Journal of General Virology (1999), **80**, 2445–2451. Printed in Great Britain

Human papillomavirus type 6: classification of clinical isolates and functional analysis of E2 proteins

Robert Kovelman,[1] Graham K. Bilter,[1] Ann Roman,[2,3] Darron R. Brown[2,4] and Miguel S. Barbosa[1]

[1] Virology Program, Signal Pharmaceuticals Inc., 5555 Oberlin Drive, San Diego, CA 92121, USA

[2] Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[3] The Walther Cancer Institute, Indianapolis, IN 46202, USA

[4] Division of Infectious Diseases, Department of Medicine, Richard L. Roudebush VA Medical Center and Indiana University School of Medicine, Indianapolis, IN 46202, USA

Human papillomaviruses (HPVs) cause a variety of clinical manifestations, including the most prevalent viral sexually transmitted disease, genital warts. HPV-6 is found in a greater number of genital warts than any other HPV. To increase our understanding of the structural and functional relationships between HPV-6 isolates and to provide information for epidemiological studies, the sequences of the E2, E6 and E7 coding regions of HPV-6 genomes in clinical samples were determined. This sequence analysis was performed on isolates originally designated HPV-6a on the basis of analysis of patterns generated by restriction enzyme digestion. It was found that the designation of subtype on the basis of restriction enzyme digestion correlated poorly with the designation of subtype on the basis of sequence comparison; in fact, the clinical isolates were clearly categorized into HPV-6a and HPV-6b groups, with the previously described HPV-6vc being a member of the HPV-6a group. It was also found that the HPV-6a E2 protein is a much less potent activator of transcription than the HPV-16 E2 protein, generalizing our previous results with the HPV-6b E2 protein to this second HPV-6 E2 protein. These studies indicate that the amino acid differences observed between these natural variants of the HPV-6 E2 protein do not affect its function.

Introduction

The human papillomaviruses (HPVs) are a diverse family of DNA viruses that infect and cause disease in a wide variety of epithelial cell types. Different families of HPV types infect distinct subsets of epithelial cells; for example, genital epithelial cells are commonly found to be infected by HPV-6, HPV-11, HPV-16 and HPV-18, while HPV-1 and HPV-2 infect non-genital epithelia. In addition to this tissue tropism, infection by some of the genital HPV types, such as HPV-16 and HPV-18, is a significant risk factor for the development of cervical cancer, and these HPVs have therefore been termed 'high-risk' (reviewed by zur Hausen, 1991). However, some variants of the 'low-risk' HPV-6 have also been identified in malignancies of the genital tract and other tissues (Rando *et al.*, 1986; Kasher & Roman, 1988; Oft *et al.*, 1993; Wilczynski *et al.*, 1993). Thus, further information on the association of low-risk HPVs with the progression to malignancy is needed.

A better understanding of such an association first requires a clear system of classifying HPVs. Originally, the classification of papillomaviruses into types was performed by hybridization analysis, with more detailed subtype designation being assigned on the basis of differences in restriction enzyme digestion patterns. For HPV-6, these studies led to the categorization of the genomes into a number of subtypes (Gissmann *et al.*, 1983; Boshart & zur Hausen, 1986; Rando *et al.*, 1986). More recently, sequencing analysis has been performed on different clinical isolates or clones of HPV-6. The focus of these studies has been on certain portions of the genome, particularly parts of the upstream regulatory region (URR), which contains control elements for transcription and

Author for correspondence: Robert Kovelman.
Fax +1 619 623 0870. e-mail rkovelma@signalpharm.com

The GenBank accession number for the nucleotide and deduced amino acid sequences of HPV-6vc is AF092932.

replication, and the coding regions for L1, L2, E6, E7 and E1 ∧ E4 (Kasher & Roman, 1988; Farr *et al.*, 1991; Icenogle *et al.*, 1991; Rübben *et al.*, 1992; Yaegashi *et al.*, 1993; Kitasato *et al.*, 1994; Heinzel *et al.*, 1995; Roman & Brown, 1995). On the basis of URR sequence analysis, it has been suggested that isolates of HPV-6 should be grouped into HPV-6a-related and HPV-6b-related variants (Heinzel *et al.*, 1995; Grassmann *et al.*, 1996).

Only limited sequence information is available for the E2 coding region. It is relatively divergent between the two previously sequenced HPV-6 genomes, HPV-6b (Schwarz *et al.*, 1983) and HPV-6a (Hofmann *et al.*, 1995). Thus, sequencing of the E2 coding region of genomes isolated directly from clinical samples would provide detailed, epidemiologically useful information on the relatedness of those HPVs. In addition, interest in knowing more about the sequence divergence in the E2 coding region is increased by our previous results, which showed that the E2 proteins encoded by the high-risk HPV-16 and HPV-18 are much more potent transcriptional activators than the E2 proteins encoded by the low-risk HPV-6b and HPV-11 (Kovelman *et al.*, 1996). In particular, we wanted to know whether the low activity of the HPV-6b E2 protein would also be found with other isolates of HPV-6. We found that the HPV genomes in the clinical isolates used for this study were clearly categorized into two groups and that the E2 proteins encoded by these two groups were both of lower activity in stimulating transcription than the E2 proteins from high-risk HPVs. Thus, this study shows that a lower level of transcriptional activation is a common property of E2 proteins from HPV-6.

Methods

■ **Materials.** Chemicals were obtained from Sigma or Fisher Scientific, enzymes from New England Biolabs or Perkin-Elmer and custom oligonucleotides from Ransom Hill Bioscience or Great American Gene Company. Normal human epidermal keratinocytes (neonatal) were obtained from Clonetics. The isolation and preparation of the clinical samples from condylomata acuminata of patients in Indiana, USA, were described previously (Roman & Brown, 1995).

■ **Plasmids.** The plasmid pSK-HPV-6vc was generated by removing the viral genome from pBR-HPV-6vc (Rando *et al.*, 1986) by digestion with *Bam*HI and inserting it into the *Bam*HI site of pBluescript SK (Stratagene). Construction of pCMV-16E2 and pCMV-6bE2 (previously named pCMV-6E2) has been described elsewhere (Kovelman *et al.*, 1996). The expression vector pCMV-6vcE2 was constructed in a manner such that all sequences outside the E2 coding regions in pCMV-6bE2 and pCMV-6vcE2 were identical. The reporters used to assay E2-dependent transcriptional activation (p2x2xE2BS-luc) and repression [p6bURR-(promoter)-luc] have also been described previously (Kovelman *et al.*, 1996).

■ **Sequencing.** The sequence of the HPV-6vc genome was determined by using appropriately spaced oligonucleotide primers with pSK-HPV-6vc as the template. In order to sequence the E2 coding region from the clinical isolates, DNA oligonucleotides with the sequences 5′ AAATGGGAATGCAGTGTATGA 3′ and 5′ GATGTGTACACAATAAACTCA 3′, which respectively hybridize to the HPV-6 genome approximately 100 base pairs upstream and 200 base pairs downstream of the E2 gene, were used to amplify a fragment by PCR with *Taq* polymerase, and the fragment was purified by use of the QIAquick PCR purification kit (Qiagen). This purified fragment was then used directly as the template for sequencing reactions. Sequencing of the E6/E7 region of the clinical isolates was performed in a similar manner, with DNA oligonucleotides with the sequences 5′ GGTTTAAAAAATAGGAGGGACCGA 3′ and 5′ CCCACTGTCCTCCACCTCCTCATC 3′ having been used to amplify a fragment 948 base pairs in length encompassing the E6/E7 coding regions and flanking sequences. All DNAs were sequenced at least twice on each strand. Sequencing was performed on ABI 373 or 377 automated sequencers (Perkin Elmer–Applied Biosystems), with reactions and procedures being performed according to the manufacturer's instructions. All nucleotide sequence positions described are numbered according to the HPV-6b sequence.

■ **Assays.** Transfection of C33-A cells by the calcium phosphate method and keratinocytes by lipofection was performed as described previously (Kovelman *et al.*, 1996). Briefly, cells were lysed 2 days after transfection and luciferase and *β*-galactosidase activities were determined. Except where noted, the data shown reflect luciferase activities normalized to *β*-galactosidase activities, with the results for lysates of control cells transfected with the reporter and an empty expression vector being set to a value of 1.

Results

Sequence relationships between HPV-6 genomes from clinical samples

The partial characterization of HPV-6 genomes obtained from ten clinical isolates and two previously cloned genomes, HPV6-T70 and HPV6-W50, was reported previously (Farr *et al.*, 1991; Roman & Brown, 1995). In order to determine relationships between these HPV-6 genomes in greater detail, we determined the sequence of the E2 coding region. Interestingly, the E2 sequences in these samples were clearly categorized into two major groups. As shown in the summary chart in Fig. 1, one of these groups included two of the clinical samples, 1110 and 1125, and the previously cloned HPV6-W50 and HPV6-T70, while the second group comprised the eight other clinical samples. Within each group, one or two minor variants were present (C at position 3730 for sample 1110 and HPV6-W50 for the first group and C at position 3634 in sample 1084 for the second group). The E2 sequences of the first group were essentially identical to that of HPV-6b (Schwarz *et al.*, 1983), with only the one change in two of the samples relative to the HPV-6b reference clone. The second group was closer in sequence, but not identical, to the E2 sequence of a published clone of HPV-6a (Hofmann *et al.*, 1995).

Since the E2 coding region has not commonly been used as a standard for determining relationships between HPV genomes, we also determined the sequence of the E6 and E7 coding regions in order to ascertain whether a similar grouping would be obtained. As shown in Fig. 2, we found that all 12 of the clinical samples and clones analysed were categorized into

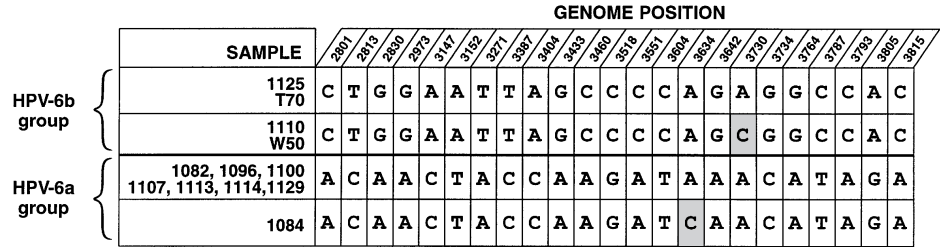

Fig. 1. Categorization of HPV-6-containing clinical isolates and clones according to sequence differences in the E2 coding region. Positions where sequences within the E2 coding region differed between HPV-6 samples and the nucleotides found at those positions are shown. Numbering of positions is in accordance with the original HPV-6b designation.

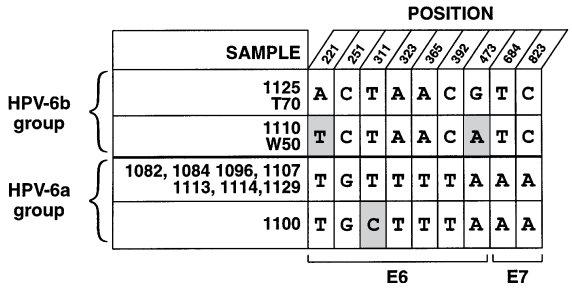

Fig. 2. Categorization of HPV-6-containing clinical isolates and clones according to sequence differences in the E6 and E7 coding regions. Experimental findings are summarized as for the E2 region in Fig. 1. Sequence data for T70 and W50 are from Farr *et al.* (1991).

the same HPV-6a and HPV-6b groups on the basis of E6/E7 sequences.

Positions of divergence between the amino acid sequences of the E2, E6 and E7 proteins encoded by these two groups of HPV-6 and the previously sequenced isolate of HPV-6a (Hofmann *et al.*, 1995) are shown schematically, relative to the known functional elements or domains of these proteins, in Fig. 3 (the nucleotide changes within a group shown in Figs 1 and 2 did not alter the amino acids encoded at those positions). In the case of the E2 proteins, for which there was amino acid divergence at a number of positions, differences were found in all three domains (activation, hinge and DNA-binding/dimerization), with a slight clustering of changes at the C terminus of the DNA-binding domain. These differences were not correlated in any obvious way with the E2 protein of the high-risk HPV-16; in other words, none of these three groups had an amino acid sequence more closely related to HPV-16 than the others. The amino acid differences detected for E6 and E7 were identical to those reported by Krige *et al.* (1997) and were a subset of those reported by Grassmann *et al.* (1996).

Sequence of the HPV-6vc genome

We found it interesting that the majority of these samples were closely related but not identical to a previously sequenced clone of HPV-6a. During analysis of other cloned HPV-6 genomes, we found identity between the sequence of the E2 coding region of the HPV-6a group described above and that of HPV-6vc, the genome of which had been partially characterized but not fully sequenced. We also found that this sequence identity extended to the E6/E7 region. Since genomes containing sequences identical to that of HPV-6vc were the most common in the set of clinical samples being characterized, we determined the sequence of the remainder of the HPV-6vc genome. A comparison of the percentage differences at the nucleotide level between HPV-6b, our isolates of HPV-6a and the previously sequenced clone of HPV-6a is shown in Table 1. Sequence divergence at the nucleotide level between these three was in general 1·5% or less, except for the E2, E4 and E5 coding regions. Nonetheless, the nucleotide sequences clearly showed that HPV-6vc is a member of the HPV-6a group.

HPV-6vc was originally thought to be a novel, potentially oncogenic form of HPV-6 because of its isolation from a verrucous carcinoma (Rando *et al.*, 1986). On the basis of its previously reported restriction enzyme digestion pattern, HPV-6vc would be considered an HPV-6a subtype (Rando *et al.*, 1986). As summarized in Table 1, our sequencing results clearly demonstrate that the entire HPV-6vc genome is closely related to that of a previously sequenced clone of HPV-6a isolated from benign tissue and confirm that HPV-6vc is a member of the HPV-6a group. As shown above in support of this classification, we isolated HPV genomes from benign condylomata acuminata that were identical to that of HPV-6vc in their E2, E6 and E7 coding regions.

Transcriptional regulation by HPV-6 E2 proteins

We showed previously that the E2 proteins from HPV-6b and HPV-11 are much weaker transcriptional activators than the E2 proteins from HPV-16 or HPV-18 (Kovelman *et al.*, 1996). Since most of the clinical isolates we have analysed are categorized as HPV-6a, not HPV-6b, we undertook a comparison of the transcriptional regulatory activities of the HPV-6a and HPV-6b E2 proteins. We cloned the E2 coding regions from HPV-6b and, as a representative of the HPV-6a group, HPV-6vc downstream of the CMV immediate early promoter and we transfected these plasmids and an E2-dependent reporter plasmid into a genital epithelial cell line, C33-A. Using a reporter plasmid (p2x2xE2BS-luc) that we had shown

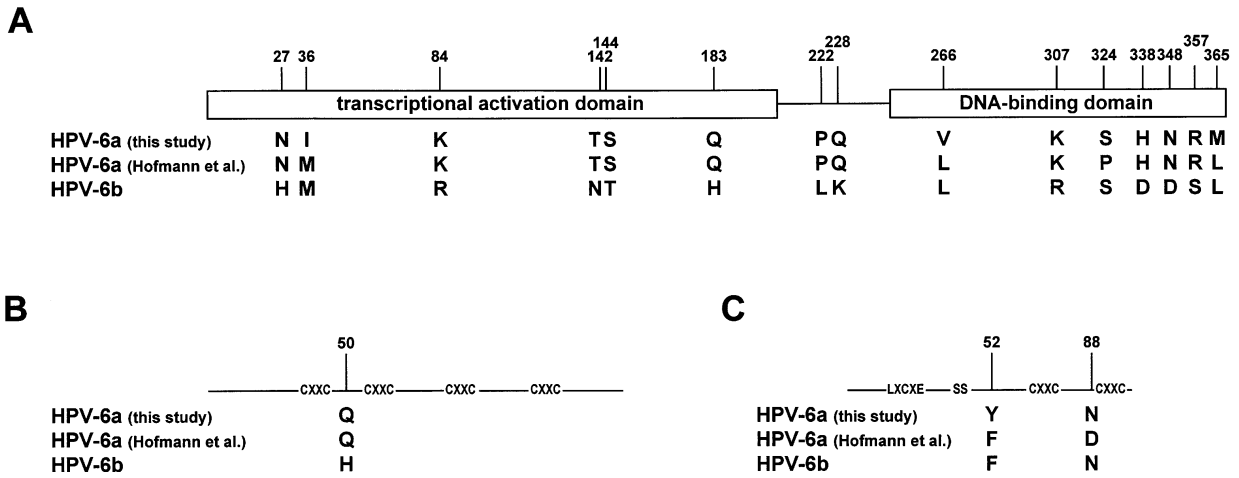

Fig. 3. Amino acid differences between HPV-6 E2, E6 and E7 proteins. Amino acids that are divergent between the E2 (A), E6 (B) and E7 (C) proteins encoded by the two groups of HPV-6a-related variants and the HPV-6b group are shown below schematic drawings of the significant motifs and domains of these proteins. Designation of groups is as described in the legend to Table 1. Motifs shown include those for metal binding (CXXC), binding to the Rb protein (LXCXE) and a consensus site for casein kinase II phosphorylation (SS). Numbers shown correspond to amino acid positions for each of the respective proteins.

Table 1. Percentage sequence divergence between coding regions of HPV-6a and HPV-6b genomes

6a refers to the group of HPV-6a-related isolates (including HPV-6vc) that we describe in this study and 6a (Hofmann) refers to the clone sequenced by Hofmann *et al.* (1995).

Coding region	6a vs 6a (Hofmann)	6a vs 6b	6a (Hofmann) vs 6b
E6	0·7	1·3	1·3
E7	0·7	0·7	0·7
E1	0·5	1·1	1·1
E2	0·6	1·9	1·7
E4	0·3	1·8	1·5
E5A	1·8	4·3	2·5
E5B	1·8	2·7	4·6
L2	0·5	1·2	1·2
L1	0·4	0·7	0·5

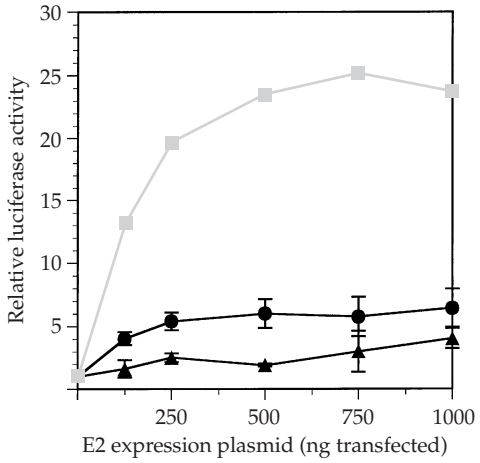

Fig. 4. Transcriptional activation by the HPV-6a (●) and HPV-6b (▲) E2 proteins in C33-A cells. C33-A cells were co-transfected with pCMV-6vcE2 (as a representative of the HPV-6a group), pCMV-6bE2 or pCMV-16E2, a luciferase reporter plasmid containing four E2-binding sites and pCMV-β-gal. The results represent luciferase activity normalized to β-galactosidase activity. The results of transfections shown in this figure are from a single experiment, but all such experiments were repeated at least three times to confirm the results. Error bars are shown for the HPV-6a and HPV-6b samples because plasmids prepared on different dates were assayed, whereas the curve for the HPV-16 E2 protein (■) included for comparative purposes resulted from transfection of only one representative plasmid preparation.

previously to be sensitive in differentiating the relative activities of E2 proteins (Kovelman *et al.*, 1996), we found that the HPV-6a and HPV-6b E2 proteins were of roughly comparable activities (Fig. 4). Slightly enhanced activity with the HPV-6a protein was observed with some of the amounts of input DNA tested in this transfection, but this difference in activity was very small when compared with the difference in activities between either of the HPV-6 E2 proteins and the HPV-16 E2 protein (Fig. 4), in accordance with our previous findings from comparisons of HPV-6b and HPV-16 E2 proteins (Kovelman *et al.*, 1996).

Papillomavirus E2 proteins can also repress transcription, particularly from the promoter directly upstream of the E6 gene of HPVs (Thierry & Yaniv, 1987; Chin *et al.*, 1988; Bernard *et al.*, 1989; Dostatni *et al.*, 1991). In order to determine whether the HPV-6a and HPV-6b E2 proteins differed in their ability to repress transcription from this promoter, we performed transfections similar to those described above but with the reporter p6bURR(promoter)-luc (Kovelman *et al.*, 1996), which contains the entire HPV-6b

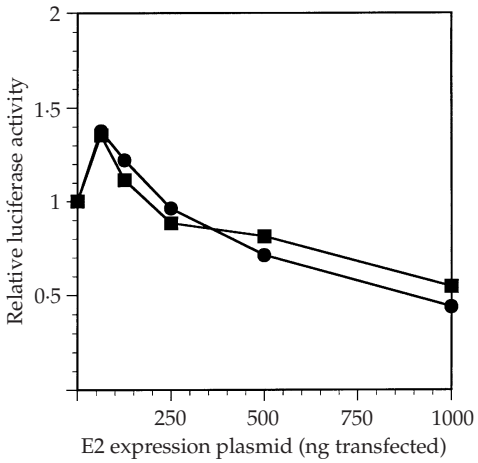

Fig. 5. Transcriptional regulation of a URR-based reporter by the HPV-6a (●) and HPV-6b (■) E2 proteins in C33-A cells. Transfections and subsequent analysis were performed as described for Fig. 4, except that the luciferase reporter contained the entire HPV-6b URR cloned directly upstream of the luciferase coding region.

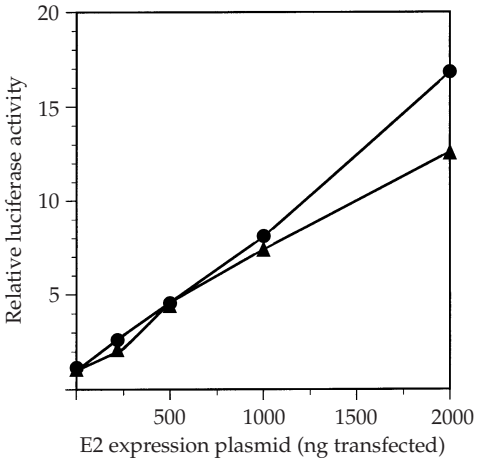

Fig. 6. Transcriptional activation by the HPV-6a (●) and HPV-6b (▲) E2 proteins in primary keratinocytes. Transfections and subsequent analysis were performed as described for Fig. 4, except that the values shown were not normalized to β-galactosidase activity. In other experiments, the results were found to be similar with or without normalization to β-galactosidase activity.

URR cloned upstream of the luciferase gene without other intervening promoter sequences. As shown in Fig. 5, the two E2 proteins were virtually identical in regulating transcription from this reporter, with both resulting in a small increase in transcription with small amounts of input E2 expression vector and up to twofold repression with larger amounts of input effector DNA.

Finally, we wanted to determine whether there was any difference in transcriptional activation by the HPV-6a and HPV-6b E2 proteins in normal human keratinocytes, the natural host cells for HPVs. We transfected the E2 expression vectors and the same reporter plasmid used to assay E2-dependent transcriptional activation in C33-A cells for the experiment shown in Fig. 4 into keratinocytes. As shown in Fig. 6, these two E2 proteins were virtually identical in their ability to activate transcription after expression in normal keratinocytes. Thus, using the HPV-6a and HPV-6b E2 proteins, we have observed very similar activities in transcriptional activation and repression assays in primary keratinocytes as well as in an epithelial cell line.

Discussion

Our analysis shows that the designation of HPV-6 subtype based on restriction enzyme digestion patterns correlated poorly with the designation of subtype based on direct sequence comparison. From this first report of HPV-6 E2 sequence analysis from clinical isolates, we conclude that isolates subtyped as HPV-6a on the basis of restriction analysis can be categorized into groups identical or closely related to either HPV-6b or the previously sequenced isolate of HPV-6a (Hofmann *et al.*, 1995). We found the same relationship on the basis of E6 and E7 sequencing. A corollary to this observation is that the E2, E6 and E7 proteins encoded by the cloned HPV-6a and HPV-6b genomes are representative of the proteins encoded by clinical isolates. Interestingly, there was no correlation between the categorization based on sequence and the clinical state of the lesion from which the genomes were isolated, as samples from carcinomas (HPV-6vc and HPV6-T70) were in both groups, as were the remaining genomes isolated from benign lesions.

The E2 sequences in these clinical samples, originally categorized as HPV-6a on the basis of *Pst*I digestion pattern (Roman & Brown, 1995), fell into two groups. The sequence of one group was essentially identical to that of HPV-6b in the E2 coding region. The sequence of the E2 coding region of the other group was essentially identical to that of HPV-6vc, which we also sequenced from a previously cloned genome (Rando *et al.*, 1986), and it was closely related to that of a previously sequenced clone of HPV-6a (Hofmann *et al.*, 1995). Categorization of these clinical samples correlated with their previous grouping according to the sequence of the upstream portion of the URR (Roman & Brown, 1995), with one exception. Although samples 1084 and 1125 were in the same group in the previous study, they are clearly distinguished into the HPV-6a and HPV-6b categories, respectively, according to their E2 sequences. This difference and related sequencing results with the cloned HPV6-W50, which has an HPV-6a *Pst*I digestion profile but an HPV-6b E2 sequence, reveal a divergence between HPV subtype designations based on restriction digestion and those based on E2 sequences.

Chan *et al.* (1992) suggested previously that designating HPV-6 subtypes on the basis of restriction enzyme digestion was inappropriate when one considered that the sequence divergence was, in fact, of the order of 1% and that the term 'variant' would be more descriptive. To establish phylogenetic

associations, Heinzel *et al*. (1995) used the presence of a 20 bp insertion at nt 7719 to separate HPV isolates into HPV-6a variants (those containing this insertion) and HPV-6b variants (those which did not). Sequence comparisons indicated that HPV-6vc was more closely related to the previously sequenced isolate of HPV-6a than to HPV-6b. On the basis of an analysis of URR sequences and the presence or absence of the 20-mer, Grassmann *et al*. (1996) have also divided HPV-6 isolates into two groups, one containing HPV-6b, HPV6-W50 and HPV6-T70 and the other containing HPV-6a and HPV-6vc. Interestingly, our E2, E6 and E7 data on these cloned genomes are consistent with this grouping.

Our data also indicate that there is no correlation between HPV-6vc-related genes and malignant progression. In fact, the E2 coding regions of eight of ten samples from condylomata acuminata fell into the same category as HPV-6vc, even though HPV-6vc was originally isolated from a carcinoma (Rando *et al*., 1986). In addition, HPV6-T70, which was also isolated from malignant tissue, was found to be similar to HPV-6b according to our E2/E6/E7 sequencing, further underlining the lack of correlation between the type of lesion and the presence of particular HPV coding sequences.

This study also extends our initial observation that the HPV-6b E2 protein is less active than the HPV-16 E2 protein, both as a positive and as a negative regulator of transcription (Kovelman *et al*., 1996), to a second HPV-6 E2 protein. A corollary to this finding is that a number of amino acid changes throughout the three domains of the E2 protein do not affect its activity. The E2 protein is a critical regulator of transcription and replication and any changes in its function are likely to have important consequences for the life-cycle of the virus as well as the clinical manifestation of the infection. Previously, we described a sensitive reporter system to detect a difference in activity between the E2 proteins from high-risk and low-risk HPV types (Kovelman *et al*., 1996). In the current study, use of this system demonstrated that the HPV-6a E2 protein was similarly weak as a transcriptional activator in comparison with the HPV-6b E2 protein (Fig. 4). In addition to these experiments in C33-A cells, we also assayed transcriptional activation by the two proteins in primary human keratinocytes, the normal host cell for HPVs. The HPV-6a and HPV-6b E2 proteins were virtually identical in their ability to stimulate transcription from the E2-dependent reporter in these primary cells (Fig. 6). Thus, our previous finding that the activity of the HPV-6b E2 protein is much lower than that of E2 proteins from high-risk HPVs (Kovelman *et al*., 1996) was not a result of the HPV-6b E2 having undergone a deleterious mutation *in vitro*. In addition, we found that the HPV-6a and HPV-6b E2 proteins were indistinguishable in their ability to repress transcription *in vivo* (Fig. 5). Thus, we have demonstrated significant conservation of E2 function in HPV-6 from clinical samples, confirming the relevance *in vivo* of previous studies that employed the HPV-6b E2 protein as the prototype for its class. It will be of interest to extend this comparative analysis of the function of these categories of E2 protein to include its other functions, in particular its key role in controlling papillomavirus replication in conjunction with the E1 protein.

We thank Emilia Glezer for expert cell culture assistance and Nathan Eller for assistance with the preparation of the figures. This work was supported in part by grants CA55703 from the National Cancer Institute to M.S.B., R43 AI41262 from the National Institute of Allergy and Infectious Diseases to M.S.B. and AI31494 from the National Institute of Allergy and Infectious Diseases to D.R.B. (Project 4) and A.R. (Project 5).

References

Bernard, B. A., Bailly, C., Lenoir, M. C., Darmon, M., Thierry, F. & Yaniv, M. (1989). The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. *Journal of Virology* **63**, 4317–4324.

Boshart, M. & zur Hausen, H. (1986). Human papillomaviruses in Buschke-Löwenstein tumors: physical state of the DNA and identification of a tandem duplication in the noncoding region of a human papillomavirus 6 subtype. *Journal of Virology* **58**, 963–966.

Chan, S. Y., Bernard, H. U., Ong, C. K., Chan, S. P., Hofmann, B. & Delius, H. (1992). Phylogenetic analysis of 48 papillomavirus types and 28 subtypes and variants: a showcase for the molecular evolution of DNA viruses. *Journal of Virology* **66**, 5714–5725.

Chin, M. T., Hirochika, R., Hirochika, H., Broker, T. R. & Chow, L. T. (1988). Regulation of human papillomavirus type 11 enhancer and E6 promoter by activating and repressing proteins from the E2 open reading frame: functional and biochemical studies. *Journal of Virology* **62**, 2994–3002.

Dostatni, N., Lambert, P. F., Sousa, R., Ham, J., Howley, P. M. & Yaniv, M. (1991). The functional BPV-1 E2 trans-activating protein can act as a repressor by preventing formation of the initiation complex. *Genes & Development* **5**, 1657–1671.

Farr, A., Wang, H., Kasher, M. S. & Roman, A. (1991). Relative enhancer activity and transforming potential of authentic human papillomavirus type 6 genomes from benign and malignant lesions. *Journal of General Virology* **72**, 519–526.

Gissmann, L., Wolnik, L., Ikenberg, H., Koldovsky, U., Schnurch, H. G. & zur Hausen, H. (1983). Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. *Proceeding of the National Academy of Sciences, USA* **80**, 560–563.

Grassmann, K., Wilczynski, S. P., Cook, N., Rapp, B. & Iftner, T. (1996). HPV6 variants from malignant tumors with sequence alterations in the regulatory region do not reveal differences in the activities of the oncogene promoters but do contain amino acid exchanges in the E6 and E7 proteins. *Virology* **223**, 185–197.

Heinzel, P. A., Chan, S. Y., Ho, L., O'Connor, M., Balaram, P., Campo, M. S., Fujinaga, K., Kiviat, N., Kuypers, J., Pfister, H., Steinberg, B. M., Tay, S. K., Villa, L. L. & Bernard, H. U. (1995). Variation of human papillomavirus type 6 (HPV-6) and HPV-11 genomes sampled throughout the world. *Journal of Clinical Microbiology* **33**, 1746–1754.

Hofmann, K. J., Cook, J. C., Joyce, J. G., Brown, D. R., Schultz, L. D., George, H. A., Rosolowsky, M., Fife, K. H. & Jansen, K. U. (1995). Sequence determination of human papillomavirus type 6a and assembly of virus-like particles in *Saccharomyces cerevisiae*. *Virology* **209**, 506–518.

Icenogle, J. P., Sathya, P., Miller, D. L., Tucker, R. A. & Rawls, W. E. (1991). Nucleotide and amino acid sequence variation in the L1 and E7 open reading frames of human papillomavirus type 6 and type 16. *Virology* **184**, 101–107.

Kasher, M. S. & Roman, A. (1988). Characterization of human papillomavirus type 6b DNA isolated from an invasive squamous carcinoma of the vulva. *Virology* **165**, 225–233.

Kitasato, H., Delius, H., zur Hausen, H., Sorger, K., Rösl, F. & de Villiers, E.-M. (1994). Sequence rearrangements in the upstream regulatory region of human papillomavirus type 6: are these involved in malignant transition? *Journal of General Virology* **75**, 1157–1162.

Kovelman, R., Bilter, G. K., Glezer, E., Tsou, A. Y. & Barbosa, M. S. (1996). Enhanced transcriptional activation by E2 proteins from the oncogenic human papillomaviruses. *Journal of Virology* **70**, 7549–7560.

Krige, D., Mills, H. R., Berrie, E. L., Doherty, N. C., Jones, D. K., Ryan, C. A., Davies, H., Myint, S., McCance, D. J., Layton, G. T. & French, T. J. (1997). Sequence variation in the early genes E1E4, E6 and E7 of human papilloma virus type 6. *Virus Research* **49**, 187–191.

Oft, M., Bohm, S., Wilczynski, S. P. & Iftner, T. (1993). Expression of the different viral mRNAs of human papilloma virus 6 in a squamous-cell carcinoma of the bladder and the cervix. *International Journal of Cancer* **53**, 924–931.

Rando, R. F., Groff, D. E., Chirikjian, J. G. & Lancaster, W. D. (1986). Isolation and characterization of a novel human papillomavirus type 6 DNA from an invasive vulvar carcinoma. *Journal of Virology* **57**, 353–356.

Roman, A. & Brown, D. (1995). Sequence variation in the extreme 5′ end of the human papillomavirus type 6a long control region. *Journal of Infectious Diseases* **171**, 697–700.

Rübben, A., Beaudenon, S., Favre, M., Schmitz, W., Spelten, B. & Grussendorf-Conen, E.-I. (1992). Rearrangements of the upstream regulatory region of human papillomavirus type 6 can be found in both Buschke-Löwenstein tumours and in condylomata acuminata. *Journal of General Virology* **73**, 3147–3153.

Schwarz, E., Dürst, M., Demankowski, C., Lattermann, O., Zech, R., Wolfsperger, E., Suhai, S. & zur Hausen, H. (1983). DNA sequence and genome organization of genital human papillomavirus type 6b. *EMBO Journal* **2**, 2341–2348.

Thierry, F. & Yaniv, M. (1987). The BPV1-E2 trans-acting protein can be either an activator or a repressor of the HPV18 regulatory region. *EMBO Journal* **6**, 3391–3397.

Wilczynski, S. P., Oft, M., Cook, N., Liao, S. Y. & Iftner, T. (1993). Human papillomavirus type 6 in squamous cell carcinoma of the bladder and cervix. *Human Pathology* **24**, 96–102.

Yaegashi, N., Xi, L., Batra, M. & Galloway, D. A. (1993). Sequence and antigenic diversity in two immunodominant regions of the L2 protein of human papillomavirus types 6 and 16. *Journal of Infectious Diseases* **168**, 743–747.

zur Hausen, H. (1991). Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* **184**, 9–13.

Received 6 April 1999; Accepted 25 May 1999