JOURNAL OF VIROLOGY, Jan. 1986, p. 1–6
0022-538X/86/010001-06$02.00/0

Vol. 57, No. 1

E5 Open Reading Frame of Bovine Papillomavirus Type 1 Encodes a Transforming Gene

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Received 7 May 1985/Accepted 16 September 1985

We have previously shown that the early region of the bovine papillomavirus type 1 genome contains two nonoverlapping segments that can independently induce the morphological transformation of cultured cells. The transforming gene from the 5′ end of the early region is encoded by the E6 open reading frame. The second transforming segment was previously localized to a 2.3-kilobase fragment (2.3T) from the 3′ end of the early region. To determine which of the four open reading frames (E2, E3, E4, and E5) located within 2.3T encodes a transforming gene, we have now introduced a series of insertion and deletion mutations into a clone (pHLB1) in which 2.3T is activated by the Harvey viral long terminal repeat, and we tested the mutants for their ability to induce focal transformation. Our results indicate that the E5 open reading frame, which could encode a low-molecular-weight hydrophobic peptide, is required for pHLB1-induced transformation of NIH 3T3 cells, but that the E2, E3, and E4 open reading frames are not.

Bovine papillomavirus type 1 (BPV) virions or the cloned BPV genome can induce the morphologic transformation of certain cultured cell lines such as mouse NIH 3T3 and C127 (6). The double-stranded DNA genome is maintained as a multicopy autonomously replicating plasmid in the transformed cells (11); however, no infectious virions are produced in cultured cells, since the sequences encoding the major viral structural proteins are not transcribed into RNA (7). The sequence of the 8-kilobase (kb) genome has revealed at least 10 potential protein-coding sequences or open reading frames (ORFs), all on the same strand of the DNA (3) (Fig. 1). All of the viral transcripts that have been detected are encoded by this strand. The viral genome has been divided into early and late regions. The ORFs that are transcribed only in papillomas (warts) that produce virion proteins have been designated late (L1 and L2). The early ORFs are transcribed in nonproductively transformed cells as well as in papillomas (E1 through E8) (1, 7, 10, 22). Two TATA boxes that may function as transcriptional promoters are located upstream from the eight ORFs of the early region (2), and a polyadenylation signal (3) and a transcriptional enhancer element are found 3′ to the early ORFs (2, 14) (Fig. 1).

A 5.6-kb *Hin*dIII-to-*Bam*HI fragment (69T), containing the entire early region (12) (Fig. 1), has the ability to both transform and replicate extrachromosomally. It is therefore probable that the early ORFs encode the proteins that perform the transforming and replicative functions of the virus. 69T contains at least two genes that can independently transform cultured cells. This conclusion was suggested by the observation that all mutations in 69T that interrupted only one ORF retained at least some transforming activity on C127 cells and verified by the recent identification of two nonoverlapping subgenomic fragments with transforming potential (19). One of the transforming genes is encoded by the E6 ORF, which is located at the 5′ end of the early region. A second is located at the 3′ end, since the 2.3-kb *Eco*RI-to-*Bam*HI fragment of the early region (2.3T) is also transforming when it is ligated to a retroviral long terminal repeat (LTR), either in the promoter-positive orientation (pHLB1, Fig. 1) or the opposite orientation (16). The two independently transforming segments have distinguishable transforming activities. Clone pXH800, which contains E6 promoted by a retroviral LTR, transforms C127 but not NIH 3T3 cells, whereas pHLB1 transforms both cell lines (19). In agreement with these results, two different BPV cDNA clones with transforming activity have recently been reported; one includes E6 as its 5′ ORF, whereas the other contains only the 3′ ORFs that are intact in pHLB1 (23).

2.3T contains the entire E2, E3, E4, and E5 ORFs as well as sequences from the extreme 3′ end of the E1 ORF (Fig. 2). E1 is probably required for the maintenance of the extrachromosomal state of the genome, since E1 mutants integrate. However, there is no evidence that E1 is directly involved in the transformation process (15, 18). Mutations that interrupt E2 reduce the transforming activity of the full-length genome (18). In addition, the transforming cDNA from the 3′ end of the early region encodes an intact E2 at its 5′ end (23). This has led to the speculation that E2 may encode the transforming gene of 2.3T. No functions have yet been ascribed to the E3, E4, or E5 ORF.

To determine which of the ORFs of 2.3T encode(s) a gene that can independently transform cultured cells, we have introduced a series of *Xho*I linker insertion and deletion mutations into 2.3T. We have decided to analyze the transforming activity of 2.3T in the pHLB1 construction rather than in the context of the full-length genome because the pHLB1 construction eliminates several features of BPV genetics that may complicate the interpretation of mutants with reduced transforming activity in the full-length genome. The absence of the E6 transforming gene from clone pHLB1 removes the possibility that this other transforming gene might contribute to the focal transformation induced by this clone (19). By placing the expression of 2.3T under the control of the LTR rather than under BPV controlling elements, its transforming activity is less likely to be affected by mutations in viral genes whose products positively regulate the expression of the transforming genes but are not involved directly in cellular transformation. Also, pHLB1 does not have an intact E1 gene or the *cis*-acting plasmid maintenance sequences which are required for autonomous replication of the genome (15, 18); as predicted, it is found

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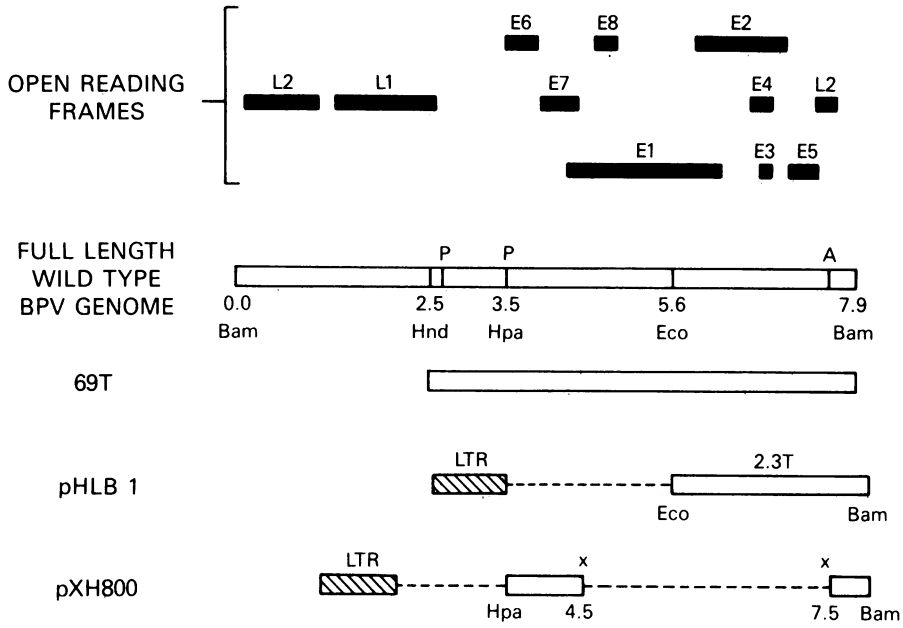

FIG. 1. BPV subgenomic transforming clones. The genome of BPV, linearized at the unique *Bam*HI site in which it was cloned into the pBR322 derivative pML2d, is shown; the linked pML2d is not shown. The locations of ORFs are indicated above the genome. The early ORFs are designated E1 through E8, and late ORFs are designated L1 and L2. The numbers below the BPV genome indicate the distance in kilobases from the *Bam*HI site at 0.0. The locations of the putative early promoters and polyadenylation signal are designated P and A, respectively. Open bars represent BPV sequences included in the clones, and dashed lines indicate deleted sequences. Abbreviations: X, site of *Xho*I linker insertion; Bam, *Bam*HI site; Hnd, *Hin*dIII site; Hpa, *Hpa*I site; and Eco, *Eco*RI site.

integrated in the host chromosomal DNA in transformed cells (16). Therefore, its transforming activity should not be influenced by mutations in genes that are specifically involved in the replication of the viral genome but that might indirectly affect the transforming activity of the full-length viral genome.

We have chosen NIH 3T3 cells for the genetic analysis of pHLB1 because they are more efficiently transformed by

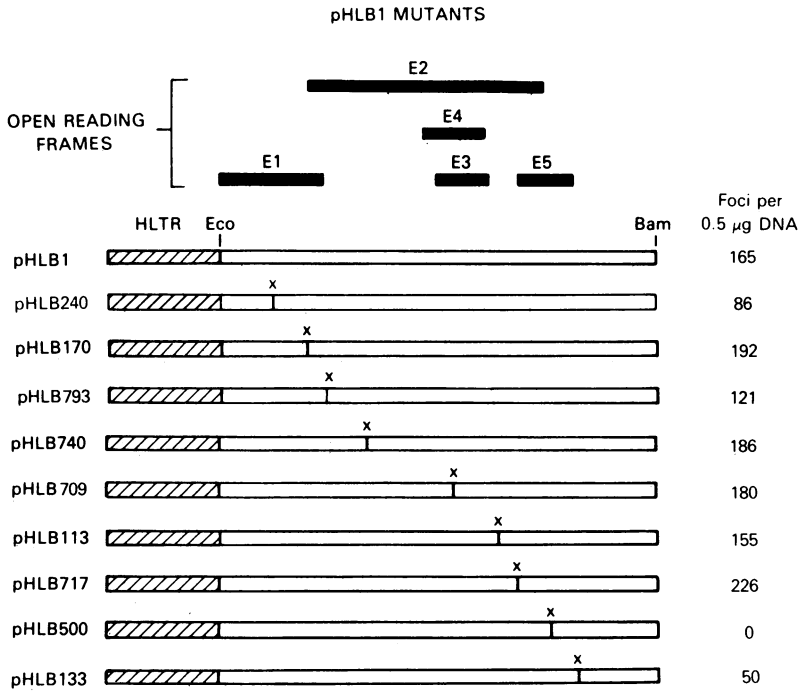

FIG. 2. Transforming activity of *Xho*I linker insertion mutants of pHLB1. Designations are as in Fig. 1. Only the C terminus of E1 is present in the 2.3-kb BPV fragment of pHLB1. pHLB240 and pHLB793 were tested in separate experiments, and the number of foci was normalized with pHLB1, which induced 291 and 486 foci, respectively, as a control.

this construction than are C127 cells and the foci are easier to distinguish from the background cells. Our genetic analysis of pHLB1-induced transformation of NIH 3T3 cells indicates that, contrary to previous expectations, E5 encodes the primary transforming sequences of 2.3T and that expression of the other ORFs is not required for the induction of transformation by this clone.

MATERIALS AND METHODS

Construction of *Xho*I linker insertion mutants of pHLB1. *Xho*I linker insertion mutants 170, 793, 740, 709, 113, 717, and 133 were initially isolated in pdBPV-1, the full-length BPV genome cloned into the *Bam*HI site of the pBR322 derivative pML2d, as previously described (19). These mutations were then introduced into the clone pHLB1 (Fig. 1), which contains the 2.3-kb *Eco*RI-to-*Bam*HI fragment from the 3′ end of the early region of BPV-1 and the 4.0-kb *Eco*RI-to-*Bam*HI fragment of pBR322 with a 0.6-kb permuted Harvey retroviral LTR inserted into the *Eco*RI site in a promoter-positive orientation relative to the BPV sequences (16). The linker insertion mutants were generated by substituting the 2.05-kb *Bst*EII (nucleotide [nt] 2405) to *Bam*HI (nt 4450) BPV fragment of each of the appropriate *Xho*I linker insertion mutant of pdBPV for the corresponding fragment of pHLB1.

The clone pHLB240 was generated by the conversion of the *Bst*EII site of pHLB1 to an *Xho*I site through addition of an *Xho*I linker after filling in the cohesive ends of the *Bst*EII site with the Klenow fragment of *Escherichia coli* polymerase I. The clone pHLB500 was generated by the addition of an *Xho*I linker to the *Bst*XI site (nt 3881) of pHLB1 after digestion of the single-strand ends of the *Bst*XI cleaved DNA with T4 DNA polymerase. The locations of the *Xho*I linkers in these mutants are listed in Table 1.

Deletion derivatives of pHLB1 were constructed by removing the sequences between pairs of *Xho*I linker insertion mutants of pHLB1. This was accomplished by the ligation of the larger *Bam*HI to *Xho*I fragment, containing the pBR322 sequences, from the *Xho*I linker insertion mutant that donated the 5′ end of the recombinant *Xho*I site with the smaller *Xho*I-to-*Bam*HI fragment from the linker insertion mutant that donated the 3′ end of the *Xho*I site.

Sequence analysis of the *Xho*I linkers and the surrounding nucleotides was determined after subcloning of appropriate fragments into an M13 vector and sequencing by the method of Sanger et al. (17).

Cells and DNA transfection assay. NIH 3T3 and C127 cells have been described previously (13). In the transfection experiments, undigested DNAs were precipitated with calcium chloride (9), and 0.2 ml was added to 35-mm dishes seeded on the previous day with 2.5×10^5 NIH 3T3 or C127 cells. NIH 3T3 DNA (25 μg/ml) was used as the carrier; 0.5 μg of mutant DNA was used per dish. The cells were treated as previously described (13), except that dimethyl sulfoxide was not used. Foci were counted 3 weeks later. The dishes containing the clones that failed to yield foci were kept at least 1 month, at which time they were still negative.

TABLE 1. Coordinates of *Xho*I linker insertion mutations

Mutant	Location[a] (nt)	ORFs interupted	Frame shift
240	2410	E1	ND[b]
170	2598–2592	E1, E2	No
793	2693–2743	E2	Yes
740	2869–2911	E2	No
709	3353–3354	E2, E3, E4	Yes
113	3600–3635	E2	Yes
717	3675–3741	E2, E5	No
500	3884–3889	E5	Yes
133	4020–4040		

[a] Nucleotides adjacent to the *Xho*I linker. Locations of the linkers were determined by DNA sequence analysis, except for 240, which was located by the loss of the *Bst*EII restriction site into which the linker was cloned.
[b] ND, Not determined.

TABLE 2. Coordinates of ORFs

ORF	First nt[a]	Last nt	First ATG
E1	813[b]	2663	849
E2	2581	3837	2608
E3	3267	3551	
E4	3173	3526	3191
E5	3714	4010	3878

[a] Coordinates are given in nucleotides from the first nucleotide of the unique *Hpa*I site in BPV-1.
[b] Sequences in pHLB1 begin at nt 2113.

RESULTS

***Xho*I linker insertion mutants of pHLB1.** Nine *Xho*I linker insertion mutants of pHLB1 were constructed, each with an octonucleotide *Xho*I linker inserted into a single site in 2.3T. Sequence analysis indicated that short deletions or insertions of BPV sequences had often been generated at the site of the linker insertion, with the result that not all of the 8-base-pair (bp) linker insertions induced frameshift mutations (Table 1). The nine mutations were distributed throughout the 2.3-kb BPV fragment, 2.3T (Fig. 2). The focus-forming activity of the mutants in a calcium chloride DNA precipitation transfection assay on NIH 3T3 cells is tabulated on the right-hand side of Fig. 2. The mutations outside of the E5 ORF did not significantly effect the transforming efficiency of the clone. This includes mutations that interrupted the 3′ end of E1 (pHLB240 and pHLB170) and those that interrupted the E2 ORF (pHLB170, pHLB793, pHLB740, pHLB709, pHLB113, and pHLB717). Two of these mutants (pHLB709 and pHLB110) also interrupt the E3 and E4 ORFs, which overlap with E2. Two of the inserts introduced frame shifts in the E2 ORF, pHLB793 in the 5′ end and pHLB709 in the middle of the ORF. The insertion in pHLB709 also introduced a frame shift in the E3 and E4 ORFs.

Two mutants interrupted the E5 ORF. In pHLB717 the first 25 bp from the 5′ end of E5 were deleted. This deletion is upstream from the first AUG translation start codon, which is near the middle of the ORF (Table 2); it did not affect the transforming activity of the clone. The insertion in pHLB500 is at the *Bst*XI site, which is 5 bp downstream from the first AUG in E5 and 47 bp downstream from the 3′ end of E2. This mutation produced a frame shift in the E5 ORF and, in contrast to the other insertions tested, rendered pHLB1 nontransforming. An insertion 11 bp downstream from the end of E5 (pHLB133) did not significantly affect the transforming efficiency. The somewhat reduced activity of pHLB133 in the experiment shown in Fig. 2 was not observed in repeat assays (data not shown).

C127 cells appeared to be more resistant to transformation by pHLB1. The number of foci induced in C127 cells by pHLB1 was usually about 20% of the number of foci induced in NIH 3T3 cells (30 to 100 foci versus 150 to 500 foci per 0.5 μg of DNA). In C127 cells, pHLB1 normally induced only about one-half as many foci as the full-length BPV clone,

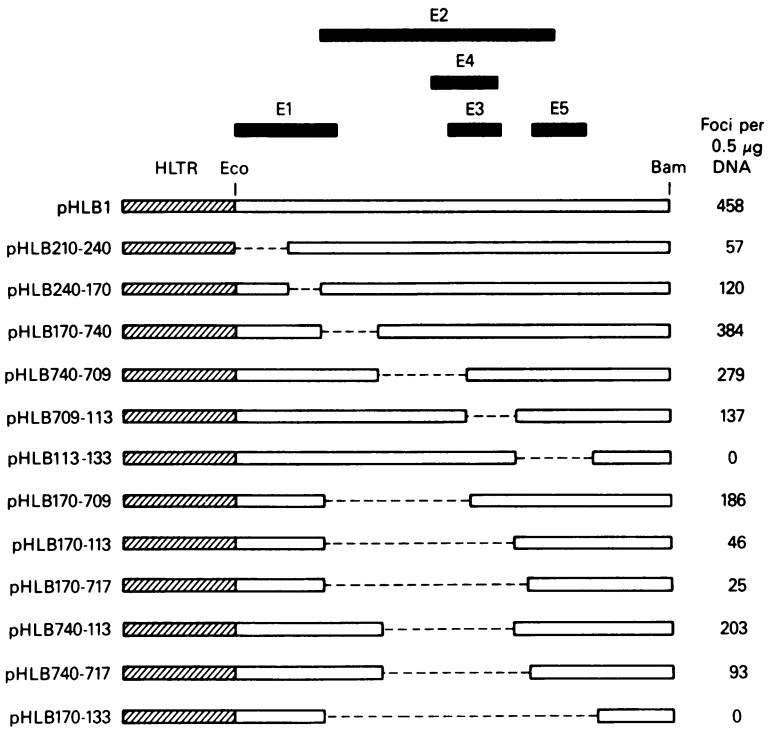

FIG. 3. Transforming activity of deletion mutants of pHLB1. Designations are as in Fig. 1. pHLB210-240 was tested in a separate experiment; the number of foci was normalized with pHLB240-170, which induced 148 foci in the same experiment, as a standard.

whereas in NIH 3T3 cells pHLB1 induced two to three times as many foci as did the full-length BPV clone (data not shown). The foci induced in C127 cells by pHLB1 were generally less distinct than those induced in these cells by the full-length clone. This was not the case with the NIH 3T3 foci. Therefore it appears that, although C127 cells are readily transformed by the E6 transforming gene, they are less susceptible to pHLB1-induced transformation than are NIH 3T3 cells. The pHLB1 mutants that interrupted the E2 ORF often appeared to have reduced transforming activity on C127 cells, but this reduction was difficult to quantitate because the contrast between the induced foci and the background was often diminished. pHLB500, the E5 mutant that was nontransforming on the NIH 3T3 cells, never induced foci on C127 cells.

Deletion mutants of pHLB1. The above results suggested strongly that the transforming gene product in pHLB1 includes sequences within the E5 ORF and that, in this construction, the E2, E3, and E4 ORFs probably do not independently encode a transforming gene product. However, these results did not exclude the possibility that short coding segments upstream of the E5 ORF might be spliced onto the E5 coding sequences to generate the functional transforming gene product. To test this possibility, a series of deletion mutants of pHLB1 was constructed by deleting the sequences located between the *Xho*I linker inserts in pairs of the mutants discussed above. The 12 deletion mutants tested are diagrammed in Fig. 3. The mutants are designated by the numbers of the linker insertions that served as the 5′ and 3′ junction of the deletion (see Table 1 for the locations of the *Xho*I sites). The focus-forming activities of these mutants on NIH 3T3 cells are listed to the right of Fig. 3. The foci induced in NIH 3T3 cells by the transformation-competent derivatives of pHLB1 were indistinguishable morphologically from the foci induced by the parental clone. The two mutants with deletions in the 3′ end of E1 (pHLB210-240 and pHLB240-170) were able to induce focal transformation, although the deletion of the sequences of E1 immediately downstream from the LTR had an unexpectedly large effect on the transformation efficiency. Constructions in which relatively short adjacent segments were deleted from the 5′ end (pHLB170-740), the middle (pHLB740-709), or the 3′ end (pHLB709-113) of E2 were highly transforming (Fig. 3). Mutants in which the entire E3 and E4 ORFs were deleted (pHLB740-113 and pHLB740-717) were also transforming. These results make it unlikely that the transforming gene contains coding sequences upstream of the E5 ORF.

Mutants with larger deletions in E2 appeared to have less transforming activity (compare pHLB170-740 with pHLB170-709, pHLB170-110, and pHLB170-717). This reduced activity is probably not due to the inactivation of an E2 gene product, since the two frameshift mutations in E2 and the smaller deletions of consecutive segments in the same region did not have this effect. We speculate that the larger deletions, and the deletion adjacent to the LTR, may have affected the transforming activity by altering the expression of the E5 mRNA.

As expected, the deletion of the entire E2, E3, E4, and E5 ORFs (pHLB170-133) rendered pHLB1 nontransforming, confirming that the retroviral LTR and BPV enhancer by themselves did not have transforming potential in this assay. The only other nontransforming deletion mutant was pHLB113-133, in which the sequences from 113 bp upstream of the E5 ORF to 11 bp downstream of it were deleted. As described above, the two insertions that served as the junctions of this deletion (113 and 133) do not by themselves significantly affect the transforming potential of pHLB1 (see

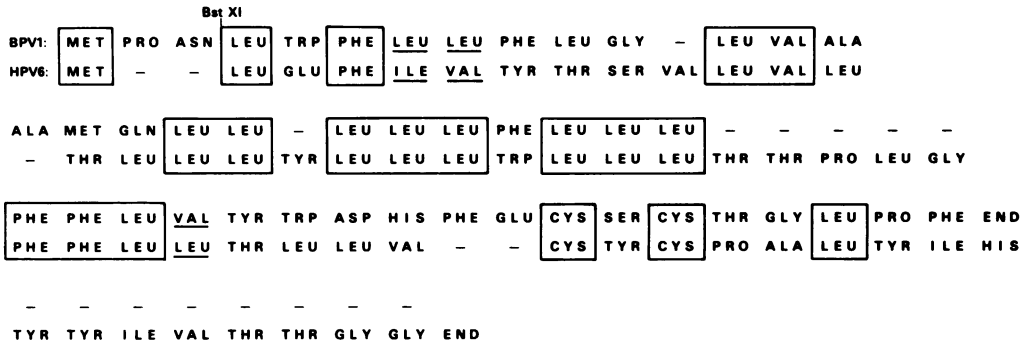

FIG. 4. Comparison of the predicted BPV E5 and HPV6 E5a peptides. The amino acid sequences from the first methionine codon in the two ORFs are shown. Dashed lines indicate gaps introduced into the sequences to optimize alignment. Identical amino acids are boxed, and conserved changes are underlined. The location of the *Bst*XI recognition site, in which the inactivating *Xho*I linker was inserted, is shown above the sequence.

pHLB113 and pHLB133 in Fig. 2). This confirms that sequences in the E5 ORF are required for pHLB1-induced transformation.

DISCUSSION

We have previously found that mutants of BPV DNA from which E6, E7, and most of E1 have been deleted can still transform C127 and NIH 3T3 cells. The transforming activity of these mutants was localized to a 2.3-kb fragment (2.3T) at the 3′ end of the early region by the ligation of this segment to a retroviral control element in the promoter-positive (pHLB1) or -negative orientation (16). Our analysis of pHLB1 indicates that only the expression of E5 is required for focal transformation of NIH 3T3 cells. An *Xho*I linker insertion immediately downstream from the first ATG of the E5 ORF and deletions that eliminated the E5 ORF eliminated the transforming activity of the clone. In contrast, insertions that interrupted the E1, E2, E3, or E4 ORF did not significantly reduce the transforming activity of pHLB1. Similarily, deletions that removed segments of these four ORFs were also transforming, although some of the deletions, particularly the larger ones, did affect the transforming efficiency, perhaps by altering the expression of the putative transforming protein.

Since any of the sequences upstream of the E5 ORF can be removed without abolishing the transforming activity of pHLB1, our working hypothesis is that the transforming gene is encoded by the sequences between the first AUG of the ORF and the end of the ORF, from which a short peptide of only 44 amino acids could be synthesized. However, since neither an E5-specific transcript nor a protein product has been unequivocally identified, we cannot rule out the possibility that the putative E5-encoded transforming peptide is a functional domain of a larger BPV protein that, in our constructions, has transforming activity in a truncated form.

It has recently been determined that the E2 ORF encodes a function that can act in *trans* to stimulate the activity of a BPV transcriptional enhancer located 5′ to the early region (21). If this enhancer plays a role in the expression of early region genes, the reduced transforming activity of E2 mutants seen in the context of the full-length genome may be due to a reduction in the expression of the E6 or E5 transforming genes. Consistent with this interpretation is our finding that E2 insertion mutants have no effect on the transforming activity of pHLB1, in which the expression of E5 is activated by the retroviral LTR rather than by BPV elements.

The nontransforming insertion mutant in E5 leaves the E2, E3, and E4 ORFs intact, so it is unlikely that these latter ORFs encode independently transforming genes. However, we do not know whether these ORFs are functionally expressed in pHLB1 or whether the mutation in E5 affects their expression. Therefore, the possibility that these ORFs encode transforming genes that might be active in other constructions or in other cell types cannot be excluded.

ORFs that may be analogous to the E5 of BPV have been described in some, but not all, other papillomavirus genomes that have been sequenced (5). The E5 ORFs of HPV1a (4) and CRPV (8) do not appear to share homology with the BPV E5. Unlike BPV E5, they overlap the L2 ORF and extend past the early polyadenylation site. HPV6b encodes two overlapping ORFs designated E5a and E5b. E5a is adjacent to E2, and E5b is adjacent to L2. The E5a ORF shares homology with the E5 ORF of BPV (20). The amino acid sequences of BPV E5 and HPV6b E5a, from the first AUG of the ORFs, are aligned in Fig. 4 to emphasize their similarities. These short peptides are very hydrophobic, especially in the conserved regions. It is therefore possible that these two E5 ORFs encode low-molecular-weight, membrane-associated proteins.

ACKNOWLEDGMENT

This investigation was supported in part by Public Health Service grant 5-F32-CA-07237 awarded to J.T.S. by the National Cancer Institute.

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