrated viral DNA. A comparative analysis of the topographical distribution of cytokeratin 1 RNA and HPV 16 E6-E7 RNA in these tumors should support the observations presented in this paper.

FIG. 5. RNA-RNA in situ hybridization of tissue sections of cysts and tumors induced in nude mice after subcutaneous injection of HPK II (a), HPK II-EJras (f), and CaSki cells (l). (b and c) Light and dark field views, respectively, of a hematoxylin- and eosin-stained section of an HPK II cyst after hybridization with the HPV 16 URR-E6-E7 probe. (d and e) Serial section of the same biopsy specimen hybridized with human cytokeratin 1 as a probe. The arrangement of photomicrographs for the other biopsy specimens is the same as that for the HPK II cyst. Specific hybridization signals are seen as white spots by dark field microscopy and, if present in high concentrations, also as black grains by light field microscopy. Magnification, panels a, f, and l ×70; other panels ×140.

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