Integration and Transcription of Human Papillomavirus Type 16 and 18 Sequences in Cell Lines Derived from Cervical Carcinomas

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SUMMARY

Five cell lines, SKG-I, SKG-II, SKG-IIIb, QG-U and QG-H derived from cervical carcinomas of Japanese patients, were examined for the presence of human papillomavirus (HPV) DNA and the expression of viral mRNA. The DNA of HPV type 16 was shown to be linked covalently with SKG-IIIb, QG-U and QG-H cell DNA, and HPV 18 DNA with SKG-I and SKG-II cell DNA. Although different regions of the HPV genome were integrated in these cell lines, the non-coding region and an early region including the E6 and E7 open reading frames (ORFs) were conserved in all cell lines. The complete genome of HPV 16 was found in QG-H cells by digestion of the DNA with a single-cut restriction enzyme. The other early region ORFs E1, E2, E4 and E5 were interrupted by flanking host cell DNA, suggesting that the integration into host cell DNA occurs preferentially in this region. HPV-specific mRNA species were detected in all five cell lines. In the three cell lines containing the HPV 16 genome, mRNAs hybridized with the early region of the genome, covering the entire E6 and E7 ORFs and a minor part of the E1 ORF, although the amount and size of the major mRNAs varied in these cell lines. These mRNAs did not hybridize with the late region of the HPV genome containing the L1 and L2 ORFs. In SKG-II, SKG-IIIb and QG-H cells we also detected c-myc and c-Ha-ras mRNA expression at about nine times the level of that in normal cells.

The human papillomaviruses (HPVs) induce epithelial proliferative lesions of skin or mucosa and have been classified into more than 40 types on the basis of their DNA sequence homology. Complete nucleotide sequences and genome structures of at least six types of HPV DNA have been reported so far (Schwarz et al., 1983; Seedorf et al., 1985).

HPV type 16 and 18 DNAs, which have been molecularly cloned from cervical carcinomas, are closely associated with cervical dysplasia and cervical carcinoma (Dürst et al., 1983; Boshart et al., 1984; Tomita et al., 1986; Shirasawa et al., 1986) and parts of the genomes have been found integrated in host cell DNA from these lesions, whereas HPV 6, 11 and 16 DNAs in benign lesions such as condylomata acuminata and Bowenoid papulosis are present exclusively as monomeric or oligomeric episomes (Boshart et al., 1984; Dürst et al., 1985; Lehn et al., 1985; Matsukura et al., 1986; Shirasawa et al., 1986). Integrated genomes of HPV 16 and 18 have also been observed in cell lines derived from cervical carcinomas (Boshart et al., 1984; Schwarz et al., 1985; Yee et al., 1985; Pater & Pater, 1985; Tsunokawa et al., 1986a). These results strongly support the hypothesis that integration of these viral DNAs plays a key role in the development of cervical carcinoma and maintenance of the malignant state.

Viral RNA transcripts have been detected in some cell lines as well as cervical carcinoma tissues (Schwarz et al., 1985; Yee et al., 1985; Lehn et al., 1985; Tsunokawa et al., 1986a), indicating that the integrated viral genomes are transcriptionally active. The viral transcripts, however, have not been studied in detail. The mechanism by which intraepithelial neoplasia
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progresses to carcinoma is still unknown, although HPV 16 DNA sequences were recently reported to have transforming activity (Tsunokawa et al., 1986b). This progression may involve cooperation between viral oncogenes and cellular oncogenes (Riou et al., 1985).

In this work we analysed the physical state of integrated HPV genomes, the expression of mRNA from these viral genomes and mRNA expression from proto-oncogenes in cell lines derived from cervical carcinomas of Japanese patients. We found that integration of the HPV genome into host cell DNA occurs preferentially, disrupting the early region including the E1, E2, E4 and E5 open reading frames (ORFs), but the non-coding region and ORFs E6 and E7 are well conserved and are transcribed. We discuss these results in connection with malignant transformation and its maintenance.

SKG-I (Taguchi, 1981), SKG-II (Ishiwata et al., 1978), SKG-IIIb (Nozawa et al., 1983), QG-U and QG-H (Matsunaga et al., 1979) are established cell lines derived from cervical carcinomas of Japanese patients. These five cell lines, and HeLa and FL cells were cultured in Eagle's basal medium supplemented with 10% foetal calf serum. High molecular weight total cellular DNA was extracted using phenol/chloroform/isoamyl alcohol as described before (Tomita et al., 1986; Shirasawa et al., 1986). Total RNA was prepared by the guanidinium thiocyanate/caesium chloride method. The cells were lysed in a solution containing 6 M-guanidinium thiocyanate, 5 mM-sodium citrate, 0.5% sodium lauryl sarcosinate and 0.1 M-2-mercaptoethanol. The lysates were layered on 5.7 M-caesium chloride solution containing 0.1 M-EDTA and centrifuged at 36000 r.p.m. in an SW50-1 rotor at 20 °C for 14 h. The pellet was washed with 95% ethanol, suspended in distilled water and RNA was then precipitated with ethanol. Polyadenylate [poly(A)+]-containing RNA was obtained by two cycles of chromatography on oligo(dT)-cellulose (type 7, Pharmacia). Southern blotting was carried out as described previously (Tomita et al., 1986; Shirasawa et al., 1986). For Northern blotting poly(A)+ RNA was suspended in 6·3% formaldehyde, 50% formamide, 20 mM-MOPS pH 7·0, 75 mM-sodium acetate, 5 mM-EDTA and incubated at 60 °C for 15 min. The denatured RNA was then electrophoresed on a 0·8% agarose gel containing 6·3% formaldehyde, 20 mM-MOPS pH 7·0, 75 mM-sodium acetate and 5 mM-EDTA, followed by transfer onto nitrocellulose filters. The filters were baked at 80 °C for 2 h. Ribosomal RNA and lambda DNA digested with HindIII were used as markers. The baked filters were soaked in 3 × SSC and 0·1% SDS at 42 °C and prehybridized at 42 °C overnight in 50 mM-phosphate buffer pH 6·5, 50% formamide, 5 × SSC, 10 × Denhardt's solution and 250 μg/ml sheared denatured calf thymus DNA. Hybridization was carried out in the same solution with the addition of 32P-labelled probe, at 42 °C overnight. The filters were washed four times in 2 × SSC and 0·2% SDS for 5 min then twice in 0·1 × SSC and 0·2% SDS for 30 min. The filters were dried and autoradiographed (Tomita et al., 1986). The Clal/EcoRI 1·5 kb fragment of human c-myc 3' exon DNA (Shibuya et al., 1985) and the SacI DNA fragment of human c-Ha-ras containing four exons (Oncor, Md., U.S.A.) were purified from gels, labelled and used as probes.

To find whether HPV DNA was present, the total high mol. wt. DNA extracted from SKG-I, SKG-II, SKG-IIIb, QG-U, QG-H and HeLa cells was subjected to dot blot hybridization. This revealed that SKG-IIIb, QG-U and QG-H cells harboured HPV 16 DNA sequences and SKG-I, SKG-II and HeLa cells harboured HPV 18 DNA sequences (data not shown). By Southern blot analyses it was shown that the viral DNA in these cells comigrated with high mol. wt. cell DNA (data not shown). Furthermore, Southern blotting after digestion of DNA with BamHI (which cuts HPV 16 DNA once and HPV 18 DNA twice) showed the presence of fragments larger than 7·9 kb (full-length genome size) except in SKG-IIIb DNA. These results suggest that the viral DNA is integrated into host cell DNA (Fig. 1a, c). In addition, in QG-H cells an intense band of 7·9 kb that might represent the viral full-length genome was observed. Digestion of DNA from QG-U and QG-H cells with PstI (a multiple cut enzyme) yielded typical PstI fragments of HPV 16, but no such fragment was detected in SKG-IIIb DNA, suggesting that multiple rearrangements or deletions of the HPV sequence had occurred in this DNA (Fig. 1b).

To determine which parts of the HPV 16 and 18 genomes were present in these cells, Southern blot hybridizations were carried out using subgenomic fragments as probes. For HPV 16 the 2·8 kb, 1·8 kb, 1·5 kb, 1·1 kb and 0·5 kb PstI fragments were prepared and for HPV 18 five
Fig. 1. Hybridization analysis of DNA from cell lines harbouring HPV. (a, b) DNAs from SKG-IIIb (lanes 1), QG-U (lanes 2) and QG-H (lanes 3) cells were digested with BamHI (a) or PstI (b), then analysed by Southern blot hybridization with HPV 16 DNA. (c) DNAs from SKG-I (lane 1), SKG-II (lane 2) and HeLa (lane 3) cells were digested with BamHI, then subjected to Southern blot hybridization with HPV 18 DNA. The bars indicate HindIII fragments of lambda DNA of 23, 9.4, 6.6, 4.4, 2.3 and 2.0 kb. The arrowheads indicate the positions of PstI fragments of HPV 16 DNA.

BamHI, EcoRI and HindII fragments were prepared following the method of Schwarz et al. (1985) as shown in Fig. 2. The blot hybridization data are shown in Fig. 2(a) to (c) and the results are schematically summarized in Fig. 2(d) and (e). In SKG-I, SKG-II, QG-U and QG-H DNAs, most or even all of the genome DNA was retained, but in SKG-IIIb DNA only subgenomic regions which hybridized with the 1.5 kb and 1.8 kb PstI fragments were retained Fig. 2(a).

In QG-H DNA it appears that viral genomic DNA was integrated in two patterns, one multimeric and the other monomeric. As shown in Fig. 2(b) the 7.9 kb fragment produced by digestion with the single-cut enzyme BamHI hybridized with all PstI subgenomic probes and is thought to represent a complete genome. The F3 fragment showed lower signal intensities than did F4 with the 1.5 kb probe, probably because of rearrangements of the viral genome. The virus-cell DNA junction is estimated to be within the 0.5 kb PstI fragment, because the F3 fragment showed a faint signal with the 0.5 kb probe. This faint signal did not result from contamination with other probes because no similar faint band was observed in lane U with the 0.5 kb probe. Thus, the F3 fragment is assumed to be an integrated monomeric viral genome that has lost the BamHI site.

SKG-I and SKG-II originated from cervical carcinomas of different patients, but the Southern blot patterns were identical as reported by Tsunokawa et al. (1986 a). The organization of the integrated viral genomes was also identical (Fig. 2c).

As shown in Fig. 2(d) and (e) the integration sites deduced from blot hybridization experiments were localized within the E1, E2, E4 and E5 ORFs. Each cell line retained the non-coding region and the L1, E6 and E7 ORFs.
To analyse transcription of the integrated viral genomes, poly(A)+ RNAs from SKG-IIIb, QG-U and QG-H cells were analysed by Northern blotting using the 1-3 kb EcoRI/PstI fragment (see Fig. 3a; non-coding region, E6 and E7 ORFs) and the 1.5 kb PstI fragment (see Fig. 3b; L1 and L2 ORFs) of HPV 16 as probes. As shown in Fig. 3(a) and (b), RNA transcripts hybridizing with the 1.3 kb fragment were detected in each cell line, whereas no RNA transcript hybridized with the 1.5 kb fragment. The sizes of these transcripts were characteristic of each cell line. Major mRNA species measured 6-4 kb, 3-3 kb and 1.9 kb in SKG-IIIb cells, 6-0 kb, 2.7 kb and 1.8 kb in QG-U cells, and 8.4 kb, 6.5 kb and 4.8 kb in QG-H cells. Minor mRNA species larger than 8-0 kb were observed in QG-U and QG-H cells (Fig. 3a). Tsunokawa et al. (1986a) have previously detected mRNA expressed by HPV 18 in SKG-I and SKG-II cells, with whole genome DNA as the probe. However, the Northern blot patterns were not identical with those of the mRNAs from HPV 16 expressed in SKG-IIIb, QG-U and QG-H cells. We tested the expression of the early region of the HPV 18 genome in SKG-I, SKG-II and HeLa cells by dot blot hybridization analysis. As shown in Fig. 3(c), RNA transcripts in each of these cells hybridized with the 2.4 kb fragment (see Fig. 3a; non-coding region, E6, E7 and E1 ORFs). The levels of the RNA transcript in SKG-II and HeLa cells were about twice and 20-fold higher than that in SKG-I.

The expression of c-myc and c-Ha-ras proto-oncogenes in these five cell lines as well as in HeLa and FL cells was tested by dot blot RNA–DNA hybridization analysis. As shown in Fig. 4, in SKG-II, SKG-IIIb and QG-H cells ninefold elevation of c-myc and c-Ha-ras mRNA levels was observed compared to normal lymphocytes, but in HeLa cells it was about 30-fold higher than that of normal cells.

HPV 16 or 18 genomes were found integrated in all the cells examined here, but the retained regions of the genomes were variable. Most or all of the HPV genome was retained in SKG-I, SKG-II, QG-U and QG-H cells. Particularly in QG-H cells, a multimeric tandem repeat of the whole genome seems to be retained, because a 7-9 kb genome-size fragment which hybridized with all PstI fragments was detected. However, in SKG-IIIb cells, only a small part of the genome including the non-coding region and ORFs E6 and E7 was retained and the other part of the early region (E1, E2, E4 and E5 ORFs) had been deleted (Fig. 2d). From these observations, it appears that maintenance of the malignant state may require a specific HPV DNA region.

A site at which recombination with host cell DNA occurred could not be specified precisely, but the region disrupted by host cell DNA generally falls into the early region including the E1, E2, E4 and E5 ORFs, suggesting that the recombination and integration occur preferentially at these ORFs. These patterns of integration coincide with those previously reported in cervical carcinomas (Lehn et al., 1985; Matsukura et al., 1986) and in HeLa, C4-1 and 756 cells (Schwarz et al., 1985). In addition, conservation of intact ORFs E6 and E7 and of the non-coding region seems to be a general phenomenon, because this conservation has been observed not only in the five cell lines examined in this work but also in other cell lines (Schwarz et al., 1985) and in cervical carcinoma tissues (Lehn et al., 1985, Matsukura et al., 1986).

Fig. 2. Organization of HPV genomes integrated in cervical carcinoma cell lines. (a to c) Southern blot analysis of cell DNAs with subgenomic fragments of HPV DNA. DNAs from SKG-IIIb (a: lanes Illb), QG-U (b: lanes U), QG-H (b: lanes H), SKG-I (c: lanes I) and SKG-II (c: lanes II) cells were digested with PstI (a) or BamH1 (b, c), then subjected to Southern blot analysis with PstI subfragments of HPV 16 DNA (a, b) or BamH1/EcoRI/HincII subfragments of HPV 18 DNA (c) as probes. The sizes of the fragments used as probes are indicated at the bottom in kb. The bars indicate size markers as shown in Fig. 1. Individual fragments detected in QG-H DNA (b: lane H) are indicated by arrowheads (F1, F2, F3 and F4). (d, e) Schemes for the probable integration patterns of HPV genomes. HPV 16 DNA (d) or HPV 18 DNA (e) retained in each of the indicated cell lines is represented by solid lines. Cellular DNAs are indicated by zigzag lines and possible regions of virus–cell DNA junctions are shown by dashed lines. The lines F1, F2, F3 and F4 represent the fragments of QG-H DNA indicated in (b) lanes H, and the vertical bars indicate the BamH1 sites. The ORFs are given on the top [d: HPV 16 ORFs; e: HPV 6b ORFs assigned to HPV 18 restriction fragments (Schwarz et al., 1985)]. The restriction maps of HPV 16 (d) and HPV 18 (e) are presented in the middle, indicating the cleavage sites for PstI (●), BamH1 (○), HincII (□) and EcoRI (■). The sizes of subgenomic fragments are given in kb.
Fig. 3. Hybridization analysis of RNA from cervical carcinoma cell lines. (a, b) Poly(A)+ RNA from SKG-IIIb (lanes 1), QG-U (lanes 2) and QG-H (lanes 3) cells was subjected to Northern blot hybridization with probe a, the subgenomic 1.3 kb EcoRI/PstI fragment (a) or probe b, the 1.5 kb PstI fragment (b) of HPV 16 DNA. 28S and 18S ribosomal RNAs were used as size markers. The ORFs and restriction map of HPV 16 DNA are given at the bottom, indicating the cleavage sites for PstI (■) and EcoRI (●). (c) Dot blot analysis of total RNA from cells. Total RNA (9, 3 and 1μg) from SKG-I, SKG-II and HeLa cells was blotted onto a nitrocellulose filter and hybridized with the 2.4 kb EcoRI/HincII fragment of HPV 18 DNA as probe.

Schwarz et al. (1985) reported that two of three cervical carcinomas contained virus-specific transcripts. Lehn et al. (1985) also reported that viral mRNA species were expressed in only one of four cervical carcinoma tissues that contained exclusively integrated HPV 16 DNA. From these observations they speculated that the maintenance of the malignant nature of cervical carcinoma does not necessarily depend on continuous transcription of the viral genome. In contrast, viral mRNAs are transcribed in most other cell lines reported so far (Schwarz et al., 1985; Yee et al., 1985; Tsunokawa et al., 1986a) and in our five cell lines. Therefore, a continuous activation of viral promoters or expression of gene products from the early region could be required at least for the maintenance of malignant cells that are able to grow in vitro.

Variability of mRNA size in cervical carcinoma cell lines and in tissues has been observed (Schwarz et al., 1985; Yee et al., 1985; Tsunokawa et al., 1986a). In SKG-IIIb, QG-U and QG-H cells, the major mRNAs differed in size, and some transcripts larger than 6.0 kb hybridized with the early region of the viral genome. These facts suggest that transcription continues into
Fig. 4. Dot blot analysis of total RNAs from cells with proto-oncogenes as probes. Total RNA (9, 3 and 1 μg) from SKG-I, SKG-II, SKG-IIIb, QG-U, QG-H, FL, normal lymphocyte (NL) and HeLa cells was blotted onto a nitrocellulose filter and hybridized with 32P-labelled proto-oncogene c-myc (a) or c-Ha-ras (b).

flanking cellular sequences and produces chimeral mRNA encoding viral and cellular sequences. This could be the case also in the HeLa, 756 and C4-1 cell lines (Schwarz et al., 1985).

It is known that the expression of late genes L1 and L2 which are thought to encode capsid proteins is suppressed in cultured cells, whether the viral genomes are stably maintained as plasmids or integrated (Schwarz et al., 1985; Smotkin & Wettstein, 1986). This was confirmed with QG-H cells in which the whole HPV genome seemed to be retained tandemly repeated. In SKG-IIIb and QG-U cells, cellular genes located upstream of the integrated HPV genome do not show any ability to cause expression of the downstream L2 and L1 ORFs.

Tsunokawa et al. (1986b) showed that a subgenomic fragment of HPV 16 DNA integrated in cervical carcinoma cell DNA had a transforming activity. The fragment was 3.5 kb in size and included the non-coding region and the E6 and E7 ORFs. Recently, direct identification by immunological methods of early gene products in cultured cells has been reported, for example the E6 gene product of bovine papillomavirus type 1 in mouse cells (Androphy et al., 1985) and the E7 gene product in the cervical carcinoma cell line CaSki (Smotkin & Wettstein, 1986). From these results it is possible to postulate that a product of the 5'-proximal early region (E6 and E7 ORFs) plays an important direct or indirect role in the development and maintenance of cervical carcinoma. In addition, amplification of c-myc and c-Ha-ras proto-oncogenes and enhanced expression of their products might cooperate in the advance of the carcinoma (Riou et
al., 1985). However, in three out of five cell lines examined in this work only slightly enhanced expression of c-myc and c-Ha-ras was observed. Thus, these oncogene products may not necessarily be required for these cells to maintain their growth in vitro. There remains, however, a possibility that HPV 16 sequences, especially enhancer elements or viral promoters, activate other cellular genes and induce transformation. If viral gene products are required for the maintenance of the transformed state and growth in vitro, the E6 and E7 gene products might be the most probable candidates, because these ORFs are intact and transcriptionally active in all five cell lines examined.

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