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NOTES

Genetic Assignment of Multiple E2 Gene Products in Bovine Papillomavirus-Transformed Cells

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The E2 open reading frame of bovine papillomavirus type 1 has been shown genetically to encode at least three transcriptional regulatory factors, and three E2 specific proteins have been recently identified in virally transformed rodent cells. In this study, the genes encoding these E2 specific proteins have been determined. The 48-kilodalton (kDa) protein was identified as the product of a full-length E2 open reading frame cDNA, which confirmed that this polypeptide is the E2 transactivator. The 31-kDa E2 protein species, which is the most abundant E2 specific polypeptide, was identified by analysis of both bovine papillomavirus type 1 mutants and cDNAs to be the previously identified E2 transcriptional repressor, E2-TR, which results from translation initiation at an internal E2 ATG codon. The smallest E2 protein species, the 28-kDa polypeptide, was identified as the product of the E8/E2 fusion gene which results from translation of a spliced mRNA species.

Bovine papillomavirus type 1 (BPV-1) can readily transform a variety of rodent cells in tissue culture (3), and the development of a quantitative focus transformation assay (7) has provided an in vitro system for studying papillomavirus molecular biology. On the basis of the replicative state of BPV-1 as a multicopy plasmid (19) and the specific and tightly regulated expression of viral early genes (2, 12), these virally infected rodent cells are thought to resemble the nonproductively infected cells of a fibropapilloma. Expression of the viral early genes is modulated by *trans*-acting factors encoded by the viral E2 open reading frame (ORF). This property is conserved among the many papillomaviruses (5, 8, 15, 23, 28). The full-length E2 gene encodes a transcriptional transactivator (27, 29) that activates transcription of several viral promoters (10, 14, 26) through the viral conditional enhancer element (9, 11, 25–27). Located within this enhancer are multiple copies of a DNA sequence, 5′-ACCN$_6$GGT-3′, to which the transactivator binds specifically (1, 22). The E2 transactivator is transcribed at least in part from the P_{2443} promoter, which is itself an E2-responsive promoter (14). An N-terminally truncated E2 ORF gene product (E2-TR) is a transcriptional repressor that inhibits the function of the E2 transactivator (17). Expression of E2-TR results from translation initiation at an internal ATG codon (nucleotide [nt] 3091) within the E2 ORF, positioned downstream of the P_{3080} promoter (17). A third E2 gene, the E8/E2 gene, was predicted to result from the fusion of the upstream E8 ORF to the 3′ region of the E2 ORF via an mRNA splice event between nt 1234 and 3225 (18). The E8/E2 gene product also acts as an E2 transcriptional repressor (4; P. Lambert, B. Monk, and P. Howley, manuscript in preparation; this study, see below) and is transcribed by an upstream promoter, possibly the newly defined P_{890} promoter (4). Since the DNA-binding domain of the E2 transactivator protein is contained within the carboxy-terminal 100 amino acids, the E2-TR and E8/E2 proteins that contain this coding domain (see Fig. 3) can also bind to the 5′-ACCN$_6$GGT-3′ DNA sequence recognized by the E2 transactivator (10a, 21). Furthermore, each of the repressor proteins can potentially form heterodimer complexes with the E2 transactivator (6, 20). On the basis of these shared biochemical properties, the repressors are hypothesized to inhibit E2 transactivation by competitive DNA binding at the viral enhancer, by subunit mixing, or by both.

With the use of E2-specific antisera, Hubbert et al. (16) recently identified three E2 specific polypeptides, present in BPV-1-transformed rodent cells, with apparent sizes of 48, 31, and 28 kilodaltons (kDa). The relative abundance of these proteins ranged between 1:10:2 and 1:20:2, respectively. On the basis of preliminary genetic analysis, the two smaller species were identified as N-terminally truncated E2 species, and the 48-kDa protein was identified as the full-length gene product. Our study was designed to unambiguously assign the gene encoding each of these proteins and to confirm the identity of the E2 gene products encoded by specific E2 cDNAs that had previously been used to identify the E2 transcriptional regulatory activities.

Immunoprecipitations were performed with E2-specific antisera, using extracts from cells harboring different BPV-1 cDNAs or full-length genomic clones of BPV-1 containing point mutations designed to disrupt the expression of specific E2 genes. These mutants, along with previously identified viral cDNAs that encode specific E2 activities, are listed in Fig. 1. The mutation in BPV-1 mutant p1472-1 replaces a C for a T at BPV-1 nt 3092, causing a Met → Thr codon substitution. This missense mutation removes the E2 ORF internal translation initiation codon utilized by the E2 repressor gene, E2-TR. Independent studies have shown that this mutation significantly reduces the level of E2 repressor activity expressed by BPV-1 (D. Riese, J. Settleman, and D. DiMaio, manuscript in preparation; Lambert et al., in preparation). The mutation in BPV-1 mutant p1471-1 replaces an A for a G at BPV-1 nt 1235, which alters the consensus 5′ splice sequence, 5′-AGGT-3′, to 5′-AGAT-3′. This mutant is

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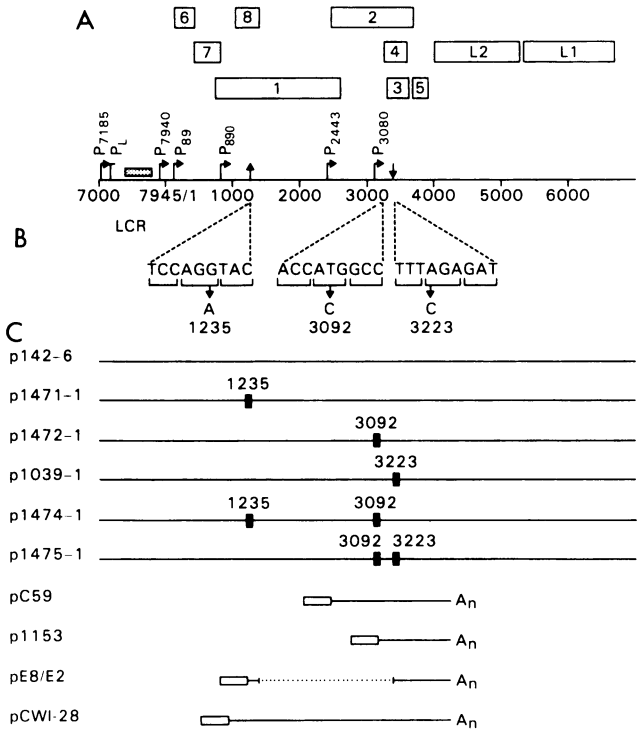

FIG. 1. BPV-1 constructs. (A) Map depicting the circular 7,945-base-pair BPV-1 genome linearized at nt 7000. The long control region (LCR) contains several transcriptional promoters, P_{7185}, P_L, P_{7940}, and P_{89} (→), and the E2 conditional enhancer, $E2RE_1$ (□). ↑, position of the 1234 5′ splice site; ↓, 3225 3′ splice site; □, early (1 to 8) and late (L1 and L2) translational ORFs. (B) DNA sequences surrounding the sites of three independent base substitution mutations, at nt 1235, 3092, and 3223. Codon triplets for the E8 and E2 ORFs are bracketed. The 1235 (Lambert et al., in preparation) and 3223 (13) base substitutions were engineered by using oligonucleotide site-directed mutagenesis. (C) Line drawings indicating the BPV-1 sequences present within each of the BPV-1 plasmids used in this study. Each plasmid contained the full-length BPV-1 genome inserted into pML2d at the unique *Bam*HI site. Plasmid p142-6 is the wt BPV-1 clone (24). Indicated are presence of the base substitution mutation (■) and positions of the simian virus 40 early transcriptional promoter and splice signals (□). Also shown are the BPV-1 cDNA structures. The 5′ endpoints of the BPV-1 sequences present in each cDNA are nt 2360 for pC59, nt 3014 for p1153, and nt 845 for pCW1-28. The 3′ endpoint of the BPV-1 sequence for each of the cDNAs is nt 4203 (A_n, position of the BPV-1 early polyadenylation signal). cDNA pE8/E2 was created by reconstructing the 1234/3225 splice by using synthetic oligonucleotides. This cDNA has BPV-1 sequences from nt 1194 to 1234 contiguous to BPV-1 sequences for nt 3225 to 4203. cDNA pC59 has the capacity to encode both the E2 transactivator and the E2 repressor gene. cDNA p1153 can encode only the E2 repressor gene. The synthetic cDNA pE8/E2 can encode only the E8/E2 fusion gene, whereas cDNA pCW1-28 can potentially encode all three E2 genes.

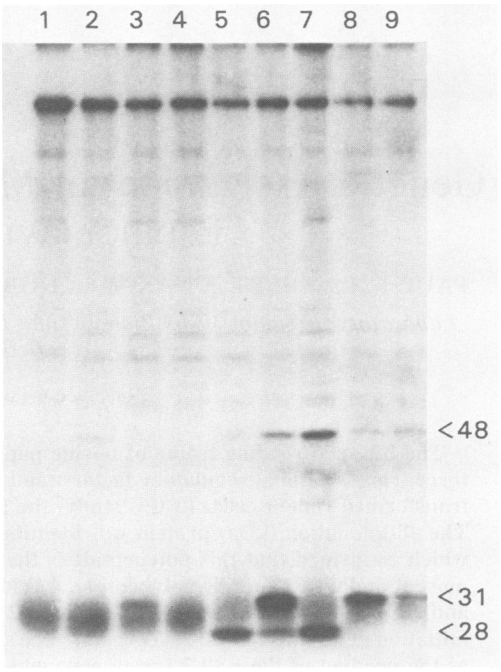

FIG. 2. Immunoprecipitations using E2-specific antisera. C127 cells were cotransfected with the BPV-1 plasmids listed in Fig. 1 along with pMMTneo as previously described (17). Cells were selected for G418 resistance, and resistant colonies were pooled at 2 weeks. Subconfluent 75-cm^2 flasks were labeled with [^{35}S]cysteine, and immunoprecipitations were performed by using the SP20 antibody as described previously (16). Immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were visualized by fluorography. Shown are control C127 cells harboring pMMTneo only (lane 1) or C127 cells cotransfected with pMMTneo and pC59 (lane 2), p1153 (lane 3), pE8/E2 (lane 4), pCW1-28 (lane 5), p142-6 (lane 6), p1472-1 (lane 7), p1471-1 (lane 8), or p1039-1 (lane 9). Indicated at the right are positions of the 48-, 31-, and 28-kDa E2 specific polypeptides.

silent in the E8 ORF and causes a conservative amino acid substitution in the overlapping E1 ORF. BPV-1 mutant p1039-1 has been previously described (13) and contains an A-to-C transversion at BPV-1 nt 3223, which alters the consensus 3′ splice sequence, 5′-TTAGA-3′, to 5′-TTCGA-3′. This mutant is silent in the E2 ORF and causes a conservative Leu-to-Phe substitution in the overlapping E4 ORF. Both p1471-1 and p1039-1 are disrupted for the 1234^3225 mRNA splice that is necessary for the generation of an E8/E2 mRNA species. BPV-1 mutants p1474-1 and p1475-1 are double mutants containing both substitutions present in p1471-1 and p1472-1 or in p1472-1 and p1039-1, respectively (Fig. 1). Each of these double mutants has mutated the internal ATG initiation codon at nt 3091 utilized by the E2-TR gene and either the 1234 5′ splice signal (p1474-1) or the 3223 3′ splicing signal (p1475-1) utilized by the E8/E2 gene. They are predicted, therefore, to encode only the E2 transactivator. The BPV-1 cDNAs used in this study have been described previously for expression of either the E2 transactivator (pC59; 29) or E2 repressor (p1153 [17], pCW1-28 [17], and pE8/E2) [Lambert et al., in preparation] activities.

Mouse C127 cells were cotransfected with pMMTneo, a plasmid encoding the Tn*5* neomycin resistance gene under the control of the mouse metallothionein gene promoter, and each of the BPV-1 plasmids listed in Fig. 1. Cell populations were selected for G418 resistance and labeled with [^{35}S]cysteine, and the protein extracts were immunoprecipitated by using E2-specific antisera (Fig. 2). As previously shown (16), wild-type (wt) BPV-1 (p142-6)-transfected cells expressed three E2 specific polypeptides with sizes of 48, 31, and 28 kDa (Fig. 2, lane 6). The BPV-1 mutants (lanes 7 to 9) each encoded a subset of the E2 specific polypeptides present in wt BPV-1-transfected cells. The E2 gene encoding each polypeptide can thus be assigned. The 31-kDa protein is identified as the product of translation initiation at the

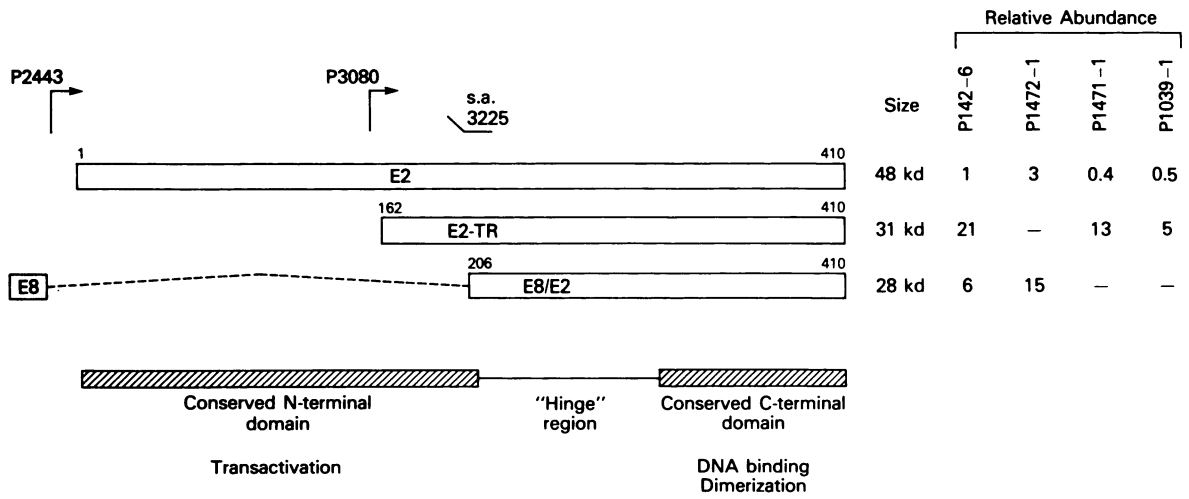

FIG. 3. Gene structures and relative abundances of the multiple E2 proteins. Line drawings indicate the coding domains of the three E2 genes. Numbers refer to amino acid residues in the E2 ORF from the first methionine. The protein size of each gene is assigned on the basis of the conclusions drawn from this study. At the right is shown the relative abundance of each protein for each BPV-1 genomic clone. Quantitation was performed by densitometric scanning of the autoradiograph shown in Fig. 2, with correction for the number of cysteine residues present in each protein. For comparison, the value of 1 was assigned to the abundance of the 48-kDa protein present in wt BPV-1-transfected cells.

internal E2 ATG codon, given its absence in p1472-1-transfected cells (Fig. 2, lane 7). The absence of the 28-kDa protein in both of the 1234 and 3225 splice mutants, p1471-1 and p1039-1, establishes it as the E8/E2 gene product (Fig. 2, lanes 8 and 9). Furthermore, the 28- and 31-kDa proteins were missing in the double mutants p1474-1 and p1475-1, consistent with these assignments (data not shown).

BPV-1 cDNAs were analyzed for the capacity to express E2 specific polypeptides. The C59 cDNA containing the full-length E2 ORF, which was previously shown to encode the E2 transactivator function (29), expressed predominantly the 48-kDa species (Fig. 2, lane 2), consistent with the previous assignment of this polypeptide as the full-length E2 ORF gene product. Low levels of the 31-kDa polypeptide were also detected in some immunoprecipitation experiments with pC59 (data not shown). This is likely to reflect P_{3080} promoter activity in pC59 or internal translation initiation off the full-length messenger species. The P_{3080} cDNA, p1153, which was previously shown to encode the E2 transcriptional repressor, E2-TR, initiating at the internal E2 ATG codon at nt 3091, expressed the 31-kDa E2 species (lane 3), consistent with the conclusions based on analysis of BPV-1 mutant p1472-1. A synthetically constructed E8/E2 cDNA was tested for expression of E2 specific proteins (Fig. 2, lane 4), and no E2 specific peptides were detectable. This result may simply reflect the low levels of E2 expression seen with our cDNAs or the presence of a diffuse cell-specific band (see untransfected control, lane 1) of similar mobility that obscured low-level presence of the 28-kDa polypeptide. The 28-kDa protein was expressed by the cDNA pCW1-28 (Fig. 2, lane 5). This cDNA can potentially encode all three E2 peptides, since it has the contiguous BPV-1 sequences from nt 845 to 4203; in fact, low levels of the 48-kDa protein were also detected (Fig. 2, lane 5). The initial genetic analyses of pCW1-28 did not resolve whether the repressor activity encoded by this cDNA mapped principally to the E8/E2 gene or to the E2-TR gene (17, 18). The data obtained from this immunoprecipitation, however, indicate that E8/E2 is the major E2 species encoded by pCW1-28 and therefore strongly suggest that E8/E2 is, like E2-TR, an inhibitor of E2 specific transactivation.

In addition to the absence of specific E2 polypeptides, the full-length genomic BPV-1 mutants exhibited changes in the relative abundance of those E2 peptides which were expressed. The relative abundance of the E2 specific proteins for each mutant genome is indicated in Fig. 3. The most striking changes were the increased levels of both the 48- and 28-kDa species in cells containing p1472-1. Overall, the ratio of E2 repressor protein to E2 transactivator protein decreased from 28 in wt BPV-1-transfected cells to 5 in the p1472-1-transfected cells. This dramatic change would be predicted if the 31-kDa protein, which is both the most abundant E2 protein and the more abundant repressor protein in wt BPV-1 cells, represses the expression of both the 28- and 48-kDa proteins. The change in abundance of the transactivator to the repressor proteins with this mutant is associated with increases in viral transformation and viral plasmid replication (Lambert et al., in preparation). In the case of BPV-1 mutants p1471-1 and p1039-1, loss of the 28-kDa species had a significant but reduced effect on the abundance of the expressed E2 proteins and the overall ratio of repressor to transactivator, presumably because the 28-kDa protein was the less abundant repressor protein. Overall, these changes in the abundance of E2 proteins seen with the BPV-1 mutants further implicate a role for autoregulation by these transcriptional factors on their own expression (14).

In summary, we have identified the specific E2 gene encoding each of the E2 proteins present in BPV-1-infected cells. The 31- and 28-kDa E2 proteins represent the gene products of the E2-TR and the E8/E2 transcriptional repressor genes, respectively, whereas the 48-kDa protein is the E2 transactivator gene product. Several observations can be made on the basis of these assignments. First, the relative abundance of the E2 repressor gene products over the E2 transactivator could account for the very low levels of viral gene expression in transformed rodent cells (12). Second, the presence of the E8/E2 gene product confirms earlier predictions that the 3225 3′ splice site could provide a

mechanism for the virus to encode additional E2 gene products (13) and that such gene products would share certain biochemical properties with the transactivator, including DNA binding (10a, 21) and dimerization (6, 20). Third, why does the virus express both the 31- and 28-kDa transcriptional repressors? Given that the E2-TR and E8/E2 genes are expressed from different promoters, it is possible that they are differentially regulated at the level of transcription. Thus, while it is clear that the E2-TR gene product is the predominant repressor in steady-state transformed rodent cells, the E8/E2 protein could play an important role under other conditions. Alternatively, the biochemical activities of these repressor proteins could differ in a manner not yet discernible. The BPV-1 mutants defective in repressor expression are now being studied to provide some insight into the role that the two E2 transcriptional repressors play in maintaining the nonproductive infective state.

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