Identification of a second transforming region in bovine papillomavirus DNA

(Gene expression/retrovirus long terminal repeat/insertional mutagenesis)

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ABSTRACT Bovine papillomavirus type 1 (BPV-1) has been used as a model for studying papillomavirus genes because BPV-1 virions or BPV-1 genomic viral DNA efficiently induce morphologic transformation of certain cultured cells. Previous studies of BPV-1-induced transformation have found that a cloned 5.4-kilobase (kb) fragment (69T) of the genome is transforming and that a 2.3-kb segment from the 3’ end of this fragment is also transforming if activated by a retroviral regulatory element (the long terminal repeat). We now report that 69T contains another transforming segment near its 5’ end that can also be activated by a long terminal repeat. Since this second segment does not overlap the 3’ transforming segment, we conclude that BPV-1 encodes at least two genes that can independently transform cultured cells. Mutational analysis of the 5’ transforming segment suggests that the transforming gene of this segment lies within the E6 open reading frame. The two transforming segments differ in their biological activity in that the E6-containing fragment can transform C127 mouse cells but not NIH3T3 mouse cells, whereas the 3’ fragment can transform both cell lines.

Papillomaviruses induce benign epithelial tumors (warts) of the skin and mucous membranes. In some cases these tumors can undergo malignant degeneration (1, 2). Interest in papillomaviruses has been stimulated further by the accumulating evidence that human genital cancer is frequently associated with infections of certain human papillomavirus (HPV) types (reviewed in ref. 3). For example, in one survey of genital tumors, HPV16 or HPV18 (or both) was detected in >70% of cervical carcinoma and premalignant high-risk lesions (4).

Molecular and genetic studies of papillomaviruses have been limited by the lack of a cell culture system suitable for virus propagation. Bovine papillomavirus type 1 (BPV-1), which induces fibropapillomas in its natural host, has been studied because it can efficiently induce morphologic transformation of certain cultured cells, such as mouse C127 and NIH3T3 cell lines (5, 6). BPV-1-transformed cultured cells exhibit a fully transformed phenotype: they form foci in monolayer culture, colonies in agar, and tumors in nude mice. The full-length BPV DNA genome replicates in these cells as an unintegrated multicopy episome (7). However, the transformed cells do not synthesize the major structural viral antigens and hence do not produce infectious virions. Autonomous replication of viral DNA is not absolutely required for transformation, since mutant genomes have been constructed that still transform despite integrating into the host chromosomal DNA after transfection (8, 9).

Previous genetic studies of BPV-induced transformation, a 5.4-kilobase (kb) subgenomic transforming fragment (69T, representing 69% of the full-length viral genome) that can replicate autonomously was identified (6). A 2.3-kb segment from the 3’ end of 69T was also shown to have transforming potential when activated by a retroviral long terminal repeat (LTR) (8). Sequence analysis has revealed that 69T encodes at least eight open reading frames (ORFs) (10). The functions encoded by these potential protein coding sequences have not yet been determined nor have protein products encoded by them been identified. Since 69T is transcribed in papillomas and transformed cells, it is presumed that the ORFs of 69T encode the genes responsible for autonomous replication of the genome and cellular transformation (11-13).

To determine which ORFs encode the genes that are responsible for BPV-1-induced transformation of cultured cells, we have examined the transforming potential of a large number of single-site insertion mutants of the full-length BPV genome and identified subgenomic fragments with transforming activity. The results indicate that BPV encodes at least two transforming genes that are located at opposite ends of 69T and that the transforming gene of the newly identified transforming segment is encoded by the E6 ORF.

EXPERIMENTAL PROCEDURE

Generation of Linker Insertion Mutants. Clone pdBPV-1 (142-6) was used as the source of BPV DNA into which linkers were inserted (14). This clone represents the full-length BPV genome inserted at its unique BamHI site in the plasmid pML2d. Plasmid pML2d is a 2.6-kb derivative of pBR322 from which sequences that inhibit simian virus 40 replication in mammalian cells have been deleted (15). The BPV genome linked to this plasmid is transforming (14).

Linker insertion mutations were introduced into the full-length BPV genome by the procedure of Heffron et al. (16) as follows: clone pdBPV-1 (142-6) was randomly cleaved with pancreatic DNase I in the presence of MnCl2. Blunt-end molecules were generated by incubation of the linearized full-length molecules with T4 DNA polymerase and the four deoxyribonucleotide triphosphates. The linker C-C-T-C-G-A-G-G, containing the C-T-C-G-A-G recognition sequence of Xho I (clone pdBPV-1 does not contain a Xho I site), was added to the ends by blunt-end ligation with T4 DNA ligase. Excess linkers were removed by digestion with Xho I and the molecules were recircularized by using T4 DNA ligase. The plasmids were transformed into Escherichia coli strain RR1, and individual colonies were screened for mutant plasmids containing an Xho I site. The locations of the Xho I linker insertions were mapped by digesting the plasmids with Xho I in combination with other restriction endonucleases and subjecting the digests to agarose gel electrophoresis. Sequence analysis (see below) of four of the mutants showed that the actual sites of insertion were within 50 base pairs (bp) of the locations estimated by electrophoretic mobility. Restriction endonuclease fragments of these mutants were

Abbreviations: BPV-1, bovine papillomavirus type 1; kb, kilobase(s); ORF, open reading frame; LTR, long terminal repeat; nt, nucleotide(s); bp, base pair(s); HPV, human papillomavirus.

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used to construct the deletion mutants described below.

**Construction of Deletion Mutants.** Clone pXH133 is a full-length insertion mutant that has an Xho I linker inserted 7.5 kb downstream from the left-hand BamHI site (locations of all sites are indicated by their distance in kb from this BamHI site; see Fig. 1). Clone pXH760 is a second mutant that has an Xho I linker inserted at 4.5 kb. pXH633 is a recombinant deletion mutant of BPV DNA from which the BPV sequences from 4.5 to 7.5 kb have been deleted by replacing the 5.1-kb HindIII-to-Xho I fragment of pXH133 with the 2.0-kb HindIII-to-Xho I fragment of pXH760 (Fig. 1). pXH635 was generated by the digestion of pXH633 with Hpa I and Cla I (the unique Cla I site in pXH633 is located in pML2d upstream from the 0.0 BamHI site) and intramolecular ligation of the 4.0-kb fragment after making the Cla I ends flush with the Klenow fragment of E. coli polymerase I.

Clone pM15 (17) is a nontransforming segment of Moloney murine sarcoma virus cloned in pBR322; this segment includes a 0.9-kb EcoRI-to-Bal I fragment that contains the viral LTR (the viral control element) plus 0.3 kb of downstream sequences down to the viral donor splice site. pXH800 was generated by insertion of this 0.9-kb EcoRI-to-Bal I fragment of pM15 into the 4.0-kb Hpa I-to-EcoRI fragment (the EcoRI site is located in pML2d) of pXH633. Clone pXH960 resulted from the intramolecular ligation of the 4.2-kb Hpa I-to-Sal I fragment (the Sal I site is located in pML2d) of pXH800.

**Construction of pXH800 Mutants.** Xho I linker insertions were introduced into pXH800 (Fig. 2) by replacing its 0.95-kb Hpa I-to-Xma I fragment (the Xma I site is located at 4.4 kb in BPV, downstream from Hpa I) with the corresponding fragment from five of the full-length BPV insertion mutants. (Each of these full-length mutants was biologically active.) The locations of the inserts in pXH828 and pXH855 were determined by the electrophoretic mobility of restriction fragments; their Xho I sites were mapped 600 and 750 nucleotides (nt) downstream from Hpa I, respectively. The sites of the insertions in pXH831, pXH875, and pXH975 were determined by sequence analysis (Genex, Rockville, MD) using the M13 cloning/dideoxy sequencing methods (18, 19); short deletions were found at the sites of insertion, as noted previously (16). By using the first nucleotide of the BPV Hpa I site as nt 1 (10), the coordinates of the BPV nucleotides adjacent to linkers were pXH731, 90 and 99; pXH875, 445 and 470; pXH975, 681 and 689. pXH897 has two Xho I linkers inserted in tandem; the other two clones each contain a single linker.

In mutant pXH997, the sequences between the Xho I linker in pXH897 and the Xho I linker in pXH800 at the 3' end of the transforming segment were deleted. pXH975 and pXH931 were similarly constructed by using the Xho I linker insertions in pXH875 and pXH831, respectively, as the 5' ends of the deletion. pXH888 contains an insertion, generated in the initial linker insertion experiment, that is associated with an estimated 210-nt deletion of the sequences from 540 to 750 (relative to the Hpa I site). pXH175 was generated by deletion of the segment between the linker in pXH831 and the linker in pXH875.

**Cells and DNA Transfection Assay.** NIH3T3 and C127 cells have been described previously (20). The C127 cells are a clonal nontransformed line derived by W. P. Parks and C. Chen from the mammary tumor tissue of an RIII mouse. In the transfection experiments, undigested DNAs were precipitated with calcium chloride (21), and 0.2 ml was added to 35-mm dishes seeded on the previous day with 2.5 × 10^5 C127 or NIH3T3 cells. NIH3T3 DNA (25 µg/ml) was used as carrier; instead, a control DNA was used that had been treated as described previously, except that dimethyl sulfoxide was not used (20). 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.

**RESULTS**

**Linker Insertion Mutants.** A series of single-site mutants of the full-length BPV-1 genome were generated by the random insertion of an 8-bp Xho I linker as described in Experimental Procedures. The majority of the mutants developed by this procedure (which may also have 10-20 short deletions at the site of insertion [16; see below]) should produce shifts in the reading frame when inserted into protein coding sequences. Forty of these linker insertion mutants in 69T were mapped and their transforming potential on mouse C127 cells was determined. Unexpectedly, all of the mutants with single insertions in potential protein coding segments were still able to induce focus formation, although each of the major ORFs was interrupted by at least one of the linker insertion mutations (data not shown). These results suggested that the BPV genome might contain more than one gene that can independently transform C127 cells.

**Construction of Transforming Subgenomic Clones.** To test the hypothesis that BPV-1 encodes at least two transforming genes, we constructed a series of deletion mutants by making recombinants via the unique Xho I restriction sites in the insertion mutants described above (Fig. 1). Since the 3' 2.3 kb of 69T had been shown previously to be transforming, we directed our attention to the two ORFs, E6 and E7, that are located outside this 2.3-kb segment. Clone pXH633 is a mutant that was generated by deleting the segment between an Xho I linker insertion at 4.5 kb and an insertion at 7.5 kb. The 410 nt upstream from the BamHI site at 7.9 kb were included in the construction because they contain BPV polyadenylation and enhancer sequences (22, 23). This mutant lacks all of the ORFs of clone pHLB1, the previously identified 3' transforming clone (8). Nevertheless, it was still able to induce focal transformation of C127 cells, indicating that BPV-1 contains a second transforming gene.

Clone pXH633 has a reduced transforming efficiency relative to the wild-type clone and, in comparison to wild-type foci, the foci induced by pXH633 appeared later and grew more slowly, perhaps because pXH633 lacks the pHLB1-encoded transforming gene(s). Deletion of the 3.5-kb segment between BamHI at 0.0 kb and Hpa I at 3.5 kb rendered the clone nontransforming (pXH635, Fig. 1); it is likely that this clone is unable to induce transformation because it lacks BPV transcriptional regulatory sequences (8, 10, 12, 23). We therefore inserted a promoter, known to be transcriptionally active in mouse cells, into the BPV genome immediately upstream from the ORFs. This was done by ligating the Moloney sarcoma virus LTR (17) to the Hpa I site of pXH635 (clone pXH800, Fig. 1). pXH800 induced focal transformation of C127 cells at least as efficiently as did pHLB1, the clone containing the previously identified transforming segment (Fig. 1). Since the LTR and BPV enhancer segments are not transforming under these conditions (see clones pXH875, pXH975, pXH931, and pXH175 in Fig. 2), we conclude that this 1-kb segment from the 5' end of 69T encodes a transforming gene. When this 1-kb BPV segment is promotated by the LTR, the BPV enhancer/polyadenylation segment is not required for efficient induction of transformation of C127 cells (clone pXH960, Fig. 1).

In contrast to the C127 cells, the NIH3T3 cells were not transformed by pXH633, pXH800, or the derivatives of pXH800 discussed below. However, they were transformed at high efficiency by the wild-type clone and pHLB1, indicating that these cells could function in the transfection assay (Fig. 1).

**Mutational Analysis of the 5' Transforming Fragment.** To determine which of the open reading frames contained in the
BPV fragment of pXH800 was responsible for its transforming potential, we introduced several Xho I linker insertion and deletion mutations into the clone (Fig. 2). This segment contains two complete ORFs, E6 and E7, and the 5' end of E1. The three insertions in E7 (pXH828, pXH977, and pXH855) did not significantly affect the transforming efficiency of the clone. The mutant with an insertion in the 5' end of the E6 ORF (pXH831) retained the transforming activity of pXH800. Sequencing revealed that pXH831 is a substitution mutant in which the 8-nt linker replaced 8 nt of the 4th through the 6th codon after the first ATG codon of the E6 ORF. This insertion would therefore not be expected to induce a frameshift. The mutant with the insertion in the 3' end of E6 (pXH875) was, however, nontransforming. By sequence analysis, the linker insertion in pXH875 is associated with a deletion of 24 bp, resulting in a frameshift that fuses the E6 and E7 ORFs. This same insertion in the full-length genome resulted in a reduction in its transforming activity on C127 cells to 1/10th that of wild-type BPV but had little, if any, effect on its activity on NIH3T3 cells (data not shown).

Deletions in the transforming fragment were constructed by excision of the sequences between pairs of Xho I insertions (Fig. 2). Deletions that involved only the E7 and E1 ORFs did not abolish the transforming ability of the fragment (pXH977 and pXH888), whereas those that removed all or part of E6 (pXH975, pXH931, and pXH175) rendered the clone nontransforming. These results strongly suggest that E6, which is transcribed in C127 cells transformed by BPV (11), encodes a protein that can transform mouse C127 cells. Similar conclusions have been reached independently in studies of subgenomic BPV DNA (33).

C127 foci induced by pXH800 and pXH997 were isolated, expanded, and further characterized. Cells from these clones contained BPV sequences, grew in soft agar, and formed tumors in nude mice (data not shown). Since the 3' half of the E7 ORF is deleted in pXH977, these results suggest that expression of E6 alone is sufficient for the induction of the fully transformed phenotype in C127 cells.

**DISCUSSION**

It has been shown previously that a subgenomic fragment of BPV (in clone pHLB1), located at the 3' end of the region of the genome transcribed in cultured cells, can transform mouse C127 and NIH3T3 lines (8). This segment includes several ORFs (E2, E3, E4, and E5). In this study, we have identified a second subgenomic BPV segment, located at the 5' end of this transcribed region, that can independently induce a fully transformed phenotype in C127 cells but cannot transform NIH3T3 cells. When activated by a retroviral LTR, the 5' segment induced focal transformation of C127 cells as efficiently as did the LTR-activated 3' segment.

It is likely that E6 encodes the transforming gene of the 5' segment. All but one of the mutations that interrupt the E6 ORF render the fragment nontransforming, whereas those that involve the other ORFs in this segment, E7 and E1, have little effect on its transforming activity. Sequence analysis indicated that the transforming E6 mutant, pXH831, is a substitution mutant, the 7th through the 14th nucleotides after the first ATG in the ORF having been replaced by the 8-bp linker. The transforming ability of pXH831 may indicate...
**OPEN READING FRAMES**

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Fig. 2. Mutational analysis of the 5' transforming region. Designations are the same as in Fig. 1. Only the NH₂ terminus of E1 is present in the 1-kb Hpa I-to-Xho I fragment of pXH800. DNAs were transfected onto C127 cells in both experiments. Numbers in parentheses refer to a third experiment in which pXH800 induced 149 foci per 0.5 μg of DNA.

Either that the first AUG of the E6 ORF is not used to initiate the synthesis of the presumptive E6 protein or that the three predicted amino acid substitutions in the NH₂ terminus of the putative E6 protein do not affect its transforming activity. Because none of our mutants is located in the 5' end of E7, our results have not formally ruled out the possibility that the first 75 nt of E7 might also be involved in the transforming process.

If translation of the E6 ORF of BPV-1 was initiated from the first AUG [as is true of most eukaryotic proteins (24)], the translation product would be 137 amino acids (≈16 kDa) (25). ORFs analogous to E6 of BPV-1 are also present in other papillomavirus genomes that have been sequenced (25, 26). E6 is not as well conserved as certain other papillomavirus ORFs. However, all of the sequenced E6 ORFs show a similar arrangement of four Cys-X-X-Cys tetrapeptides. (This peptide also appears twice in the COOH-terminal half of E7 that is not required for transformation in our constructions.) Interestingly, cysteine-rich repeats are also found in polyoma and simian virus 40 small t antigens, in several early proteins of adenovirus, and in glycoprotein hormones such as thyrotropin and metallothioneine (26).

Other tumorigenic papovaviruses, such as polyoma and simian virus 40, encode only one gene which have thus far been shown to induce morphologic transformation of cultured cells (27, 28). However, other early gene products of these viruses appear to be involved in the tumorigenic process (27–29). Whether the two independently transforming BPV genes act cooperatively in the in vivo induction of tumors has not yet been determined. Both transforming segments are apparently transcribed in BPV-transformed cells and in BPV-induced fibropapillomas (11–13); analogous segments are also transcribed in cottontail rabbit (Shope) papillomavirus-induced carcinomas (30). However, in HeLa and other cell lines that are derived from human cervical carcinomas and contain HPV18 DNA, sequences analogous to E6, but not to the 3' transforming segment, are transcribed (34). When cotransfected onto C127 cells, the two LTR-activated transforming segments induce foci more rapidly than either segment does alone (9 vs. 16 days), and the number of foci induced is greater (unpublished observation), suggesting that they do act synergistically in vitro.

In contrast to the 3' transforming segment, which transforms both C127 and NIH3T3 cells, the E6-containing transforming segment transforms C127 cells, but not NIH3T3 cells. It is not known what accounts for this differential sensitivity. The inability of the E6-containing clones to transform NIH3T3 cells could have several explanations. One possibility is that the gene may not be expressed at sufficient levels in these cells to induce transformation. However, the Moloney sarcoma virus LTR used in the E6 construction does promote transformation by the segment in mouse C127 cells and can efficiently promote the transcription of other genes in NIH3T3 cells (31, 32). Also, Harvey murine sarcoma virus, which is an acute transforming retrovirus, transforms both cell lines with similar efficiency (unpublished observation). Another possible explanation is that the putative E6 protein can induce transformation by a mechanism to which the NIH3T3 cells are not susceptible. This raises the possibility that C127 cells might be responsive to a class of transforming genes that are negative in the NIH3T3 transfection assay, which is used by most investigators to screen for transforming genes.