Relative enhancer activity and transforming potential of authentic human papillomavirus type 6 genomes from benign and malignant lesions

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Human papillomavirus type 6 (HPV-6) is predominantly associated with benign genital warts whereas HPV-16 and HPV-18 are detected predominantly in carcinomas of the lower genital tract; HPV-6 is found rarely in such carcinomas. Experiments were designed to discriminate between two hypotheses concerning the role of HPV-6 in the genesis of a genital tract carcinoma: (i) the HPV-6 in the carcinoma (HPV-6-T70) differs genetically from HPV-6 in a benign lesion (HPV-6-W50), giving HPV-6-T70 properties similar to those of HPV-16/18; (ii) HPV-6-T70 and HPV-6-W50 have similar biological activity, suggesting that the role of HPV-6 in oncogenesis is different from that of HPV-16/18. Restriction enzyme digestion and DNA sequence determination established that HPV-6-T70 differs from HPV-6-W50 in the upstream regulatory region (URR) but not in the proteins encoded by open reading frames (ORFs) E5, E6 or E7, ORFs implicated in oncogenesis. To determine whether the difference in the URR sequence could alter the level of expression of viral genes, the URs were cloned into the enhancer-less plasmid pSVect. Analysis of chloramphenicol acetyltransferase activity after transfection into HeLa and Vero cells showed that the URs had comparable enhancer activity. Cotransfection of baby rat kidney (BRK) cells with HPV-6-T70 and an activated ras gene indicated that, in contrast to HPV-16 and HPV-18, this HPV-6 genome could not cooperate with ras to transform BRK cells. The data suggest that HPV-6-T70 and HPV-6-W50 have similar enhancer activity and transforming potential.

Introduction

Human papillomavirus types 16 and 18 (HPV-16 and HPV-18) are found predominantly in cervical carcinomas (Boshart et al., 1984; Dürst et al., 1983; Fuchs et al., 1988; McCance et al., 1985; Schneider et al., 1985). HPV-6 and HPV-11, on the contrary, are found predominantly in benign condylomata or laryngeal papillomas (Gissmann et al., 1982, 1983). The oncogenic potential of HPV-16 and HPV-18 has been amply documented in vitro (Matlashewski et al., 1987; Pater et al., 1988; Storey et al., 1988). In vitro analysis of HPV-6 and HPV-11 indicates that they do not share with HPV-16 and HPV-18 an oncogenic potential that can be readily demonstrated (Pater et al., 1988; Storey et al., 1988) but, on occasion, HPV-6 or HPV-11 is found in a malignant lesion (Byrne et al., 1987; Dürst et al., 1983; Gissmann et al., 1982, 1983; Manias et al., 1989; Rando et al., 1986a; Sutton et al., 1987; Wu & Mounts, 1989). We have cloned an HPV-6 from a poorly differentiated, non-keratinizing squamous cell carcinoma of the vulva (Kasher & Roman, 1988a) to determine whether HPV-6 from a malignant lesion has an oncogenic potential similar to that of HPV-16 and HPV-18, which would explain its association with the malignancy. Alternatively, its oncogenic potential could be low, similar to HPV-6 from a benign lesion. In this case, other explanations for its role in the genesis of the tumour would have to be proposed. Experiments were designed to distinguish between these two alternatives.

The HPV genome is organized into open reading frames (ORFs) which encode early and late viral proteins and an upstream regulatory region (URR) which contains promoters, enhancers and possibly the origin of DNA replication. The products of the HPV-16 and HPV-18 E6 and E7 ORFs are required for efficient immortalization and restricted differentiation of primary human keratinocytes (Barbosa & Schlegel, 1989; Hawley-Nelson et al., 1989; Hudson et al., 1990; Kaur & McDougall, 1989; Munger et al., 1989) and the product of the E7 ORF can cooperate with an activated ras gene to transform baby rat kidney (BRK) cells (Matlashewski et al., 1987; Pater et al., 1988; Storey et al., 1988). The E7 ORFs of HPV-6 and HPV-11 are able to cooperate with...
ras with a much lower efficiency than the E7 ORFs of HPV-16 and HPV-18 and are able to do so only when expressed from a heterologous promoter (Storey et al., 1990). The difference in the oncogenic potential of an HPV-6 present in a malignant versus a benign lesion could therefore be due to differences in the sequences of the E6 and E7 ORFs or differences in enhancer strength which could lead to increased expression of E6 and E7 proteins.

The prototype HPV-6b genome, cloned from a condyloma (Schwarz et al., 1983), has been shown to contain a 120 bp deletion in the URR due to a cloning artefact (Boshart & zur Hausen, 1986). In addition, we have previously shown that the 5' end of the HPV-6b URR can become altered during amplification in Escherichia coli (Kasher & Roman, 1988b). We therefore cloned an HPV-6 genome from a vulvar condyloma, HPV6-W50, to compare with the HPV-6 genome cloned from the vulvar carcinoma, HPV6-T70.

Here we describe the direct comparison of HPV6-W50 to HPV6-T70 with respect to DNA sequence and two functional assays. The sequences of the URRs of the two genomes varied from the prototype HPV-6b as well as from each other; however, no sequence differences that alter the E6 and E7 proteins were found. In a transient expression assay, comparable enhancer activity was seen with the URR of HPV6-T70 and HPV6-W50. A comparison of the ability of the two HPV-6 genomes to cooperate with an activated ras gene to transform BRK cells indicated that HPV6-T70 did not have an increased oncogenic potential. The results of these two functional assays suggest that the difference in the original lesions from which the two genomes were cloned was due to changes in the cellular environment rather than differences in the oncogenic potential of the two viral genomes.

**Methods**

**Cloning of HPV-6 DNA from a condyloma.** The cloning of HPV6-W50 and restriction enzyme analysis were as previously described for HPV6-T70 (Kasher & Roman, 1988a). Briefly, total cellular DNA isolated from a vulvar condyloma, designated wart 50 (W50), was digested with EcoRI and size-fractionated by sucrose gradient centrifugation. DNA of approximately 8 kb was cloned into bacteriophage λgtWES-lambda B. HPV-6-positive plaques were identified, the recombinant phage was amplified in E. coli BE257/recA (obtained from Dr L. Laimins, University of Chicago, Ill., U.S.A.) and size-fractionated by sucrose gradient centrifugation. DNA of approximately 8 kb was cloned into bacteriophage λgtWES-lambda B. HPV-6-positive plaques were identified, the recombinant phage was amplified in E. coli BE257/recA (Rao & Rogers, 1978).

**Amplification and sequencing of W50 and T70 DNA.** HPV-6 URR sequences present in total cellular DNA isolated from W50 and T70, the vulvar carcinoma from which pH6-PV6-T70 was cloned (Kasher & Roman, 1988a), were amplified and sequenced according to the following protocol. The polymerase chain reaction (PCR) was carried out as recommended by the manufacturer (Cetus). Dideoxynucleotide sequencing of the cloned and PCR-amplified DNA was done with a Sequenase kit (US Biochemicals) following the manufacturer's instructions. All numbering refers to the sequence of the prototype HPV-6b (Schwarz et al., 1983). The oligonucleotide primers used to amplify the URR sequences were from nucleotides (nt) 7229 to 7252 on the coding strand and 7490 to 7468 on the non-coding strand. The sequence of the PCR products was determined using end-labelled primers containing nt 7259 to 7276 and 7450 to 7434. Sequencing of the cloned URR was done using primers containing nt 7229 to 7252, 7515 to 7529 and 7752 to 7766 on the coding strand, and 7450 to 7434 and 93 to 71 on the non-coding strand. The cloned E6 ORF was sequenced with primers containing nt 7866 to 7883 and 592 to 577 from the coding and non-coding strand, respectively, the cloned E7 ORF was sequenced with primers containing nt 418 to 432 and 836 to 822 from the coding and non-coding strand, respectively.

**Plasmids containing the URR of HPV6-W50 and HPV6-T70.** Plasmids pW50a, pW50b, pT70a and pT70b were constructed by digesting pH6-PV6-T70 and pH6-PV6-W50 with NdeI, gel purifying the fragments corresponding to nt 6498 to 235 (1733 bp for W50 and 1670 bp for T70), digesting with DpnI (nt 7151) and gel purifying the NdeI-DpnI fragments (1080 bp for W50 and 1017 bp for T70). NdeI linkers were added and the fragment was cloned into the NdeI site of pSVEcat upstream of the simian virus 40 (SV40) early promoter and the chloramphenicol acetyltransferase (CAT) gene (Haugen et al., 1987). To verify that the cloned URRs contained authentic HPV sequences, each new plasmid preparation was sequenced.

**Cells and transfections to analyze enhancer activity.** HeLa cells (obtained from Dr L. Laimins, University of Chicago, Ill., U.S.A.) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum and 100 μg/ml-gentamicin. Vero cells were grown in DMEM supplemented with 10% heat-inactivated calf serum and 100 μg/ml-gentamicin. Cells were seeded at 10^6 per 10 cm dish the day before transfection and the medium was changed 2 h prior to transfection. Transfections were done by the calcium phosphate coprecipitation method (Graham & van der Eb, 1973) using 10 μg test DNA, 2.5 μg pRSVflgal DNA (Thierry & Yaniv, 1987) and 7.5 μg sonicated salmon sperm DNA. The cells were incubated for 4 to 6 h at 37 °C and then shocked with 15% glycerol in 1 x HBS (25 mM-HEPES, 140 mM-NaCl, 0.75 mM-Na2HPO4 pH 7.1) for 90 s at room temperature. The cells were rinsed with 1 x TD (25 mM-Tris, 135 mM-NaCl, 5 mM-KCl, 0.7 mM-Na2HPO4 pH 7.4), fresh medium was added and the incubation was continued at 37 °C for 48 h.

**CAT and β-galactosidase assays.** Cells were harvested at 48 h post-transfection and cell extracts were made by freezing and thawing (Gorman et al., 1982). The protein content of the cell extracts was determined by the Bio-Rad protein assay. To remove potential inhibitory factors, 1 μl 0.5 M-EDTA was added to the extracts which were then heated to 65 °C for 10 min and centrifuged for 5 min to remove debris (Crabb et al., 1989). The CAT assay (Gorman et al., 1985) was modified as follows. Protein (2.5 to 25 μg) was diluted to 100 μl with 0.25 M-Tris–HCl pH 7.8, to which was added 0.025 μCi [14C]chloramphenicol, 1.5 μl water and 2.5 x 10^4 amu-acetyl-CoA, and incubated at 37 °C for 1 h. The [14C]chloramphenicol was extracted with 1 ml ethyl acetate and separated by thin-layer chromatography. Thin-layer chromatography plates were scanned using an AMBIS beta scanner and the percentage conversion to acetylated chloramphenicol was determined. To determine β-galactosidase (β-gal) activity, 20 μl unheated extract was added to 380 μl Z buffer (100 mM-sodium phosphate buffer, 10 mM-KCl, 1 mM-MgSO4, 50 mM-2-mercaptoethanol and 80 μl o-nitrophenyl β-D-galactoside (4 mg/ml in 100 mM-sodium phosphate buffer pH 7.0) and incubated at 37 °C (Miller, 1972). Relative β-gal activity was determined by measuring A420. To correct
for differences in transfection efficiency, the CAT activity was normalized with the corresponding β-gal activity for each transfection.

Transformation of baby rat kidney cells. BRK cell cultures were initiated from 3 or 8 day old Fisher rats. The medium was changed 8 to 10 h after plating. After an additional 4 h, the cells were transfected with 15 μg HPV DNA (pHPV18; pHPV6-W50, a single copy of HPV6-W50 inserted into pML2d at the unique BamHI site; or pHPV6-T70, two head-to-tail copies of HPV6-T70 inserted into pML2d at the unique EcoRI site), 10 μg pEJras DNA (Shih & Weinberg, 1982) and 5 μg pSV2neo DNA (Southern & Berg, 1982) per 6 cm dish using the modification of the calcium phosphate coprecipitation method described by Wigler et al. (1979): BBS (25 mM-BES pH 6.95, 140 mM-NaCl, 0.75 mM-Na2HPO4) rather than HBS was used in the transfection (Chen & Okayama, 1987). After overnight incubation, the cells were exposed to 15% glycerol in 1 x HBS for 60 s at room temperature, fresh medium was added and the cells were incubated overnight. Cells from one 6 cm dish were then transferred to a 10 cm dish and incubated in DMEM supplemented with 10% foetal calf serum, 200 μg/ml Geneticin (G418) and 10^-6 M-dexamethasone. Foci, colonies of morphologically transformed cells, were scored at 3 weeks.

Results

Cloning and restriction analysis of HPV6-W50 and HPV6-T70

Total cellular DNA was extracted from a vulvar condyoma (W50) and subjected to Southern blot analysis using HPV-6b as a probe. Uncut DNA showed three bands at the predicted positions for forms I, II and III DNA, suggesting that the HPV genome was present as an episome (Fig. 1). This was confirmed by digestion with BclI, a no-cut enzyme for HPV-6b (data not shown), and DpnI, a no-cut enzyme for DNA replicated in mammalian cells, which yielded bands identical to those of the uncut DNA (Fig. 1). In addition, digestion with either EcoRI or HpaI, both one-cut enzymes for HPV-6b, yielded a band of approximately 8 kb. Copy number reconstructions indicated that approximately 10000 copies of the genome per cell were present (Fig. 1, compare lanes 3 and 9). Digestion with PstI indicated the presence of an extra site, with respect to the prototype HPV-6b, between nt 6489 and nt 3912. Comparison with HPV-6b showed a loss of an AvaI site at nt 1071 and an increase of approximately 100 bp in the size of the expected 732 bp fragment (nt 7203 to 33). The EcoRI, HpaI, PstI and AvaII restriction map of HPV6-W50 was compared to that of HPV6-T70 isolated from a vulvar carcinoma (Kasher & Roman, 1988a) (Fig. 2). Differences in the AvaII restriction fragments of the two cloned DNAs were found to reside in the URR. Other restriction site variability did not result in detectable fragment size differences.

The region of the URR in which the size difference was detected was amplified from total cellular DNA isolated from the tissue specimens. Primers that flanked

Fig. 1. Southern blot of HPV6-W50 DNA. Total cellular DNA (10 μg) either uncut or cut with a restriction enzyme was run on a 1% gel, transferred to nitrocellulose and probed with 32P-labelled HPV-6b DNA. Lane 1, uncut; lane 2, EcoRI-cut; lane 3, HpaI-cut; lane 4, PstI-cut; lane 5, HpaII-cut; lane 6, AvaII-cut; lane 7, DpnI-cut; lane 8, Nsil-cut; lane 9, 100 copies of linearized HPV-6b DNA. Lanes 1 and 2 are from one gel; the arrow indicates the position of 8 kb DNA. Lanes 3 to 9 are from another gel; the position of bp size markers, from bacteriophage λ DNA cleaved with HindIII and φX174 DNA cleaved with HaeIII, are indicated at the right.

Fig. 2. Comparison of the restriction maps of HPV-6b, HPV6-W50 and HPV6-T70. Closed triangles, promoters; open triangles, poly(A); open circles, splice donors; closed circles, splice acceptors; vertical broken line, ATG; a, 94 bp insert at nt 7350; b, 24 bp insert at nt 7323; c, 58 bp insert at nt 7350 followed immediately by a 49 bp deletion; B, BamHI; A, AvaII; Ps, PstI; H, HpaI; P, PpuMl; F, FnuDII; E, EcoRI; N, NdeI.
of the HPV6-T70 product was 295 bp, approximately 35 bp larger than the prototype (Kasher & Roman, 1988a) (Fig. 3).

**Sequencing of PCR-generated and cloned DNA**

The sequences of HPV6-W50 and HPV6-T70 were compared to that of the prototype HPV-6b (Fig. 4). The numbering system for all comparisons and positions of insertions and deletions is that of the HPV-6b genome (Schwarz et al., 1983). Sequencing of the cloned HPV6-T70 DNA had revealed that the increased size of the AvaI restriction fragment was due to a 24 bp insertion at nt 7323, a 58 bp insertion at nt 7350, and a 49 bp deletion of nt 7351 to 7399 (Kasher & Roman, 1988a). The cloned HPV6-W50 DNA was sequenced to determine the identity of the extra nucleotides in the AvaII restriction fragment and a 94 bp insertion was found at nt 7350. The first 58 bp were identical to those of the insertion in HPV6-T70 with the exception of one base change. To verify the authenticity of these changes in the sequences of the cloned DNA, PCR-amplified DNA (Fig. 3) was sequenced. Sequencing of the PCR-amplified DNA confirmed that the insertions and deletions of the cloned DNA were present in the DNA extracted from the tissues. Further sequencing of HPV6-T70 did not show any other sequence changes within the URR (nt 7292 to 101) in comparison with HPV-6b. Single base pair changes were detected in the HPV6-W50 clone at nt 7373 (G to T), 7585 (A to C), 7654 (C to A), 7860 (G to A) and 7974 (T to A). Changes were detected in the HPV6-W50 clone at nt 7373 (G to T), 7585 (A to C), 7654 (C to A), 7860 (G to A) and 7974 (T to A). Changes were also sequenced to determine any differences between the two genomes. No differences existed between the HPV6-T70 DNA and HPV-6b in either of the two ORFs. Sequencing of the E6 and E7 ORFs of
Enhancer and transforming potential of HPV-6s

The HPV-16 and HPV-18 genomes can cooperate with an activated \textit{ras} gene to transform BRK cells (Matlashewski \textit{et al.}, 1987; Pater \textit{et al.}, 1988; Storey \textit{et al.}, 1988). Under similar conditions neither the HPV-6 nor HPV-11 genomes can transform BRK cells (Pater \textit{et al.}, 1988; Storey \textit{et al.}, 1988). To determine whether the oncogenic potential of HPV6-T70 was greater than that of HPV6-W50, we cotransfected BRK cells with each HPV genome under the control of its own URR and an activated \textit{ras} gene. HPV-18 was included as a positive control and pML2d was a negative control; we assumed that HPV6-W50 would also be a negative control. Analysis of HPV6-W50 was necessary since previously reported experiments (Storey \textit{et al.}, 1988) had been conducted with HPV-6b which contains a cloning artefact in the URR (Boshart & zur Hausen, 1986). The results of three experiments using BRK cultures initiated from 8 day old rats indicated that HPV6-T70 had no

Table 1. \textit{Normalized CAT activity for HeLa and Vero cell transfections*}

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<tr>
<th>Plasmid</th>
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<tbody>
<tr>
<td>pSVEcat</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>pSV2cat</td>
<td>79.5</td>
<td>42.5</td>
</tr>
<tr>
<td>pW50a</td>
<td>8.7 + 2.1</td>
<td>0.7 + 0.4</td>
</tr>
<tr>
<td>pW50b</td>
<td>5.3 + 2.9</td>
<td>1.0 + 0.7</td>
</tr>
<tr>
<td>pT70a</td>
<td>5.0 + 2.6</td>
<td>1.4 + 0.6</td>
</tr>
<tr>
<td>pT70b</td>
<td>11.3 + 2.3</td>
<td>0.8 + 0.2</td>
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* Data, normalized as in Fig. 5, represent the mean of several transfections ± standard deviation. Standard deviation was not calculated for pSV2cat since not all values for CAT conversion were within the linear range.

Analysis of the ability of HPV6-T70 and HPV6-W50 to cooperate with an activated \textit{ras} gene in the transformation of BRK cultures

The HPV-16 and HPV-18 genomes can cooperate with an activated \textit{ras} gene to transform BRK cells (Matlashewski \textit{et al.}, 1987; Pater \textit{et al.}, 1988; Storey \textit{et al.}, 1988). Under similar conditions neither the HPV-6 nor HPV-11 genomes can transform BRK cells (Pater \textit{et al.}, 1988; Storey \textit{et al.}, 1988). To determine whether the oncogenic potential of HPV6-T70 was greater than that of HPV6-W50, we cotransfected BRK cells with each HPV genome under the control of its own URR and an activated \textit{ras} gene. HPV-18 was included as a positive control and pML2d was a negative control; we assumed that HPV6-W50 would also be a negative control. Analysis of HPV6-W50 was necessary since previously reported experiments (Storey \textit{et al.}, 1988) had been conducted with HPV-6b which contains a cloning artefact in the URR (Boshart & zur Hausen, 1986). The results of three experiments using BRK cultures initiated from 8 day old rats indicated that HPV6-T70 had no

Fig. 5. Cat assay and \textbeta-gal assay results of a HeLa cell transfection. The autoradiogram of the CAT assay is shown. For the CAT assay 2.5 \textmu g protein was used. Lanes 1 and 2, pSVEcat; lanes 3 and 4, pSV2cat; lanes 5 and 6, pW50a; lanes 7 and 8, pW50b; lanes 9 and 10, pT70a; lanes 11 and 12, pT70b. Below each lane is the percentage conversion as a measure of CAT activity, the amount of \textbeta-gal activity expressed in arbitrary units, the amount of CAT activity normalized to the amount of \textbeta-gal activity, and the CAT/\textbeta-gal activity normalized for the amount of pSVEcat activity.

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HPV6-W50 revealed two base pair changes. A change at nt 221 (A to T) did not alter the coding sequence of the E6 ORF but did create an extra \textit{PstI} site, confirming the results of the restriction enzyme analysis (Fig. 2). A second change at nt 473 (G to A) destroyed the \textit{FnuDII} site at nt 472 but left the E6 ORF coding sequence intact (Fig. 2).

Determination of enhancer activity

The presence of sequence differences in the URR prompted us to compare the enhancer activities of HPV6-W50 and HPV6-T70. The URRs of HPV6-W50 and HPV6-T70 were cloned upstream of the SV40 early promoter in the enhancerless CAT expression vector, pSVEcat. pW50a and pW50b contain the entire URR of HPV6-W50 in the sense and antisense orientations, respectively; similarly, pT70a and pT70b contain the entire URR of HPV6-T70 cloned in the sense and antisense orientations, respectively. These plasmids were each cotransfected with pRSV\beta gal, encoding \textbeta-gal, into the permanent cell lines HeLa and Vero and the CAT activity was determined. The CAT activity from cells transfected with each of the plasmids was compared to that of the negative control, pSVEcat; the plasmid pSV2cat (Gorman \textit{et al.}, 1982), containing the SV40 enhancer, was included as a positive control. The CAT activity was reported both before and after normalization for \textbeta-gal activity (Fig. 5), which was used to correct for differences in transfection efficiencies. The results were then normalized to pSVEcat activity. The average normalized enhancer activity of several transfections in HeLa and Vero cells was determined (Table 1). The transfections in each of the cell lines were conducted with three different plasmid preparations of pW50a, pW50b, pT70a and pT70b. In the HeLa cells, comparable enhancer activity was seen with HPV6-W50 and HPV6-T70 (Fig. 5; Table 1). The amount of CAT activity varied between 5.0- and 11.3-fold greater than that of the enhancerless plasmid pSVEcat, regardless of orientation. In the Vero cell line, little or no enhancer activity was seen. The amount of normalized CAT activity was 0.7- to 1.4-fold that of the pSVEcat activity (Table 1).

Analysis of the ability of HPV6-T70 and HPV6-W50 to cooperate with an activated \textit{ras} gene in the transformation of BRK cultures

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Table 2. The ability of HPV6-W50 and HPV6-T70 to cooperate with ras to transform BRK cells

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Coding capacity</th>
<th>Number of foci*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHPV18, pEJras, pSV2neo</td>
<td>HPV-18, neo', ras</td>
<td>21 24 12</td>
</tr>
<tr>
<td>pHPV6-W50.1, pEJras, pSV2neo</td>
<td>HPV-6, neo', ras</td>
<td>6  13  3</td>
</tr>
<tr>
<td>pHPV6-T70.2, pEJras, pSV2neo</td>
<td>HPV-6, neo', ras</td>
<td>4  13  2</td>
</tr>
<tr>
<td>pML2d, pEJras, pSV2neo</td>
<td>Vector, neo', ras</td>
<td>4  9  4</td>
</tr>
</tbody>
</table>

* Number of foci is the number of morphologically transformed colonies per experiment.

greater ability than HPV6-W50 to cooperate with an activated ras gene; indeed, the level of activity was no greater than that of ras alone (Table 2). In contrast, the positive control, HPV-18, did give rise to a significantly greater number of foci (Table 2). Similar results were obtained using BRK cultures initiated from 3 day old rats (data not shown).

Discussion

The fact that HPV-6 is only rarely associated with malignancies but commonly associated with benign warts could have two explanations. Firstly, on rare occasions a mutation may occur in the HPV-6 genome which converts it to an oncogenic form, similar in properties to HPV-16 and HPV-18. Such mutations could be either in regulatory regions affecting the level of gene expression or in coding regions. Secondly, the HPV-6 in the malignancy may be similar to that in the benign lesion and it is rarely associated with malignancy because the malignancy arises following the rare accumulation of cellular mutations causing the loss of cell growth control. Data presented here indicate that the first alternative is not fulfilled in the case of HPV6-T70. Although sequence differences do exist between HPV6-T70 and HPV6-W50, these changes do not affect the behaviour of the viral genome when standard assays are used to analyse enhancer activity and transforming potential.

The hypothesis that sequence variation within the HPV-6 URR might be related to oncogenic potential arose from the observation that sequence duplications were present in the URRs of HPV-6 genomes isolated from an invasive verrucous carcinoma, HPV-6vc (Rando et al., 1986a), a Buschke-Löwenstein tumour, HPV-6d (Boshart & zur Hausen, 1986), and a non-keratinizing squamous cell carcinoma of the vulva, HPV-6-T70 (Kasher & Roman, 1988a). However, sequence duplications are neither consistently detected in carcinomas nor consistently absent in benign lesions. Five HPV-6s from Buschke-Löwenstein tumours did not contain duplications (Boshart & zur Hausen, 1986), whereas, HPV-6ma cloned from a benign condyloma of the mamilla did contain a duplication (Kulke et al., 1989), as did HPV-6g cloned from a benign condyloma of the genital tract (Wu & Mounts, 1988). To determine the effect of alterations in the 5' end of the URR on enhancer function, we compared the enhancer activity of HPV6-T70 to that of HPV6-W50. Our results indicate that the sequence differences between HPV6-T70 and HPV6-W50 at nt 7323 and nt 7350 did not result in differences in enhancer activity. Previous comparisons of enhancer activity have been made between the genome carrying the duplication, HPV-6vc (Rando et al., 1986b) or HPV-6ma (Kulke et al., 1989), and the prototype HPV-6b genome which has been found to have a deletion of 120 bp within the URR (Boshart & zur Hausen, 1986); or between two HPV-6s isolated from benign lesions (Wu & Mounts, 1988). The insertions of 74 bp at nt 7348, 15 bp at nt 7418 and 19 bp at nt 7720 in the URR of HPV-6vc were shown to have greater enhancer activity than the same region of HPV-6b when comparing the entire URRs (Rando et al., 1986b). The 3' half of the URR of HPV-6vc, which contains only the 19 bp insertion, was also shown to have greater enhancer activity than the same region of HPV-6b (Rando et al., 1986b). Duplications of nt 7681 to 7896 in the URR of HPV-6ma (Kulke et al., 1989) and the 136 bp tandem repeat at nt 7846 in the URR of HPV-6g (Wu & Mounts, 1988) have been shown to increase the enhancer activity of the URRs. These results may be explained by the locations of the insertions. Duplications in the 3' half of the URR may result in an increase in the number of trans-acting factors that are able to bind to the URR whereas duplications in the 5' region may not lead to such an increase. This is supported by the presence of more putative nuclear factor binding motifs in the 3' half of the URR (Fig. 4). However, other cis elements may exist in the 5' region which have not yet been identified.

The insertions and deletions in HPV6-W50 and HPV6-T70 were all located in the purine–thymidine-rich region which is immediately upstream of the late
polyadenylation signal. Differences in the length of the alternating purine–thymidine-rich sequence and putative length and strength of Z DNA did not alter enhancer activity. Alterations upstream of the polyadenylation signal may influence HPV-6 mRNA stability (Kennedy et al., 1990) rather than enhancer activity.

HPV6-W50 and HPV6-T70 URR plasmids transfected into HeLa cells exhibited similar levels of enhancer activity above the baseline of pSVEcat whereas little or no enhancer activity was seen in Vero cells; similar results are seen with an HPV-16 enhancer (Cripe et al., 1987). The ability of the HPV-6 enhancers to function in HeLa cells but not in Vero cells may be due to the origin of the cells, i.e. epithelial (HeLa) or fibroblast-like (Vero), or may be due to the presence of HPV gene products in the HeLa cells. Since the HPV-16 URR is able to act as an enhancer in uninfected keratinocytes (Cripe et al., 1987), we presume that the greater enhancer activity of the HPV-6 URR observed in HeLa cells is due to epithelium-specific factors.

Assays for enhancer activity and transforming potential showed that HPV6-T70 has no greater oncogenic potential than HPV6-W50, suggesting that the role of HPV6-T70 in the genesis of a carcinoma must be different from the role of HPV-16 and HPV-18. Since all HPV-6s have the ability to stimulate senescing cells to proliferate (Schlegel et al., 1988), perhaps the role of HPV-6 in the genesis of a carcinoma is to provide a mitogenic stimulus. Thus, we would propose that all HPV-6s have the ability to stimulate cell proliferation; such stimulated cells may accumulate mutations in growth control genes such that, on rare occasions, a malignancy arises. This hypothesis suggests that the HPV-6 genome would not necessarily be maintained in a carcinoma. This is consistent with the observation that C127 mouse cells morphologically transformed by HPV-6b do not retain the HPV-6 genome (Morgan et al., 1990). Either the infrequent accumulation of appropriate mutations in the cellular genome or the lack of required maintenance of the HPV-6 genome may account for the rare association of HPV-6 in carcinomas. This hypothesis also suggests that there need not be an alteration in the gene responsible for mitogenesis between the HPV-6 genome isolated from a benign and a malignant lesion. Recently, Chen & Mounts (1990) reported that the E5a ORF of HPV-6c, isolated from a benign lesion, transforms permanent mouse cell lines and suggested that it does so by stimulating cellular proliferation. Consistent with the hypothesis that all HPV-6s have the same mitogenic potential, the E5a ORF sequences are identical between HPV6-W50 and HPV6-T70 (data not shown).

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References


