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Transcriptional Differences of the Human Papillomavirus Type 16 Genome between Precancerous Lesions and Invasive Carcinomas

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Human papillomavirus type 16 (HPV16) genome DNA and its transcripts in biopsied cervical neoplasias were analyzed by simultaneous extraction of DNA and RNA from one biopsied sample. Southern blot analysis revealed that 5 of 20 cervical intraepithelial neoplasias (CINs) contained HPV16 DNAs existing primarily as episomes and two of seven invasive carcinomas harbored HPV16 genome sequences integrated into the host DNA. Northern (RNA) blot analysis showed that the HPV16 genome sequences were transcriptionally active in the five CINs, as well as in the two invasive carcinomas. The pattern of HPV16-specific transcripts in the CINs was uniform, and the major transcripts were 4.2, 2.2, 1.6, and 1.4 kilobases in size. However, the pattern of HPV16-specific transcripts in the invasive carcinomas was variable and different from that in CINs, suggesting that the alteration of transcriptional pattern might play a key role in the development of malignancy.

The human papillomavirus (HPV) is a heterogeneous group and induces the proliferation of human epithelium at various body sites, which are linked to specific types (4). Several types of HPV are associated with the lesions of the uterine cervix. For instance, HPV types 6 and 11 (HPV6 and HPV11) are known as causative agents of condyloma acuminata, and HPV16, HPV18, HPV31, HPV33, and HPV35 are closely associated with cervical carcinoma and its precursors, known as cervical intraepithelial neoplasias (CINs) (2, 3, 8, 11, 12, 14, 15, 21, 24). The viral DNA of HPVs associated with these cervical lesions has been molecularly cloned, and the sequences of several types, including HPV16, have been published (20).

Among HPVs associated with the CIN and cervical carcinoma, HPV16 is the most prevalent type and has been considered to be one of the candidates for a causative agent of cervical carcinoma. In CINs, HPV16 DNA is present preferentially as episomes, often coexisting with integrated viral genomes (6, 21). In contrast, in cervical carcinoma tissues, HPV16 DNA that is integrated into host DNA with or without episomal form is frequently found (9, 13, 16, 21, 23). Furthermore, most cervical carcinoma cell lines harbor exclusively integrated HPV16 or HPV18 DNA sequences (1, 3, 7, 17–19, 22, 23, 25, 26).

The HPV16-specific transcripts which are coded from the E6 and E7 open reading frames (ORFs) have been identified in invasive carcinoma tissues and cervical carcinoma cell lines (1, 22, 23), and the integrated viral sequences in invasive carcinoma tissues have been reported to be not always transcriptionally active (13, 19). However, the transcripts of the episomal HPV16 genome in the precancerous lesion, CIN, have not yet been characterized.

Since HPVs have never been successfully propagated in cell cultures, the natural transcripts of HPVs can be analyzed only with tissues infected with these viruses. However, the study of HPV transcription has been hampered by the difficulty in recovering undegraded RNA from natural warts of heavily keratinized epithelia, in which only a small percentage of the cells support viral gene expression (4). In addition, CIN is limited in a flat intraepithelial lesion, and available specimens are usually too small for simultaneous analysis of both the viral transcripts and genome DNA.

We therefore improved a technique for the simultaneous extraction of undegraded RNA and DNA from a small tissue sample, which allowed the analysis of HPV DNA and HPV-specific transcripts in CINs and invasive carcinomas. Here we report the mapping of the HPV16-specific transcripts in these cervical lesions and the transcriptional difference between CIN and cervical invasive carcinoma tissues.

MATERIALS AND METHODS

Cell culture. The human cervical carcinoma cell lines QG-H and QG-U, which harbor transcriptionally active integrated HPV16 DNA sequences, were cultured in Eagle basal medium supplemented with 10% fetal calf serum (22).

Tissue specimens. Tissue specimens were collected during colposcopy at the Chiba Anti-Cancer Society. A part of the tissue was fixed in 10% Formalin and diagnosed histopathologically, and another part was immediately stored in a liquid nitrogen container.

Isolation of DNA and RNA. Tissue specimens were ground in liquid nitrogen to a fine powder and lysed quickly in 5 M guanidinium thiocyanate–25 mM sodium citrate–1% 2-mercaptoethanol–0.5% sodium *N*-lauroyl sarcosinate (pH 7.3) at 60°C. Cesium chloride (0.2 g/ml) was added to the lysate and dissolved by gentle homogenization. Debris was removed by centrifugation at 10,000 rpm for 30 min at 20°C in an SW50-1 rotor. The supernatant was layered on a 2-ml cushion of 5.7 M CsCl–0.1 M EDTA (pH 6.8) and centrifuged at 35,000 rpm for 18 h at 20°C in an SW50-1 rotor. The banded DNA fraction was dialyzed against 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–0.1% sodium dodecyl sulfate. The DNA was further purified by digestion with proteinase K (200 μg/ml) at 37°C overnight, extraction with phenol-chloroform, and precipitation with ethanol (22). The pelleted RNA was dissolved in distilled water and precipitated with ethanol. RNAs from cell lines were extracted by

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the guanidinium-cesium chloride method as described previously (22).

DNA and RNA blot analyses. For Southern blotting, DNA from tissues was digested with an excess amount of appropriate restriction enzymes and electrophoresed on a 0.5 or 0.8% agarose gel. The separated DNA was denatured, neutralized, and transferred to a nitrocellulose filter overnight.

For Northern (RNA) blotting, total RNAs from tissues or cells were denatured at 60°C for 15 min in 6.3% formaldehyde–50% formamide–5 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0)–15 mM sodium acetate–1 mM EDTA. The denatured RNAs were separated on a 0.8 or 1.0% agarose gel containing 6.3% formaldehyde–20 mM MOPS (pH 7.0)–15 mM sodium acetate–1 mM EDTA. The separated RNA was transferred to a nitrocellulose filter overnight.

The blotted filters were baked at 80°C for 2 h. The hybridization and autoradiography were carried out as described previously (22).

HPV DNAs and DNA fragments of HPV16. HPV6, HPV11 (5, 10), and HPV16 (8) DNAs were separated from vectors through low-melting-point agarose gels. For the preparation of subgenomic fragments of HPV16, HPV16 DNA-pBR322 was cleaved with *Pst*I, and 3.8-, 2.8-, 2.0-, 1.8-, and 1.1-kilobase (kb) fragments were purified from low-melting-point agarose gels. The subgenomic probes other than the 1.1-kb *Pst*I fragments (containing E2, E5, and L2 ORFs) were purified from low-melting-point agarose gels after further digestion as follows. The 3.8- and 2.0-kb fragments were further digested with *Bam*HI; then 0.9- and 0.6-kb fragments (containing L2 and L1 ORFs) were purified. The 2.8-kb fragment was digested with *Ava*II; then 1.8-kb (containing E1 ORF) and 1.0-kb (containing E2 and E4 ORFs) fragments were purified. The 1.8-kb fragment was digested with *Eco*RI; then the 1.3-kb fragment (containing the noncoding region, E6, and E7 ORFs) was purified. The probe DNAs were labeled by nick translation with [^{32}P]dCTP as described previously (22).

RESULTS

Simultaneous extraction of RNA and DNA. By the procedure described in Materials and Methods, about 30 μg of undegraded DNA and RNA could be extracted from a piece of the cervical tissue approximately 3 mm^3 in volume. The integrity of DNA and RNA was confirmed by electrophoresis, followed by staining with ethidium bromide. Our procedure proved to work sufficiently for the detection of c-*myc* expression in a biopsied normal cervical tissue (data not shown).

Analysis of DNAs from cervical lesions. The DNAs extracted from CINs and invasive carcinoma specimens were screened for the presence of HPV16 DNA. Typical HPV16 DNA fragments were detected in the *Pst*I-digested DNA from 5 of 20 CIN specimens and from 2 of 7 invasive carcinoma specimens (Fig. 1A). The *Pst*I-digested DNAs from the two invasive carcinomas (IC1 and IC2) contained additional fragments hybridizing to HPV16 DNA; the 2.8-kb *Pst*I fragment in IC1 was submolar, and that in IC2 was lacking (Fig. 1A, lanes 6 [IC1] and 7 [IC2]). When digested with a noncutting enzyme, *Hin*dIII viral DNA was detected as forms I and II in the five CINs (Fig. 1B, lanes 1 to 5) and as a slow-migrating single fragment in the two invasive carcinomas (Fig. 1B, lanes 6 and 7). The digestion of the DNA with a single-cut enzyme, *Bam*HI, yielded the 7.9-kb fragment, full genome size in the CINs (Fig. 1C, lanes 1 to 5), the 7.9-kb fragment and an additional fragment in IC1 (Fig. 1C, lane 6), and a fragment larger than 7.9 kb in IC2 (Fig. 1C, lane 7).

These results may be interpreted to mean that the HPV16 genome DNA exists primarily as episomes in the five CINs and is integrated in the two invasive carcinomas disrupting the 2.8-kb *Pst*I subgenomic region, which contains the E1 and E2 ORFs.

Analysis of RNA from cervical lesions and cell lines. The RNA extracted from cervical lesions containing HPV16 DNA, as well as from a condyloma acuminatum and cervical

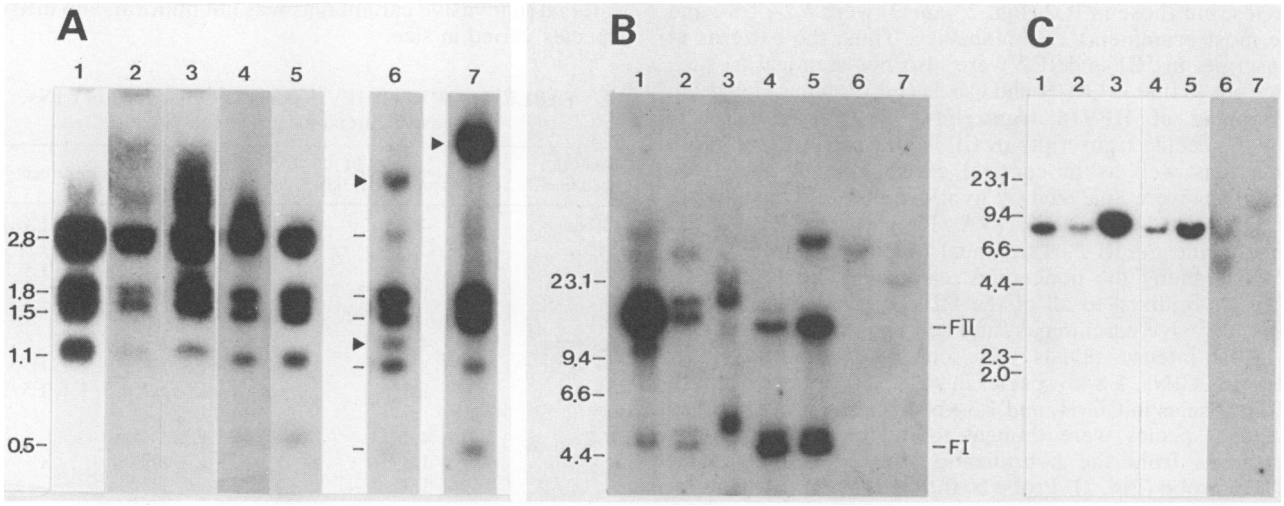

FIG. 1. Hybridization analysis of the DNA from cervical neoplasias with HPV16 DNA as a probe. DNAs (4 μg for lane 3 of panel B and 7.5 μg for the others) extracted from CINs (lanes 1 to 5) and invasive carcinomas (lanes 6 [IC1] and lanes 7 [IC2]) were digested with *Pst*I (A), *Hin*dIII (B), or *Bam*HI (C); separated on a 0.8% (A) or a 0.5% agarose gel (B and C); and subjected to Southern blot analysis. Bars indicate the positions of *Pst*I fragments of cloned HPV16 DNA (A) or fragments of lambda phage DNA digested with *Hin*dIII (B and C). FI and FII indicate the positions of forms I and II, respectively. Arrowheads indicate the additional fragments in *Pst*I-digested DNAs from invasive carcinomas. The numbers at the left of each panel represent the sizes of the fragments in kilobases.

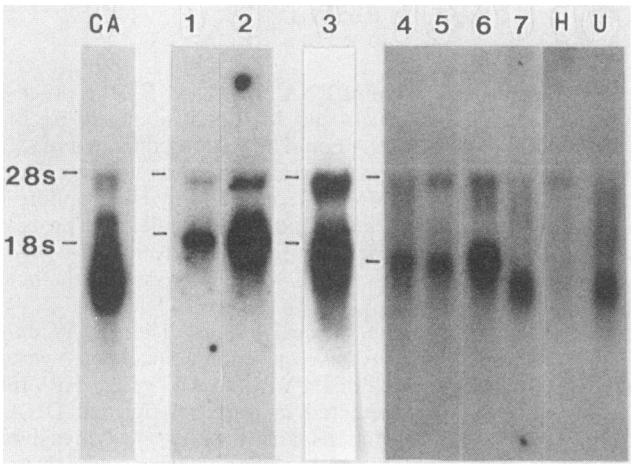

FIG. 2. Northern blot analysis of HPV-specific RNA in cervical lesions and cervical carcinoma cell lines. The total RNAs (10 μg) from the same cervical neoplasia tissues as indicated in the legend to Fig. 1 (lanes 1 to 7), from a condyloma acuminatum (lane CA), and from cervical carcinoma cell lines (lane H [QG-H] and lane U [QG-U]) were separated on a 0.8% (lanes 1 to 3) or 1.0% (lanes CA, 4 to 7, H, and U) agarose gel and transferred to nitrocellulose filters. The blotted filters were hybridized with a mixture of HPV6 and HPV11 DNAs (lane CA) or with HPV16 DNA (lanes 1 to 7, H, and U). The bars indicate the positions of 28 and 18S ribosomal RNAs.

carcinoma cell lines QG-H and QG-U, was subjected to Northern blot analysis. The HPV16-specific transcripts were detected in all CINs and invasive carcinomas which harbored HPV16 DNA. The HPV16-specific transcripts in the CINs were homogenous; they were 4.2-, 2.2-, 1.4-, and (the most prominent) 1.6-kb species (Fig. 2, lanes 1 to 5) and were different from the viral transcripts in a condyloma acuminatum, which were 4.2-, 2.6-, 1.8-, and (the most prominent) 1.4-kb species (Fig. 2, lane CA). The pattern of the HPV16-specific transcripts in the invasive carcinomas was not identical to that in CINs. The transcripts in IC1 (Fig. 2, lane 6) were 4.2-, 3.4-, and (the most prominent) 1.8-kb species, but those in IC2 (Fig. 2, lane 7) were 4.2-, 1.8-, and (the most prominent) 1.3-kb species. Thus, the patterns of transcripts in IC1 and IC2 were also not identical to each other nor to that in QG-H and QG-U (Fig. 2, lanes H and U).

Mapping of HPV16 transcripts. To characterize the HPV16-specific transcripts in CINs and the invasive carcinomas, as well as in cervical carcinoma cell lines, the transcripts were analyzed by hybridization to five subgenomic fragments of HPV16 DNA (Fig. 3A, probes a to e). Probe a, the *Eco*RI-*Pst*I fragment (nucleotides [nt] 7453 to 875) containing the noncoding region and the E6 and E7 ORFs, hybridized to all of the HPV-specific transcripts in CINs, invasive carcinomas, and cell lines (Fig. 3B, column a). Most intense signals were observed with the 1.6-kb species in CINs, 1.8-kb species in IC1, 1.3-kb species in IC2, 4.2-kb species in QG-H, and 1.3-kb species in QG-U, and all of these species were thought to be the most abundant transcripts from the hybridization to the whole-genome HPV16 probe (Fig. 2). Probe b, the *Pst*I-*Ava*II fragment (nt 875 to 2713) containing the E1 ORF, hybridized to the 4.2-kb transcripts in CINs and invasive carcinomas, though HPV-specific transcripts in cell lines failed to hybridize to this probe (Fig. 3B, column b). Probe c, the *Ava*II-*Pst*I fragment (nt 2713 to 3692) containing the E2 and E4 ORFs, hybridized to all transcripts in CINs but hybridized only to 3.4- and 1.8-kb transcripts in IC1 and to 2.4-, 2.0-, and 1.3-kb transcripts in QG-U (Fig. 3B, column c). Probe d, the 1.1-kb *Pst*I fragment (nt 3692 to 4755) containing the E5 ORF, part of the L2 ORF, and the early-region polyadenylation signal, hybridized to all of the HPV-specific transcripts in CINs. In contrast, HPV-specific transcripts in invasive carcinomas and cervical carcinoma cell lines were not hybridized to this probe except to the 3.4- and 1.8-kb transcripts in IC1 (Fig. 3B, column d). Finally, probe e, the 1.5-kb *Pst*I fragment (nt 5238 to 6787) containing the putative late region exclusively, failed to hybridize to all of the HPV-specific transcripts in CINs, as well as in invasive carcinomas (Fig. 3B, column e). This probe also did not hybridize to the transcripts in QG-H and QG-U, as we reported previously (22).

The results of these hybridization experiments are summarized in Table 1.

DISCUSSION

HPV16 genome DNA exists preferentially as episomes in CINs but primarily in the integrated state in invasive carcinomas. The different physical states of the HPV16 genome between the CINs and the invasive carcinomas may play a key role in the development of malignancy. To test this speculation, it is important to analyze the physical state of viral genomes and their transcripts simultaneously. Because CIN lesions are generally restricted to a relatively small intraepithelial area of uterine cervix, the available tissue is often too small to divide into two pieces for the extraction of DNA and RNA. To overcome this problem, a method for the simultaneous extraction of intact DNA and RNA from one sample was improved, which allowed us to successfully analyze transcripts from the episomal HPV16 genome in CINs and also from integrated viral sequences in invasive carcinomas.

The HPV16 sequences were found to be transcriptionally active in all of the tissues tested, including two invasive carcinomas, though it has been reported that HPV16 DNA is not always expressed in cervical carcinomas (13, 19). The pattern of HPV16-specific transcripts was uniform in CINs, but that in invasive carcinomas was not uniform, and mRNA species varied in size.

TABLE 1. ORFs of HPV16 genome transcribed in CINs, invasive carcinomas, and cell lines[a]

Tumor or cell line	Size of mRNA(s) (kb)	ORFs transcribed
CIN	4.2	E6, E7, E1, E2, E4, E5
	2.2	E6, E7, E2, E4, E5
	1.6	E6, E7, E2, E4, E5
	1.4	E2, E4, E5
IC1	4.2	E6, E7, E1
	3.4	E6, E7, E2, E4, E5
	1.8	E6, E7, E2, E4, E5
IC2	4.2	E1
	1.8	E6, E7
	1.3	E6, E7
Cell line		
QG-H	4.2, 2.4, 1.2	E6, E7
QG-U	5.0, 3.6	E6, E7
	2.4, 2.0, 1.3	E6, E7, E2, E4

[a] Refer to Fig. 3.

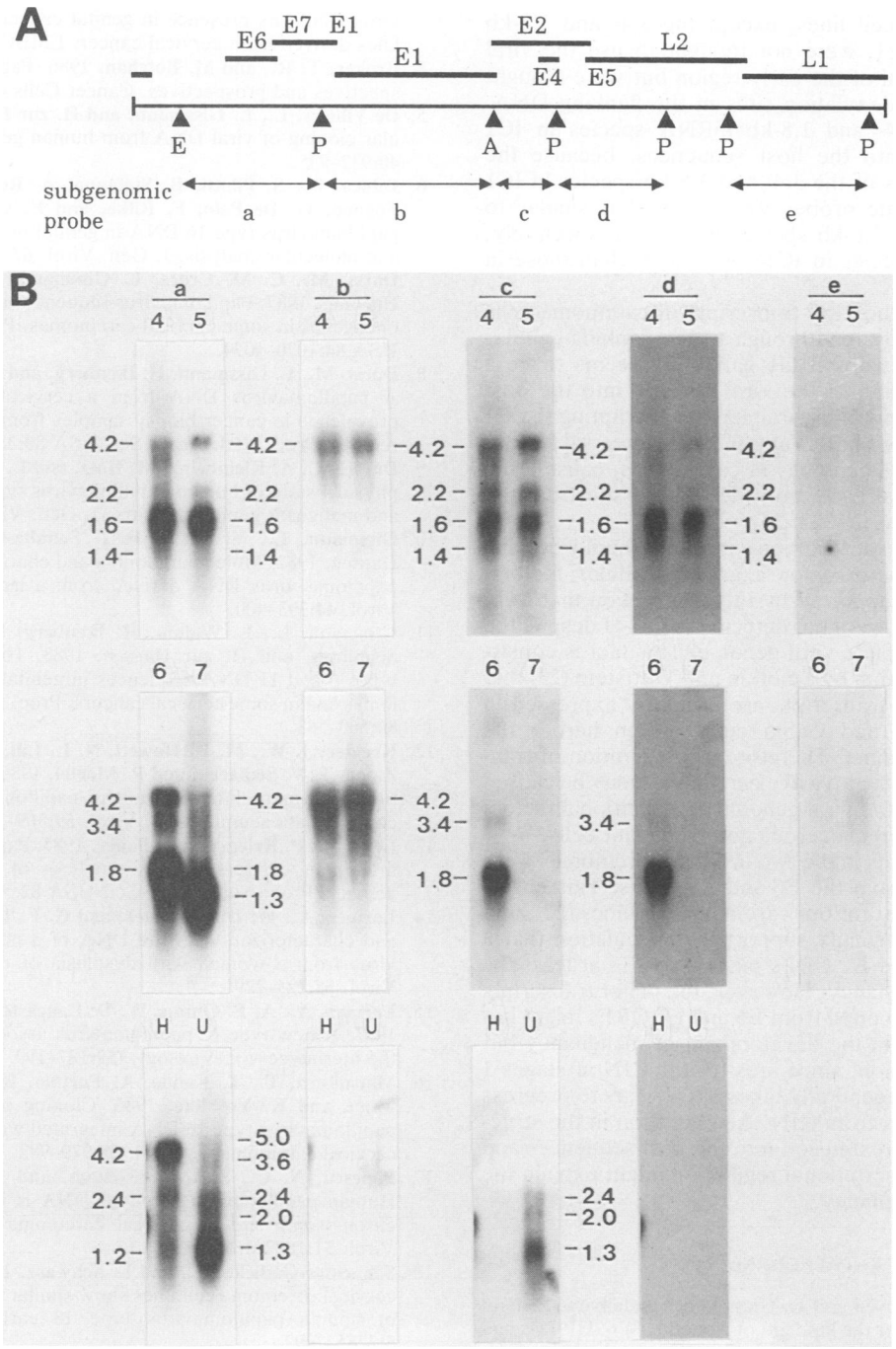

FIG. 3. Mapping of the HPV16-specific transcripts in CINs, invasive carcinomas, and cervical carcinoma cell lines by hybridization to subgenomic fragments of HPV16 DNA. (A) Restriction map of HPV16 DNA and subgenomic probes. Symbols: ▲, site of the restriction enzymes *Eco*RI (E), *Pst*I (P), and *Ava*II (A); ↔, subgenomic probes indicated (a to e). (B) Northern blotting. Total RNAs (10 μg) from the same cervical neoplasia tissues as indicated in the legend to Fig. 1 (lanes 4 and 5, CIN; lanes 6 and 7, invasive carcinoma) and from cell lines (lanes H [QG-H] and lanes U [QG-U]) were electrophoresed on 1.0% agarose gel and subjected to Northern blotting. The blotted filters were hybridized to the individual subgenomic probes indicated at the top of the columns (a to e). The bars indicate the transcripts hybridized to the probes, and the accompanying numbers represent the sizes of the transcripts in kilobases.

The most abundant (1.6-kb) mRNA species in CINs was possibly transcribed from the E6, E7, E2, E4, and E5 ORFs (Table 1), and the 4.2-kb transcript in CINs is estimated to cover all of the early ORFs. The less prominent 2.2-kb species is thought to be transcribed mainly from the E2, E4, and E5 ORFs, as well as from the E6 and E7 ORFs; the 1.4-kb transcript, which is low in quantity, is coded primarily from the E2, E4, and E5 ORFs. All of these transcripts are thought to use the putative polyadenylation signal of the early region, because all of the transcripts hybridized to probe d but not to probe e.

The HPV-specific transcripts in the two invasive carcino-

mas and carcinoma cell lines, except the 3.4- and 1.8-kb mRNA species in IC1, were not thought to use the viral polyadenylation signal of the early region but were thought to use cellular polyadenylation sites in the flanking DNA. Furthermore, the 3.4- and 1.8-kb mRNA species in IC1 might also extend into the host sequences, because the hybridization patterns of the 3.4- and 1.8-kb species in IC1 for all the subgenomic probes were somewhat similar to those of the 2.2- and 1.6-kb species in CINs, respectively, but the two RNA species in IC1 were larger than those in CINs.

The variability of the viral transcripts in carcinoma cells seems to be caused by readthrough to the flanking cellular sequences. In other words, this variability seems to be a result of the integration of the viral genome into the host DNA. The integration of the viral genome disrupting the E1 and E2 region observed in IC1 and IC2 is a universal feature of HPV16 and HPV18 genomes in carcinomas, causing the separation of the viral early polyadenylation site from the early promoter (13, 16, 19, 22, 23).

This alteration of transcriptional pattern should be also affected by other factors—for example, cellular factors. Such speculation is supported by the observation that transcripts found in CINs were not detected in QG-H despite the existence of the complete viral genome. This fact is consistent with the observation by Smotkin and Wettstein (23) that the virus-host fusion transcripts are primarily expressed in invasive carcinomas and CaSki cells, which harbor the complete HPV16 genome. Therefore, the alteration of transcriptional pattern in the invasive carcinomas may be caused by the integration of the viral genome and additional factors that are different in precancerous and malignant cells.

The major transcripts in the two invasive carcinomas were transcribed at least from the E6 and E7 ORFs. This observation agrees with that for our carcinoma cell lines (22), and our present results strongly support the speculation that a product of the E6 and E7 ORFs plays a role in at least the maintenance of malignancy. However, the major transcripts in CINs that are also coded from E6 and E7 ORFs might not be directly involved in the development of malignancy but with just transformation, since most of the CIN at stages 1 and 2 regresses and it generally takes a few years to progress to invasive carcinoma from CIN. An alteration in the structure of transcripts by extension into the host sequences and the alteration of transcriptional regulation might explain the development of malignancy.

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