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Topography of Early HPV 16 Transcription in High-Grade Genital Precancers

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The extent to which human papillomavirus (HPV) type 16 is transcribed and the nature of the transcripts produced in genital precancers has not been clearly defined. The authors analyzed 28 cases of cervical (CIN) or vulvar (VIN) intraepithelial neoplasia by RNA–RNA in situ hybridization, using probes generated from HPV 16 open reading frames (ORFs) either upstream (E6-E7) or downstream (E2-E5-L2) to the E1 ORF, where HPV 16 genomic integration most commonly occurs. Hybridization signals corresponding to one or both probes were detected in a high proportion of cells throughout the lesional epithelium of low- and high-grade CIN, including basal layers. In serial sections analyzed with the two probes, hybridization signals were obtained from both, and in similar proportion, irrespective of CIN grade. The distribution and character of hybridization signals suggests that the morphologic progression of precancers is not associated with either cessation of HPV 16 early transcription or a change in the general character of the transcripts produced. (Am J Pathol 1989, 134: 1183–1188)

Human papillomavirus type 16 has been associated with invasive genital carcinomas and their precursor lesions (cervical intraepithelial neoplasia or CIN).[1-3] The mechanism by which the virus contributes to the development of a precancer and its possible progression to invasive carcinoma are unknown. The E6/E7 open reading frames (ORFs) of human papillomavirus type 16 possess intrinsic transforming ability, and also act synergistically with an activated ras oncogene to produce transformation of established or primary cell lines.[4-9] ORFs E6 and E7 of HPV types 16 and 18 are also preferentially transcribed in some cervical cancers and cell lines from invasive cancers containing HPV 16 or 18 DNA, due to chromosomal integration and disruption of the viral genome downstream to E7 in the E1 or E2 ORF. This phenomenon effectively reduces or eliminates transcription from distal ORFs, including those that might encode gene products with autoregulatory functions.[4-13]

Although transcription of HPV 16 ORFs occurs in precancers, the relationship between HPV transcription and morphologic progression is unclear. One question concerns whether HPV transcription is required once a CIN lesion has developed. Although it is presumed that HPV DNA is present in the basal cells of these lesions, signals produced by RNA–RNA *in situ* hybridization are most frequently detected in the superficial cell layers.[15] Stoler and Broker demonstrated HPV 11 RNA in condylomata and HPV 16 RNA in precancers and cancers[14] and Crum et al[15] detected RNA corresponding to HPV-16 early reading frames in a portion of the epithelium of CIN lesions. In the latter study, however, HPV 16 RNA was not detected in all areas of the lesions studied.[15] This may be related in part to a lack of sensitivity related to the fixative used (Bouin's) or other factors. Another explanation is that high-grade CIN may contain extrachromosomal or integrated HPV DNA that is not transcribed.[16] Genomic integration of HPV 16 DNA has been reported in cancers and precancers and there is evidence that integration of papillomaviral DNA may influence the level at which HPV DNA sequences are expressed and the specific ORFs transcribed.[7,8,17] Whether this phenomenon alters the level or distribution of HPV 16 transcripts in high-grade CIN is unclear.

The purpose of this study was to determine both the extent of detectable HPV 16 early transcripts in precancers and the nature of the transcripts produced, using RNA–RNA *in situ* hybridization. The former would address the possibility that HPV transcription occurred in all cells and the latter would determine whether obvious qualitative changes occurred in transcription pattern as a function of morphologic grade. The data presented herein suggest that transcription of HPV early ORFs occurs throughout precancers of all grades, that early transcripts

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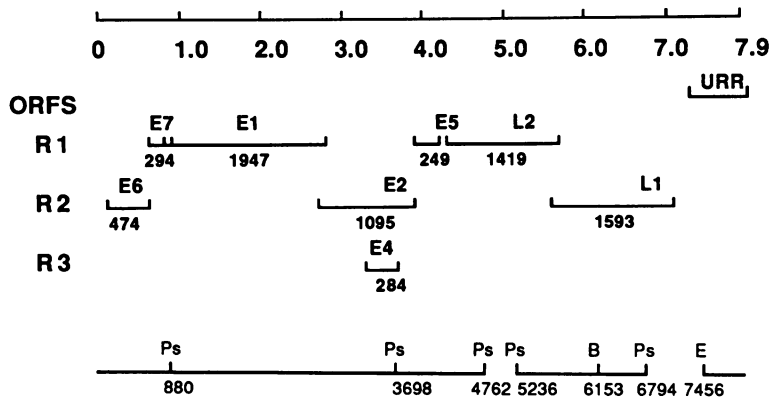

Figure 1. *Map of the HPV 16 genome, location of open reading frames, and location of restriction sites corresponding to Pst I (Ps), BamH1 (B) and EcoR1 (E) (bottom). The probe corresponding to the URR-E6-E7 ORFs was generated from an Eco-Pst fragment spanning nucleotides 7456 and 880. The probe corresponding to the 3'E2-E5-5'L2 ORFs was generated from a Pst-Pst fragment spanning nucleotides 3698 and 4762.*

originate primarily from uninterrupted extrachromosomal (plasmid) HPV DNA, and that morphologic "progression" observed to occur within individual lesions is not associated with obvious changes in the proportions of early transcripts.

Materials and Methods

Histologic Material

We selected formalin-fixed, paraffin-embedded tissues from 22 CIN lesions that contained sequences hybridizing to HPV 16 RNA probes. In addition, nine large tissue blocks from conization/excision specimens of high grade precancers of the cervix (5) and vulva (1) were obtained that also contained HPV 16 RNA. The slides were reviewed and arbitrarily classified as either CIN (or VIN) with koilocytotic atypia or surface maturation (termed "CINK" and corresponding generally to lesions classified as CIN I or CIN II) or CIN without koilocytotic atypia (termed "CIN" and corresponding to lesions classified as CIN III).[18,19]

In Situ Hybridization

The RNA–RNA *in situ* hybridization analysis was conducted in two phases. In the first, designed to determine the topographic extent of HPV RNA, sections from each case/block were incubated with a probe constructed by cloning sequences spanning nucleotides 3698-4762, spanning the 3'E2, E5, and 5'L2 ORFs of HPV 16 into a GEM-1 vector[20] (Promega, Madison, WI) (Figure 1). The insert was oriented by restriction enzyme digestion and gel analysis, and an ^{35}S RNA probe was generated using T7 RNA polymerase and 100 μCi of ^{35}S-labeled UTP. The specific activity of the probe was approximately 5×10^8 cpm/μg labeled RNA. Sections were deparaffinized, treated with Proteinase K (5 μg/ml), dehydrated in graded ethanols, and dried. A probe cocktail was then applied that consisted of 20,000 counts/μl probe, 50% formamide, 10% dextran sulfate, 1 M NaCL, 10 mM TRIS pH 8.0, 5 mM EDTA, 1× Denhardts solution, 100 μg each of yeast tRNA and denatured salmon sperm DNA and 0.1% sodium dodecyl sulfate. Sections were coverslipped and incubated overnight at 45 C in a glass dish. Coverslips were then removed and the sections were incubated sequentially in 2× SSC, 2× SSC with RNAse (20 μg/ml), then 0.2× SSC and 0.1× SSC at 50 C.[15] Sections were dehydrated in graded alcohols containing 300 mM ammonium acetate, dried, then dipped in Kodak NT3B emulsion diluted 1:1 with 600 mM ammonium acetate. Sections were incubated for 5 days in a light-tight container at 4 C, developed in D-19 developer (Kodak, Rochester, NY) stained with hematoxylin, and coverslipped.[15]

To determine if there were marked differences in hybridization signals produced by probes from different regions of the HPV early genome, serial sections from cone and excision specimens were incubated with both the probe corresponding to the E2-E5-L2 region and one spanning the upstream regulatory region and E6/E7 ORFs (nucleotides 7456-880).[20] The purpose of using larger specimens was to provide a larger area of intraepithelial neoplasia for analysis. Controls consisted of either probes generated in the opposite direction (sense probes) or a heterologous HPV RNA probe (ie, HPV 11 RNA).

Analysis of Data

In the first analysis, biopsies were evaluated for evidence of specific RNA–RNA hybridization signals by comparing the number of silver grains in the target epithelium with the background stroma. Target/background signal ratios with control probes ranged from 1.0 to 2.0, and a hybridization signal with the test probe was considered positive if the target/background signal ratio was twice control, or approximately 4.0. The horizontal extent and maximum depth of the positive signal in each case was estimated to determine the extent of HPV transcription in the epithelium (Figure 1).

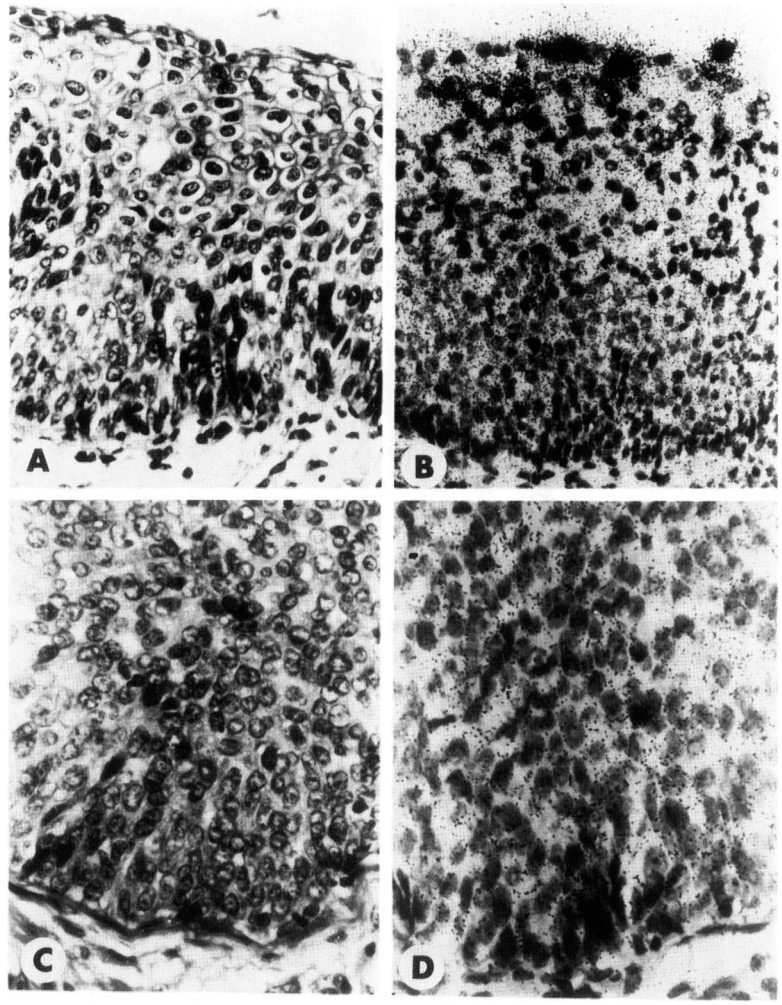

Figure 2. In situ *hybridization detection of HPV 16 RNA sequences in the basal and parabasal cells of CIN lesions using a probe spanning the E2-E5-L2 ORFs.* **A:** *H & E-stained section from a CIN with koilocytotic atypia.* **B:** *Serial section following RNA-RNA hybridization, in which the signal is diffuse with focal amplification near the surface.* **C:** *Adjacent area of crypt involvement by high-grade CIN.* **D:** *Serial section demonstrating a diffuse hybridization signal involving all cell layers, including the basal cells.*

In the second analysis, attention was directed to differences in signal intensities produced by the two probes in serial sections. Because of the fact that the probes were generated from templates of unequal length, and because the precise length of the exons corresponding to these reading frames was not known, absolute differences in hybridization signal between probes were not used to monitor changes in transcript accumulation. Rather, attention was paid to differences in the *relative* strength of the signals produced in different areas of serial sections by the probes. In this way, it would be possible to estimate the proportion of RNA signal corresponding to ORFs upstream (E6-E7) and downstream (E2-E5-L2) of the site (E1) where integration would typically occur.[7,8,20]

In serial sections, fields were selected that demonstrated identical morphology between sections, and grain counts for each probe were determined where the hybridization signals were focally strong, typical of transcripts produced during extrachromosomal replication (Figure 2A–C). In addition, areas of high-grade CIN were evaluated in which the signal was weaker and evenly distributed from cell to cell (Figure 2C–F). To standardize the counting procedure, the grains were counted electronically using a Leitz Orthoplan microscope and a Gould IP8500 Image Processing Workstation with the GRNCNT Particle Counting System. One or more fields from each area were analyzed, and the mean number of counts (minus background) was calculated for each probe within a standard area. The ratios of counts between probes in areas of prominent episomal replication (surface-related signal amplification) and in the poorly differentiated epithelium were tabulated and compared.

Results

Distribution of Transcripts

Of the 22 biopsies analyzed, 12 contained areas of unequivocal maturation or koilocytotic atypia (CIN K) and 10 lacked clear evidence of maturation in the upper cell layers (CIN). In the CIN K group, all contained foci in which the signal was more intense in the upper or more mature layers of the epithelium (Figure 2A, B). In the CIN lesions,

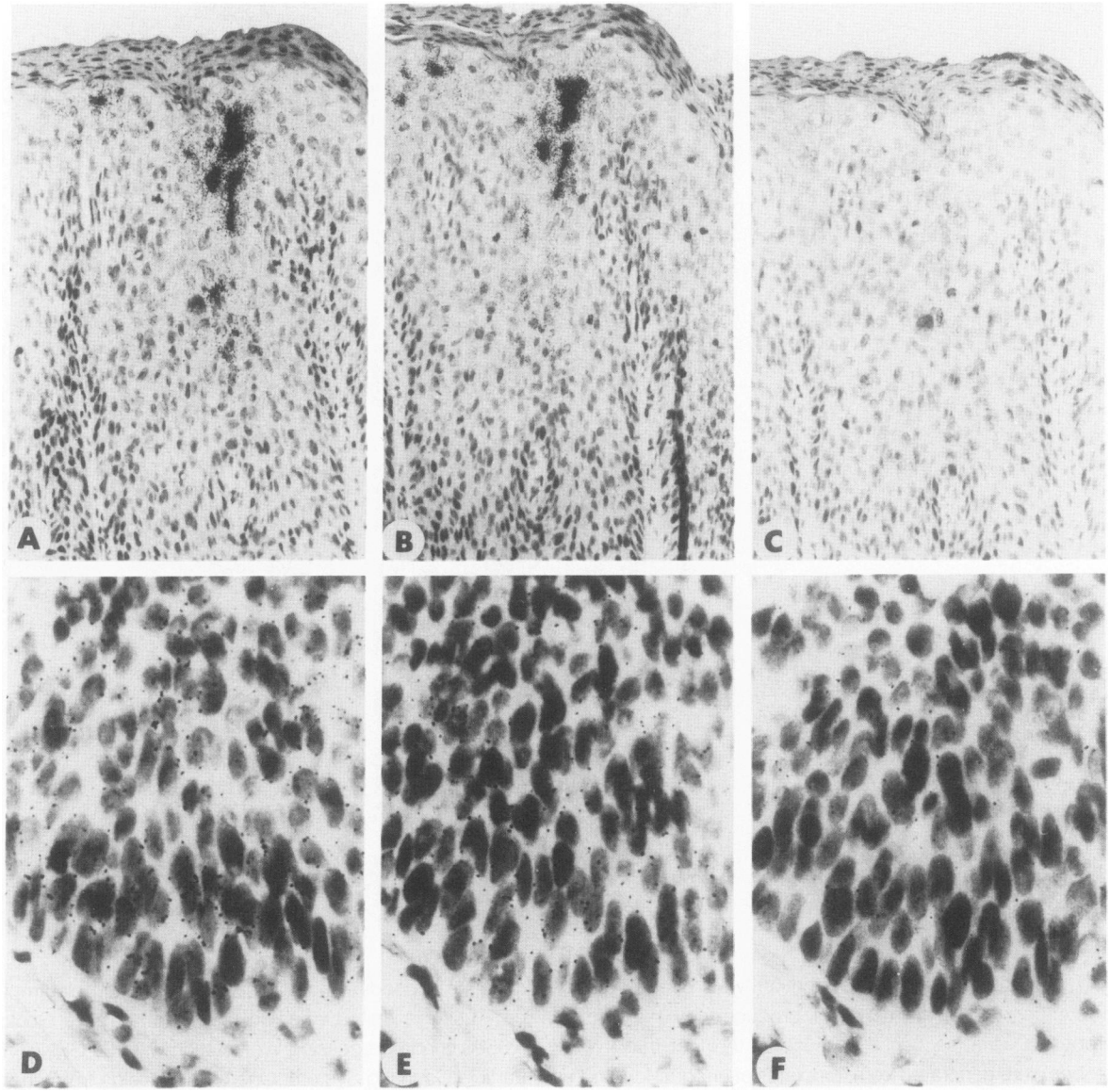

Figure 3. In situ *hybridization analysis of serial sections from a vulvar precancer (VIN III) for HPV 16 RNA using ^{35}S-labeled RNA probes derived from the URR-E6-E7* **(A, D)** *and E2-E5-L2 ORFs* **(B, E)**. *A probe generated in the sense orientation was used as a control* **(C, F)**. *In A and B the signal predominates near the epithelial surface, characteristic of transcription of extrachromosomal HPV DNA. In C and D the signal is evenly distributed throughout the immature regions of the precancerous epithelium.*

4 of 10 contained an increase in signal intensity in the upper cell layers, usually associated with subtle evidence of epithelial maturation. In 11 of 12 of the CINK lesions and 9 of 10 of the CIN lesions, the hybridization signal was present in virtually the entire epithelium (approximately 80% or greater) in both the horizontal and vertical planes (Figure 2C, D). In one of each, signals were focal (20% of the epithelium or less) and were generally present in individual cells near the epithelial surface.

Comparison of Transcription of Early ORFs

Figure 3 illustrates the comparative hybridization signals produced by both probes in areas of well (Figure 3A–C) and poorly (Figure 3D–F) differentiated epithelium. Four biopsies (from two cases) contained prominent surface-related (episomal) transcription, and the ratio of downstream to upstream signals in these areas ranged from 1.16 to 2.94 (mean, 2.16) (Figure 3A–C). The signal ratio within poorly differentiated epithelium of these and an additional 4 biopsies ranged from 0.77 to 4.94 (mean, 2.22) (Figure 3D–F). In one case a signal was detected with the E2-E5-L2 (downstream) probe, but was equivalent to background with the E6/E7 (upstream) probe. This was presumed to be a function of a low transcript level, which was more readily detected by the downstream probe rather than the absence of E6/E7 transcripts. In all cases, detectible hybridization signals obtained with the probes

derived from the E2-E5-L1 construct were observed in a large proportion of cells in the more poorly differentiated areas (Figures 2 and 3). Hybridization signals produced in these areas were comparable with those produced in the basal and parabasal cells of more mature epithelium (Figures 2 and 3).

Discussion

The patterns of hybridization observed in this analysis suggest that HPV 16 transcription occurs in the majority of cells within precancers derived from this infection. In the majority of biopsies analyzed, transcripts were found in all layers of the epithelium, irrespective of the grade of the precancer. Considering that precancers are presumably derived from a single infected clonal population, HPV DNA would be expected to be present in each neoplastic cell. However, if HPV is to be linked directly to biologic events occurring after initial infection, transcription and translation of the viral genome theoretically must occur in all cells, specifically the dividing cells in the lower epithelial strata. This pattern of hybridization was observed in all but two cases studied and the focal nature of hybridization signals in these cases is perplexing. This phenomenon may be explained by variations in probe sensitivity in a particular section, or differences in the target tissue produced by length of fixation or processing. Another explanation for focal hybridization is that the target HPV sequences were derived from an HPV other than type 16, and were detectable only in foci (ie, surface cells) containing high transcript copy number.

The principal technical reason for detecting transcripts over a broader epithelial area appears to be the use of formalin fixation. In prior experience with Bouin's fixation, we have detected HPV 16 RNA sequences in approximately 20% of CIN lesions analyzed and often the hybridization signal was extremely focal (Crum CP, Nuovo G, Silverstein S, unpublished observations). In a recent analysis of CIN lesions fixed in formalin, we have detected HPV 16 sequences in nearly 70% (Franquemont D, Ward B, Crum CP, unpublished data). Moreover, the distribution of signal with formalin-fixed preparations, in contrast to Bouin's, is more extensive. Recent studies indicate that the differences' sensitivities are related to length of fixation time in Bouin's (Nuovo G, Richart RM, personal communication, manuscript submitted).

The consistent finding of hybridization signals corresponding to both E6-E7 and E2-E5-L2 ORFs suggests strongly that the transcripts are derived principally from uninterrupted extrachromosomal HPV DNA. Chow et al demonstrated that transcription of unintegrated HPV 11 DNA in genital warts produces products that frequently consist of E6 or E7 spliced to ORFs in the E2, E4, E5, or late region, with exclusion of the intervening E1 ORF.[22] Thus, early transcripts produced from extrachomosomal HPV 16 sequences should be represented by products 5′ (ie, E6-E7) and 3′ (ie, E2-E5-L2) to E1, whereas integration at the E1 ORF would significantly alter transcription of the latter. Obviously there are limitations in the technique of *in situ* hybridization that make it impossible to assume that all the early transcripts produced in precancers are extrachromosomal in origen. Because the probe generated from the E2-E5-L2 region generally produced an equivalent or stronger signal than that of the E6-E7 probe in all areas of the epithelium, however, it would appear that most of the signal produced was extrachromosomal in origin. This observation does not refute the theory that genomic integration of HPV DNA occurs in CIN lesions, or that high-grade CIN develops from a subset of cells containing integrated DNA,[16,23] but it does indicate that such a clonal population cannot be identified readily by obvious differences in HPV 16 early transcription. The observation by Lehn et al that integrated sequences in some high-grade precancers were transcriptionally silent[16] cannot be addressed in this study. The possibility that HPV 16 transcription ceases completely in high-grade precancers, however, was not supported by our observations.

Given the wide distribution of HPV transcripts in precancers, it is conceivable that constant expression of specific HPV gene products is required to initiate and maintain the neoplastic phenotype; however, a hypothesis to explain the morphologic progression of precancerous lesions remains undeveloped. If integration of viral DNA is necessary for the progressive dedifferentiation of precancers and subsequent invasion, the potential mechanism by which this phenomenon contributes to the process is not obvious, given that transcripts derived from extrachromosomal HPV 16 DNA appear to comprise the bulk of HPV 16 transcripts produced in these lesions. The significance of extrachromosomal transcription, however ubiquitous, remains unresolved. McCance et al demonstrated recently that keratinocytes experimentally transformed with HPV 16 contained integrated HPV sequences only, but progressed through a morphologic spectrum closely resembling intraepithelial neoplasia.[24] Schneider-Maunoury and coworkers demonstrated that a cell line established from a vulvar precancer (Bowen's disease) contained only integrated HPV 16 genomes, despite the fact that the original neoplasm contained principally episomal HPV 16 DNA.[25] When transplanted into nude mice, the cell line produced nonmetastasizing lesions similar in behavior to the original neoplasm.[25] They established that viral integration was present in the original lesion as well. Le and Defendi demonstrated recently that integrated HPV 16-host DNA sequences isolated from human tumors transformed NIH 3T3 cells, whereas the host cellular or viral components of the hybrid DNA did not.[26] These studies may challenge the significance

of extrachromosomal HPV 16 replication and transcription in precancerous lesions, and pose intriguing questions about the mechanism by which viral integration influences the development and evolution of precancers.

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