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Bovine papillomavirus contains multiple transforming genes

(transcription/cDNA cloning/expression vector/papovavirus/transformation)

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ABSTRACT **Bovine papillomavirus type 1 (BPV-1) and its cloned full-length DNA can transform rodent cells *in vitro*, and the viral DNA persists as an extrachromosomal multicopy plasmid in these transformed cells. Previous studies have identified at least five discrete viral RNAs that are expressed in BPV-1 transformed cells and have shown that these transcripts share a 3′ coterminus. To further define the structure of these RNAs and to characterize the functions of individual viral transcripts, we constructed a cDNA library with mRNA from BPV-1-transformed mouse C127 cells using an Okayama and Berg plasmid. From a library of 10^5 independent clones, 200 BPV-1 specific clones were isolated and characterized. Sequence analysis has revealed differential splicing patterns for the mRNA species in BPV-1 transformed cells. In conjunction with the open reading frames (ORFs) deduced from the BPV-1 DNA sequence, it is possible to predict the structure of the potential encoded proteins. The vector used to generate these cDNA clones contains mammalian cell transcriptional regulatory elements, facilitating their functional characterization. We have identified two distinct classes of cDNA clones that can each independently transform mouse C127 cells. One class of cDNA clones contains the E2 ORF intact and the second contains the E6 ORF intact. These two putative viral functions appear to act synergistically in transforming mouse C127 cells *in vitro*.**

Bovine papillomavirus type 1 (BPV-1) causes benign fibropapillomas (warts) in cattle and also induces fibrosarcomas in heterologous hosts. The virus or its molecularly cloned DNA can transform certain mouse cells *in vitro*, and the DNA persists as an extrachromosomal multicopy plasmid in the transformed cells (1). A 69% subgenomic fragment (BPV_{69T}) bounded by the unique *Hin*dIII and *Bam*HI sites is sufficient to induce transformation *in vitro* (2), and recombinants containing this DNA segment can be maintained as free plasmids in transformed cells (3). Cell lines established from transformed foci have a fully transformed phenotype in that they are anchorage independent and are tumorigenic in nude mice (4).

The genomic organization of BPV-1 has been established from the DNA sequence (5) and from the transcription data from both BPV-1-transformed cells (6) and productively infected bovine fibropapillomas (7, 8). All of the detectable mRNA species are transcribed from a single strand, and all of the open reading frames (ORFs) of >400 base pairs are located on the same strand. As indicated in Fig. 1, several mRNAs have been mapped in the 69% transforming region where eight of the ORFs (labeled E1 through E8) are located. These viral transcripts have a common 3′ end at 0.53 map units, but they have bodies that appear to vary at their 5′ ends (6).

Recent studies from our laboratory and others have identified several regions of BPV-1 genome that influence the expression of the viral transforming functions (9–12). Deletion mutants affecting the E2 ORF are significantly impaired in their ability to transform mouse C127 cells, suggesting that the putative E2 protein is an important transforming protein (9). The expression of E2 alone, however, is not sufficient for the fully transformed phenotype. Deletion mutagenesis studies map an additional function that is critical for the fully transformed phenotype to the region between the *Hpa* I site (base 1) and the *Sma* I site (base 945) (9). These previous studies did not distinguish whether these two distinct regions of the viral genome encode separate proteins or whether they contain exons that are spliced together at the level of mRNA to generate a single transforming protein.

To further define the structure of the mRNAs in transformed cells and to characterize the functions encoded by individual viral transcripts, we constructed a cDNA library using mRNA from BPV-1-transformed mouse cells. The vector used for cDNA cloning contains the simian virus 40 (SV40) early promoter, the SV40 late region introns, and the SV40 late region polyadenylylation site, allowing expression of cDNA clones in mouse cells for functional analysis (17). In this study, expression vectors that carried individual cDNA inserts were tested for their ability to transform mouse C127 cells.

MATERIALS AND METHODS

Cells and Transformation. Mouse C127 cells (4) and BPV-1-transformed mouse C127 cells (ID13) (1) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. DNA transformation was carried out as described (14).

Construction of cDNA Library with pCD Expression Vector. Polyadenylylated mRNA from ID13 cells was prepared by methods previously described (15, 16). A cDNA library was constructed from this mRNA with the plasmids pcDV1 and pL1, using the plasmid primer method described by Okayama *et al.* (17). The bacterial transformation was carried out using *Escherichia coli* strain DH1 (13).

Isolation and Characterization of BPV-1 cDNA Clones. BPV-1 specific cDNA clones were identified by colony hybridization (18, 19). Viral cDNA clones with inserts >1 kilobase (kb) were analyzed by restriction enzyme mapping and were sequenced by Maxam and Gilbert techniques (20).

Analysis of Cellular DNA. Total cellular DNA was extracted from cDNA transformed cells according to a modified method described previously (1). Southern blot analysis was performed as described (21).

RESULTS

Screening of cDNA Library for BPV-1 Sequences. A library of ≈100,000 independent clones was made from the polya-

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Abbreviations: BPV, bovine papillomavirus; ORF, open reading frame; SV40, simian virus 40; kb, kilobase(s).

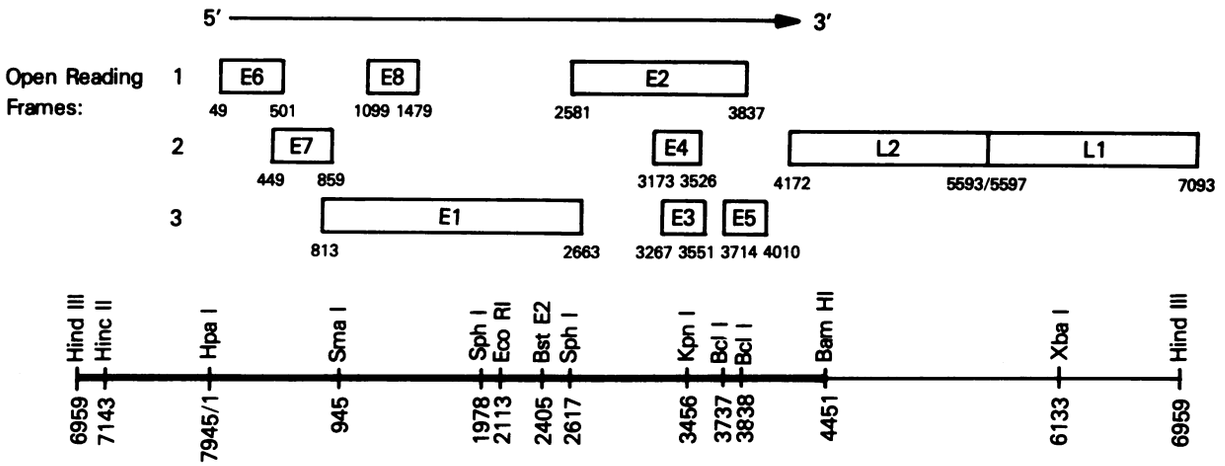

FIG. 1. Genomic organization of BPV-1 DNA. The full-length molecule (7945 base pairs) of BPV-1 opened at the unique *Hin*dIII (base 6959) is marked off with restriction sites and bases noted at the bottom of this figure. The transforming segment from *Hin*dIII to the *Bam*HI site is indicated by the heavy bar (2). The region transcribed in transformed cells and the direction of transcription are indicated by the arrow at the top of the figure (6). Open bars represent potential coding regions for BPV-1 proteins in each of the three reading frames (5). ORFs within the transforming region have been designated E1–E8. Numbers beneath ORF represent the first and last bases of the ORF. This schematic representation of the BPV-1 genome has been published previously (9) and is reproduced with permission.

denylylated RNA from ID13 cells. Using a BPV-1 specific radiolabeled probe, the library was screened and 200 BPV-1-specific clones were isolated. Positive clones were then hybridized with subgenomic fragments of BPV-1 DNA to provide preliminary mapping data and information on which to design a sequencing strategy. The insert sizes of viral cDNA clones ranged from 0.2 to 2.8 kb. Since the viral RNAs in BPV-1-transformed cells range in size from ≈1 to 4 kb (6), only clones that contained inserts ≈1 kb or larger were analyzed further.

Structural Analysis of BPV-1 Specific cDNA Clones. The data summarizing the cDNA clones that were sequenced are presented in Table 1. Seven classes of cDNA clones can be distinguished based on these sequence data. Previous analysis of the RNAs present in BPV-1-transformed cells has indicated that the different viral transcripts have a 3′ coterminus at ≈0.53 map units. Sequence analysis of several cDNAs listed in Table 1 indicates that the mRNA is polyadenylylated at base 4203, 24 bases 3′ to the first A of the polyadenylylation recognition site A-A-T-A-A-A.

In five of the seven classes of cDNAs characterized, spliced messages were identified. Splice donor sites were identified at bases 304, 864, and 2505. Splice acceptor sites were found at bases 527 and 3224. The DNA sequence of each of these splice donor and acceptor sites is presented in Fig. 2 and agrees reasonably well with the consensus splice donor and acceptor sequence (22). The 5′ end of three of the cDNAs sequenced was at base 89. This agrees with the data of Ahola *et al.* (23), who mapped this as a major 5′ end of the mRNAs present in transformed cells. There is a T-A-T-A-A-A sequence 31 bases upstream at base 58. This sequence has been shown in many instances to be part of an RNA polymerase II transcriptional promoter and to apparently function in positioning the 5′ ends of the mRNAs (24). The class III cDNA clone (C58) has a 5′ end that maps upstream from this promoter element, indicating that promoter sequences other than those with the "TATA" box at nucleotide 58 function in BPV-1-transformed cells. Many of the cDNA clones have 5′ ends mapping downstream from base 89. Whereas some of these may represent copies of truncated viral RNAs, some could be representative of full-length RNAs possibly generated from an additional viral promoter. In the case of class V cDNAs and one of the class VI cDNAs (C88) with 5′ ends mapping to base 2440, the sequence T-A-A-T-A-T-T is located 27 nucleotides upstream.

We also identified several unspliced cDNA clones and designated them as class VI or class VII clones based on size. The class VI cDNA clones ranged from 1.9 to 2.5 kb and had 5′ ends mapping from base 1889 to base 2534. The class VII cDNA clones measured from 1.1 to 1.2 kb and had 5′ ends from base 3212 to base 3309.

Potential Coding Regions for Individual cDNA Clones. Potential coding sequences for six of the seven classes of cDNAs are presented in Fig. 3. The class VII clones are not included because they are likely to consist of cDNA copies of truncated viral mRNAs. Since the viral proteins produced in BPV-1-transformed cells have not yet been identified, the likely proteins encoded by each of the cDNA classes were

Table 1. Structure of various cDNA clones

Class	Clone	Size of cDNA, kb	5′ end	Intron (±)	Donor (D) and acceptor (A) sites
I	C102	1.7	89	+	304(D), 527(A) 864(D), 3224(A)
II	C119	1.4	89	+	304(D), 3224(A)
	C211	1.4	89	+	304(D), 3224(A)
III	C58	2.1	7879	+	864(D), 3224(A)
IV	C48	1.6	429	+	864(D), 3224(A)
	C91	1.4	670	+	864(D), 3224(A)
V*	C5	1.3	2440	+	2505(D), 3224(A)
	C14	1.3	2440	+	2505(D), 3224(A)
	C37	1.3	2440	+	2505(D), 3224(A)
	C174	1.3	2440	+	2505(D), 3224(A)
	C189	1.3	2440	+	2505(D), 3224(A)
VI	C52	2.5	1889	−	
	C59	2.1	2360	−	
	C61	2.1	2360	−	
	C88	2.0	2440	−	
	C212	1.9	2534	−	
VII†	C36	1.2	3212	−	
	C122	1.2	3233	−	
	C79	1.1	3245	−	
	C32	1.1	3309	−	

*Other similar or identical clones include C9, C13, C18, C23, C134, C153, C176, C180, and C207.

†Other similar or identical clones include C125, C97, C114, C117, and C199.

Base no	Viral donor site	Base no	Viral acceptor site
304	CAG**GT**GAGG	527	TCACCTGC**AG**GA
864	AAG**GT**AGCA	3224	CTGATTTT**AG**AG
2505	CGG**GT**GAGC		

EXON — INTRON — EXON

A A
AGGT AGT
C G

C G
PyPyPyPyPyPyX AGG
T T

Consensus Sequences

FIG. 2. Comparison of BPV-1 splice junctions with consensus acceptor and donor sites.

predicted based on the general rules for protein translation in eukaryotic cells (25).

The class I cDNA clone (C102) could direct the synthesis of a 21-kDa protein that is derived from the in-frame splicing of the E6 and E7 exons, and uses bases 91–93 (AUG) and 860–862 (UAA) for the initiation and termination codons. C102 contains a second splice from base 864 to base 3224; however, these sequences are located 3′ to the termination codon that would be used. The class II clone (C119) splices the E6 and E4 ORFs together and would direct the synthesis of a 20-kDa protein using the initiation codon AUG at bases 91–93 and the termination codon UGA at bases 3527–3529. The class III cDNA (C58) has a splice donor at base 864 after the E6 termination codon UAG at bases 502–504 and, therefore, would direct the synthesis of the 15.5-kDa E6 protein. The class IV cDNA (C48) with a 5′ end at base 429 could be a truncated class III cDNA. If, however, it is representative of a class of an mRNA species in BPV-1-transformed cells, it could direct the synthesis of a 15.5-kDa E7 protein with an AUG initiation codon at bases 479–481 and the termination codon at bases 860–862.

A number of 1.3-kb clones were identical in structure and are represented by the class V cDNA clones. These each had a 5′ end at base 2440 and were spliced from base 2505 to base 3224. These do not contain a large open translation frame following an initiation methionine codon. The sequence of each of these cDNAs contains stretches of adenines around base 2440 instead of the BPV-1 genomic sequence G-A-T-A-T-T-T located at base 2440. It is possible that the cDNAs represented by the class V structures are faithful copies of RNAs within cells that have been modified in the sequences at their 5′ ends as the result of some specific RNA degradation or cleavage process. Pettersson and his group have obtained evidence by heteroduplex analysis of a class of RNA molecules in BPV-1-transformed cells with 5′ ends that map to this region (Ulf Pettersson, personal communication). Alternatively, these molecules might have been generated by the staggering effect of reverse transcriptase in a region of

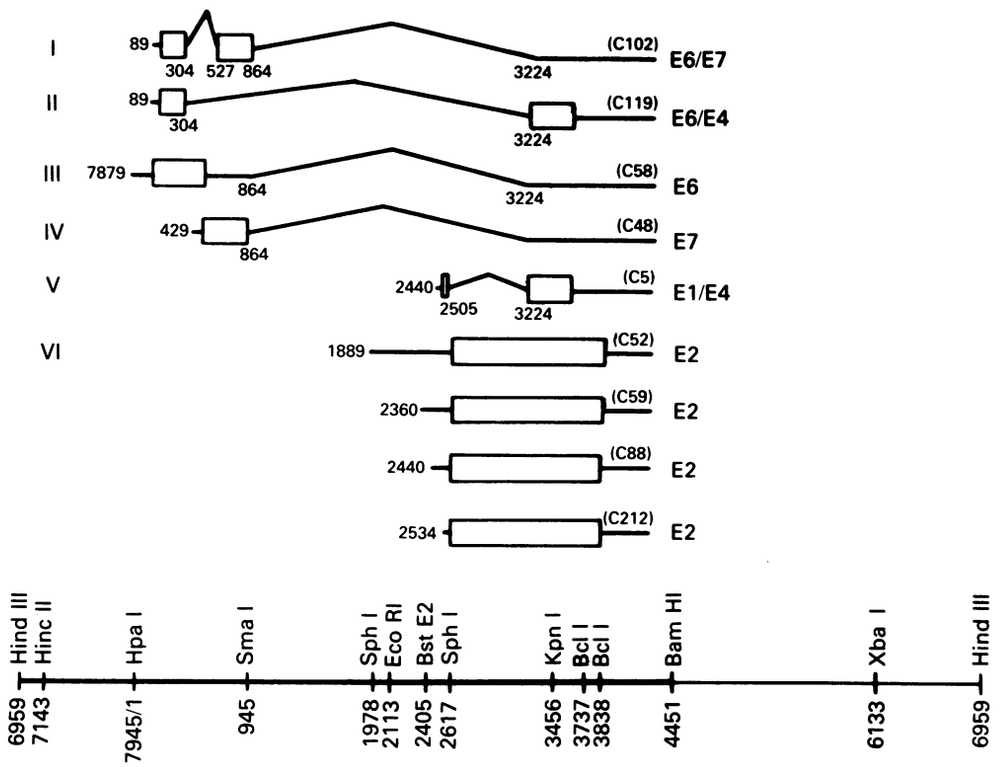

FIG. 3. Structure of various cDNA clones are indicated with the most 5′ coding region following an AUG indicated by open box except for the class V clone, which is believed to be a truncated E1/E4 clone. The putative nontranslated regions are indicated by solid line and intervening sequences are shown by slanted lines. Numbers outside the coding region represent 5′ ends and splice junctions. Potential ORFs expressed for each individual cDNA clone are designated on the right. A restriction map is at the bottom of the figure for orientation.

high secondary or tertiary structure of the mRNA. The splice from base 2505 to base 3224 results in the fusion of the E1 and E4 ORFs; however, there is no methionine initiation codon in this fused open segment. Whether class V cDNA clones represent truncated copies of transcripts that exist in BPV-1-transformed cells for the production of a protein resulting from fusion of the E1 and E4 ORFs remains to be examined.

The five cDNA clones represented by class VI were unspliced and contained the E2 ORF intact; the first AUG is located at bases 2608–2610 within the E2 ORF. Each of the class VI cDNAs could, therefore, direct the synthesis of a 48-kDa E2 protein with initiation and termination codons at bases 2608–2610 and 3838–3840, respectively. These cDNAs also contain the E3, E4, and E5 ORFs intact. However, the E3 and E4 ORFs overlap the E2 ORF and are located 3′ to the methionine initiator codon for E2, and, therefore, would be unlikely to be translated from the mRNAs represented by this class of cDNAs (25). The first AUG in the E5 ORF is at nucleotides 3879–3881. This is downstream from the E2 ORF termination codon and thus this region could potentially be translated into a 44 amino acid protein from any of the cDNAs in this study if they represented bicistronic messages.

The class VII cDNA clones are also unspliced and most likely represent cDNA copies of truncated mRNAs. These cDNA clones contain the COOH end of the E2 ORF and a truncated E4 ORF, as well as the E3 and E5 ORFs. Other than the short E5 ORF described above, there are no large open reading frame segments in these cDNA clones preceded by a methionine AUG.

Transformation of Mouse C127 Cells by Various cDNA Clones. Since each of the cDNAs was cloned into an expression vector containing SV40 transcriptional regulatory elements, it was possible to assay biological functions directly. The transforming ability of individual cDNA clones from each of the class I to class VI was tested by focus assay on mouse C127 cells, and the results are summarized in Table 2. The cloned cDNAs from classes III and VI were each able to transform mouse C127 cells. The class VI cDNA clones are nonspliced and contain the full E2 ORF, which would most likely be translated as discussed above. The class III clone (C58) is spliced and contains the complete E6 ORF as its first major open translation frame behind an initiator methionine codon.

Each of the cDNA clones tested transformed mouse cells at an efficiency less than that of the full BPV-1 genome (Table 2). There was some variability among the four class VI clones tested (Table 2), which differ by the length of the BPV-1 segment 5′ to the AUG codon in the E2 ORF. The class III cDNA clone (C58) induced foci at an efficiency of 3–4 foci per plate compared to that of the full BPV-1 genome, which induced 100–136 foci per plate in this experiment. The size and morphologic appearance of the foci induced by each of these cDNAs expressed behind the SV40 early promoter are similar. Individual foci induced by the class III cDNA (C58) and the class VI cDNA (C88) were cloned into lines and shown to contain between 10 and 20 copies of the cDNA vector integrated (data not shown).

To test whether transforming products encoded by the class III and class VI cDNA clones might have a cooperative effect in transforming mouse C127 cells, cotransformation experiments were performed. As shown in Table 2, when these cDNA clones were cotransfected, the frequency of focus formation was higher than transfection with either the class III or the class VI clones alone. This effect is more than additive, suggesting a potential cooperative effect between the putative functions encoded by these two classes of cDNA clones. In addition, the foci induced by cotransformation were larger and appeared faster than those induced by either the class III or the class VI cDNA clones alone.

Table 2. Transformation of mouse C127 cells by BPV-1 cDNA clones

Clone	Class	Potential coding region*	Foci per plate alone†	Foci per plate with C58 DNA‡
C52	VI	E2	27, 36, 50	65, 105, 112
C59	VI	E2	20, 56, 58	82, 98, 102
C88	VI	E2	7, 8, 14	27, 32, 66
C212	VI	E2	7, 13, 18	17, 41, 49
C58	III	E6	3, 4	—
C48	IV	E7	0, 0	—
C102	I	E6/E7	0, 0	—
C119	II	E6/E4	0, 0	—
C5	V	Truncated E1/E4	0, 0	—
p142-6	—	Full BPV-1 DNA	100, 120, 136	—
Salmon sperm DNA	—	—	0, 0	—

*Each of these classes of cDNAs also contains intact E3 and E5 ORFs; in addition, class VI contains an intact E4.
†Each plate received a total of 5 μg of the indicated cDNA clone.
‡Each plate received a total of 5 μg of the indicated cDNA clone and 5 μg of C58 DNA.

DISCUSSION

Transcriptional studies of the papillomaviruses have been limited because of the low abundance of viral mRNA in transformed cells (6, 23). The structural analysis of the cDNAs cloned from BPV-1-transformed cells presented in this present study indicates that the viral RNAs are generated by differential splicing. Splice donor sites map to nucleotides 304, 864, and 2505; and splice acceptor sites map to nucleotides 527 and 3224. The different classes of RNA detected and their splice junctions are in good agreement with the studies of Pettersson and colleagues who have independently mapped the polyadenylylated viral RNA species present in BPV-1-transformed cells by heteroduplex analysis (U. Pettersson, personal communication).

We map a major 5′ end to nucleotide 89, confirming the results of Ahola *et al.* (23). A T-A-T-A-A-A sequence at nucleotide 58 is likely an element of a transcriptional promoter located upstream from this transcriptional start site. The presence of ≈165 base pairs of sequences upstream from base 89 in one cDNA clone sequenced indicates that there is an upstream start site(s) and that there is an additional promoter(s) functional in BPV-1-transformed cells. Many of the 5′ ends of the cDNAs analyzed were located downstream from nucleotide 89 and many mapped to approximately nucleotide 2440. Whereas some of these may represent copies of truncated RNAs or incomplete copies of RNAs, others may be true 5′ ends, indicating that additional RNA polymerase II promoter elements may be located in this region. More detailed studies of BPV-1 transcription in transformed cells will be required to answer this question. Prevous studies have indicated that the viral mRNAs have a 3′ coterminus at 0.53 map units. Sequence analysis indicates that the site of polyadenylylation is at the guanine residue at base 4203, located 24 bases downstream from the polyadenylylation recognition sequence at 4180 (A-A-U-A-A-A). The 3′ ends of RNA transcripts in mammalian cells are polyadenylylated at sites 10–25 nucleotides 3′ to the A-A-U-A-A-A signal (26).

Previous studies have localized a transforming gene for BPV-1 to the 2.3-kb segment of the BPV-1 genome between

the *Eco*RI site and the *Bam*HI site (9, 11). Furthermore, deletion mutagenesis studies and targeted base mutagenesis studies implicate the product of the E2 ORF as having an important role in BPV-1-mediated cellular transformation (ref. 9; D. DiMaio, personal communication; M. Lusky and M. Botchan, personal communication).

Although the class VI cDNA clones that contain the E2 ORF intact could represent true copies of mRNAs that would direct the synthesis of a 48-kDa E2 protein in transformed cells, they are not spliced and their 5′ ends map in a region where no viral transcriptional promoter has yet been localized. Thus, the true structure of the putative E2 ORF mRNA remains uncertain. Each of the cDNAs in this class is, however, able to independently transform mouse cells with the expression vectors used in this study, further implicating E2 as important in papillomavirus-induced transformation.

Our previous studies have mapped a function critical for the expression of the full transformed phenotype to the segment of the genome containing the E6 and E7 ORFs (9). The present study provides the structure of a number of cDNAs with coding exons located in this region. Of these, only the clone that would direct the synthesis of the E6 ORF intact is capable of transforming C127 cells, indicating that the 15.5-kDa product of the E6 ORF is a second and independent transforming protein of BPV-1. A similar conclusion has been independently made by Schiller *et al.* (27).

The mechanism by which the putative products of the E2 and E6 ORFs may lead to transformation is not clear at this point. Comparison of amino acid sequences deduced from different papillomavirus DNA sequences has provided some interesting characteristics of these proteins. The amino terminus of the E2 protein is well conserved among BPV-1, HPV-1a, HPV-6, and CRPV (28, 29). There is also amino acid and structural homology involving ≈80 amino acids at the carboxyl terminus of the E2 ORFs of these viral genomes. In addition, this carboxyl-terminal domain has limited homology with the human c-*mos* gene product (30). There is an intriguing property of E6 ORF shared by BPV-1 and the other papillomaviruses sequenced (28, 29). Although this ORF is not well conserved among these sequenced genomes, there is a tetrapeptide sequence, Cys-X-X-Cys, that is repeated four times in the E6 ORF of each genome with conserved distances between the repeating units. Cysteine-rich repeated segments are also found in the small t antigens of the polyomaviruses including SV40, polyoma, and BK. A number of studies have indicated a role for SV40 small t antigen in transformation and anchorage-independent growth (31, 32).

In this paper, we have established that two classes of cDNA clones (class III and class VI) can independently transform C127 cells, and that they apparently act synergistically *in vitro* in a focus assay. Although other classes of cDNAs were negative by focus assay, our studies do not rule out a potential role for any of the putative viral gene products encoded by these clones in BPV-1 transformation. Furthermore, additional viral gene products not necessarily reflected by the cDNAs described in this study may also be involved in cellular transformation and may even be able to induce foci independently.

It seems likely that the transforming capability of the class III and class VI cDNA clones is due to the different viral proteins they encode. These classes of cDNAs could encode the putative E6 and E2 proteins, respectively, and deletion studies have mapped functions involved in transformation to the regions of the BPV-1 genome containing these ORFs (9). Our interpretation that the transforming functions are due to these putative proteins, however, must be tempered because of our current inability to directly define the viral proteins present in cells transformed by each of these classes of cDNAs. A potential role of the downstream ORFs present in these cDNA clones (i.e., E3, E4, or E5) cannot be ruled out at this time. Deletion mutagenesis studies using the cDNA molecules described in this paper should permit a resolution of this issue.

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