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# Role of Transcriptional Repressors in Transformation by Bovine Papillomavirus Type 1

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**Transformation of rodent cells by bovine papillomavirus type 1 (BPV-1) has been shown to require the direct contribution of the viral oncogenes encoded by the E5, E6, and E7 translational open reading frames (ORFs). It is also known that the viral E1 and E2 ORFs contribute indirectly to cellular transformation through their transcriptional modulation of these viral oncogenes. A mutant BPV-1 disrupted in two of the proteins encoded by the E2 ORF, the E2 transcriptional repressors, has a complex transformation phenotype. In this paper, we provide evidence that (i) this phenotype is not attributable to the absence of other viral genes that overlap the E2 repressor genes, (ii) the acquisition of transformation over time in cells harboring the E2 double-repressor mutant correlates with reversions of the mutations that disrupt one of the E2 repressor genes, E8/E2TR, and (iii) the initial transformation defect in the E2 double-repressor mutant can be rescued by disruption of the full-length protein encoded by the E1 ORF, the E1 transcriptional repressor. We propose a model by which the combination of E1 and E2 transcriptional repressors functions to modulate the transforming capacity of the virus.**

The discovery that the bovine papillomavirus type 1 (BPV-1) can readily transform immortalized rodent cells in tissue culture has allowed for the genetic dissection of papillomavirus functions (3). BPV-1 encodes three oncogenes that function to alter cellular growth directly; they are derived from the E5, E6, and E7 translational open reading frames (ORFs) (4, 21, 24, 26, 32). In addition, viral genes derived from the E1 and E2 ORFS also contribute to the transformation efficiency of BPV-1. The contribution of the latter genes to the transforming potential of BPV-1 is thought to be indirect, since they encode proteins that can regulate the promoters responsible for the production of the E5-, E6-, and E7-specific mRNAs. In this study, we attempt to dissect the role that proteins encoded by the E1 and E2 ORFs play in the modulation of cellular transformation by BPV-1.

The E2 ORF encodes three gene products, E2TA, E2TR, and E8/E2TR. E2TA is a transcriptional transactivator (28) that activates multiple viral promoters, including $P_{89}$ and $P_{2443}$ (6, 7, 27), two promoters that direct expression of the viral oncogenes. E2TA, together with the E1 protein, is necessary and sufficient to support BPV-1 plasmid DNA (30, 31). In addition to E2TA, the E2 ORF encodes two transcriptional repressors, E2TR and E8/E2TR, that inhibit the multiple activities of the E2TA protein (1, 2, 14, 15). The more abundant repressor protein in BPV-1-transformed cells is the 31-kDa E2TR protein, synthesis of which is initiated from an internal E2 ORF initiator codon (8, 13). The less abundant repressor is the 28-kDa E8/E2TR protein synthesized from a spliced mRNA species that fuses the upstream E8 ORF to the 3′ half of the E2 ORF. Both repressor proteins contain the E2 DNA-binding and dimerization domains found in the E2 transactivator protein (17–20). Two possible mechanisms by which the repressors might mediate E2TA repression are competitive binding for sites on the virus where E2TA binds and formation of inactive heterodimers with E2TA.

Apparently contradictory results have led to confusion as to how the E2 repressors might function to modulate cellular transformation by BPV-1. When overexpressed in *trans*, both E2TR and E8/E2TR can independently repress focus formation by wild-type BPV-1 in mouse C127 cells (2, 14, 15). However, when both repressor genes are mutated in the context of the full-length viral genome, transformation by this mutant virus is severely impaired in focus and soft agar colony transformation assays. Interestingly, though, cells harboring this E2 double-repressor mutant virus can acquire a transformed phenotype upon continued culturing (14). The reason for this complex transformation phenotype is not understood. In this paper, we demonstrate that genetic reversion of mutations in the E8/E2TR gene correlates with the delayed onset of transformation observed in a fraction of rodent cells harboring the E2 double-repressor mutants. In addition, we describe a set of results indicating that increases in the activity of the E1 protein, itself a transcriptional repressor, may be responsible for the initial transformation defect in rodent cells transfected with the E2 double-repressor mutant.

**The complex transformation phenotype of the original double-repressor mutant is independent of the 23-kDa E1N protein.** The initial phenotypic analysis of E2 repressor mutants (14) utilized an E2 double-repressor mutant, p1474-1, referred to in this study as o-BPV-$1^{E8/E2TR,\ E2TR}$. This mutant is a derivative of p142-6 (23), a bacterial recombinant plasmid that contains the full-length wild-type BPV-1 viral genome (p142-6 is referred to as BPV-$1^{WT}$ in this study). o-BPV-$1^{E8/E2TR,\ E2TR}$ contains point mutations at BPV-1 nucleotide (nt) 3092 (T→C), disrupting the internal E2 ORF ATG initiation codon for the E2TR gene (13), and at BPV-1 nt 1236 (G→A), disrupting the nt 1235^3225 mRNA splice event required for production of E8/E2TR-specific mRNA (Fig. 1). However, in addition to being deficient in the synthesis of both E2 repressor species, o-BPV-$1^{E8/E2TR,\ E2TR}$ is predicted to be defective in the synthesis of the 23-kDa E1N (also termed E1M) protein, since the nt 1235 5′ splice signal is used for the

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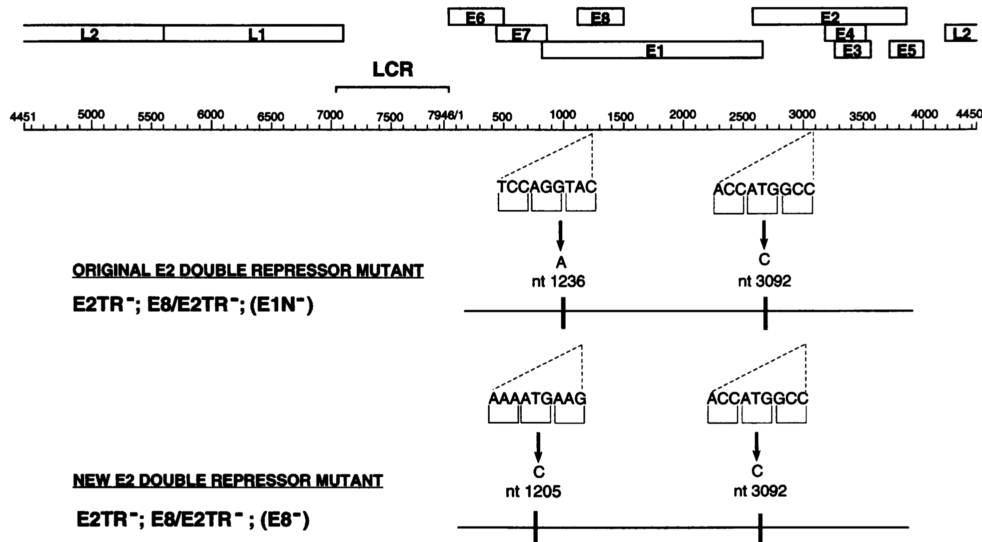


FIG. 1. Linearized map of BPV-1 depicting mutations present in the original o-BPV-$1^{E8/E2TR,\ E2TR}$ and new n-BPV-$1^{E8/E2TR,\ E2TR}$ E2 double-repressor mutant plasmids. The BPV-1 genome is shown linearized at the unique *Bam*HI site at BPV-1 nt 4450. Each ORF is represented by a rectangular box and is positioned above the numbered line at its approximate location in the viral genome. The long control region (LCR) is denoted by a bracket. The positions of individual base substitutions for each mutation discussed in the text are indicated by arrows drawn below the wild-type sequence. The brackets under the wild-type sequences denote codons in the E8, E1, and E2 ORFs surrounding the positions of the nt 1205, 1236, and 3092 base substitutions, respectively. The BPV-1 nucleotide number for each mutation is indicated.

generation of E1N-specific mRNAs (9, 29). At this time, no biological function is known for the E1N protein (9). To address the possibility that the absence of E1N protein contributes to the transformation defect of the E2 double-repressor mutant, a second E2 double-repressor mutant plasmid was created and analyzed. This plasmid, referred to as n-BPV-$1^{E8/E2TR,\ E2TR}$ (Fig. 1), was built in the background of p1472-1 (referred to as BPV-$1^{E2TR}$ in this study), a derivative of p142-6 containing the single point mutation (T→C) at BPV-1 nt 3092. n-BPV-$1^{E8/E2TR,\ E2TR}$ was constructed by digesting pCGEag 1197 (30), containing a point mutation at BPV-1 nt 1205 that changes an ATG codon (methionine) to an ACG codon (threonine), with *Xma*I (BPV-1 nt 943) and *Bst*BI (BPV-1 nt 1470). This 527-bp fragment was isolated and ligated to the 10-kb restriction fragment of p1472-1 generated by digestion with the identical enzymes. The mutation at nt 1205, which disrupts the ATG initiation codon for the E8/E2TR gene, causes a silent change in the E1 ORF (Fig. 1). Mutation of the E8 ORF ATG could disrupt expression of any other E8-specific translation products. Although no such proteins have been identified, a control plasmid, BPV-$1^{E8,\ E2TR}$, was made for this new E2 double-repressor mutant. To construct BPV-$1^{E8,\ E2TR}$, the 527-bp *Xma*I (BPV-1 nt 943)-to-*Bst*BI (BPV-1 nt 1470) fragment isolated from the double-stranded replicative intermediate of phage PL5 was ligated to the 10-kb p1472-1 restriction fragment described above. Phage PL5 is a derivative of M13mp18 that contains the 1,170-bp *Xma*I (BPV-1 nt 943)-to-*Eco*RI (BPV-1 nt 2113) BPV-1 DNA fragment inserted in the polylinker within the *lacZ* gene of M13mp18. This BPV-1 insert has a T→A base substitution at BPV-1 nt 1286, which was generated through site-directed mutagenesis (10) with an oligonucleotide having the sequence 5′GCAATAA GTCATC3′, which is partially complementary to BPV-1 nt 1281 to 1293. This mutation introduces a TTA (leucine)→TAA (stop) nonsense mutation in the E8 ORF and creates an ATT (leucine)→ATA (leucine) silent change in the overlapping E1 ORF. This mutation is downstream of the 5′ splice signal at BPV-1 nt 1235 and therefore should not affect E8/E2TR expression, but it would cause premature translational termination of any full-length E8 gene product.

The transformation phenotypes of the two double-repressor mutants o-BPV-$1^{E8/E2TR,\ E2TR}$ and n-BPV-$1^{E8/E2TR,\ E2TR}$ were compared in mouse C127 cells by using two short-term (acute) transformation assays, focus (15) and soft agar colony formation (12). In both assays, the new E2 double-repressor mutant, n-BPV-$1^{E8/E2TR,\ E2TR}$, like the original mutant, o-BPV-$1^{E8/E2TR,\ E2TR}$, was impaired in its ability to transform cells, whereas the control plasmid, BPV-$1^{E8,\ E2TR}$, was transformation competent (data not shown). These data indicate that absence of the two E2 repressor genes is sufficient to confer the nontransforming phenotype of BPV-1 E2 double-repressor mutants in these acute transformation assays.

Previous studies have shown that cells harboring the original BPV-1 E2 double-repressor mutant could acquire a transformed phenotype after long-term passage in tissue culture (14). To determine whether cells harboring the new E2 double-repressor mutant are also capable of acquiring a transformed morphology over time, BPV-1 plasmid DNAs were individually cotransfected with the plasmid p302-3 (16), which carries the neomycin resistance gene (confers resistance to geneticin [G418]), into C127 cells and the cells were grown in the presence of G418 for up to 9 weeks. At different times, plates were stained with methylene blue and the transformed characters of individual colonies were scored under a light microscope. Whereas at 3 weeks, both E2 double-repressor mutants failed to induce morphological transformation of C127 cells, by 9 weeks of continuous culture, both E2 double-repressor mutants induced morphological transformation in a fraction of the colonies (data not shown). By comparison, BPV-$1^{WT}$ was capable of efficiently inducing transformation at both early and late times. Because n-BPV-$1^{E8/E2TR,\ E2TR}$ has the same complex transformation phenotype as o-BPV-$1^{E8/E2TR,\ E2TR}$, we

conclude that defects in expression of the E2 repressors are also sufficient to induce the complex transformation phenotype.

**Transformation of C127 cells harboring E2 double-repressor mutants can be attributed to a subset of the viral DNAs present in these cells.** Since the delayed onset of transformation in cells harboring E2 double-repressor mutants is seen only in a fraction of the G418-resistant (G418[r]) colony clones derived, it is possible that specific changes to the viral DNA might be responsible for this acquired phenotype. To investigate this possibility, we performed a bacterial rescue experiment in which viral DNA from C127 cells, morphologically transformed by the E2 double-repressor mutants, was cloned through bacteria and then reevaluated for its capacity to transform C127 cells in acute transformation assays. The presence of viral DNA in these clonal populations was verified by Southern analysis (Fig. 2A). On the basis of comparison of Southern analyses performed on Hirt DNA (data not shown), only a subset of the major BPV-1-specific bands arose from extrachromosomal viral genomes; others therefore must be derived from integrated copies of the viral genome. To isolate C127 cells transformed over time by the E2 double-repressor mutants, the clonal populations characterized in Fig. 2A were placed into soft agar for 4 weeks. Soft agar colonies developed efficiently from fewer than half of the clonal populations tested (Table 1). Individual soft agar colonies were expanded in tissue culture for 3 weeks, and total genomic and Hirt DNAs were isolated from these soft agar clonal populations and analyzed by Southern analysis (Fig. 2B [data summarized in Table 2]). Different-sized BPV-1-specific bands were again detected in all of the clones analyzed, a subset of which were derived from extrachromosomal viral genomes on the basis of additional analysis of Hirt DNAs (data not shown).

The transformation competence of the viral DNAs isolated from the soft agar colonies was then assessed by performing a focus formation assay with DNA rescued in bacteria. *Escherichia coli* was transformed with the Hirt DNA analyzed in Fig. 2B; the resulting ampicillin-resistant colonies were expanded, and BPV-1 plasmid DNAs were isolated. It was predicted that extrachromosomal BPV-1 replicons present in the C127 cells could be rescued in bacteria by virtue of the presence of the bacterial vector sequences in *cis*. DNAs from multiple soft agar colonies were successfully rescued through bacteria (Table 2). The majority of DNAs that were rescued were 10.6 kb in size, representing intact BPV-1 recombinant DNA. Other rescued DNAs were smaller than 10.6 kb, representing a fraction of the deleted BPV-1 recombinant DNAs detected by Southern hybridization in Fig. 2. The ability of these rescued plasmid DNAs to induce focus formation was assessed with an acute transformation assay; BPV-1 DNA rescued from bacteria was transfected into C127 cells, and the cells were allowed to grow on plastic for 17 days. The intact BPV-1 plasmid DNAs rescued in bacteria from multiple independently derived soft agar clonal C127 cell populations were capable of forming foci on plastic (Table 2). This phenotype is in contrast to the transformation-defective property of the double-repressor mutant DNAs used for the original transfection that gave rise to the soft agar colonies. Two plasmid DNAs rescued in bacteria that contained gross deletions, however, behaved similarly to the parental double-repressor mutant plasmids in this short-term transformation assay (Table 2). Thus, a subset of the BPV-1 viral DNAs present in the soft agar colonies have reverted to a transformation-competent state. This finding supports our hypothesis that changes in the viral DNA accounted for the transformed phenotype acquired by cells harboring the E2 double-repressor mutant.

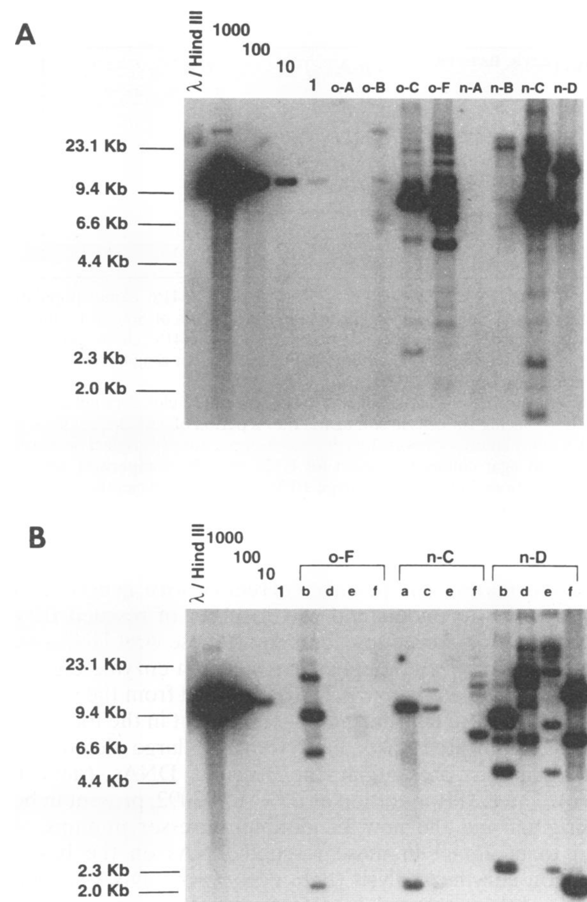


FIG. 2. Southern blots of BPV-1 viral DNA isolated from transformed C127 cells harboring E2 double-repressor mutants. Shown are Southern blots of total genomic DNAs that were digested with *Hin*dIII, run on 1% agarose gels, transferred to nitrocellulose, and probed with $^{32}$P-radiolabeled full-length BPV-1 DNA. To the left of each blot in panels A and B are indicated the sizes in base pairs of the molecular weight markers run on these gels (λ/*Hin*dIII). Lanes labeled 1,000, 100, 10, and 1 are copy number reconstructions of wild-type BPV-1 in which 2 μg of total genomic DNA from C127 cells and different amounts of recombinant 142-6 plasmid were digested with *Hin*dIII such that 1,000 copies, 100 copies, 10 copies, or 1 copy of BPV-1 is represented on the gel. Shown are long exposures (4 days) to permit detection of BPV-1 DNA present at low copy number. (A) Lanes o-A, o-B, o-C, and o-F contain DNAs isolated from G418[r] clonal populations expanded from the transfection of C127 cells with o-BPV-$1^{E8/E2TR,\ E2TR}$. Lanes n-A, n-B, n-C, and n-D contain DNAs isolated from G418[r] clonal populations expanded from the transfection of C127 cells with n-BPV-$1^{E8/E2TR,\ E2TR}$. (B) Lanes under bracket o-F contain DNAs isolated from soft agar colonies expanded from the G418[r] clone o-F. Lane b contains DNA from colony o-$F_b$, lane d contains DNA from colony o-$F_d$, lane e contains DNA from colony o-$F_e$, and lane f contains DNA from colony o-$F_f$. Lanes under bracket n-C contain DNAs isolated from soft agar colonies expanded from the G418[r] clone n-C. Lane a contains DNA from colony n-$C_a$, lane c contains DNA from colony n-$C_c$, lane e contains DNA from colony n-$C_e$, and lane f contains DNA from colony n-$C_f$. Lanes under bracket n-D contain DNAs isolated from the soft agar colonies expanded from the G418[r] clone n-D. Lane b contains DNA from colony n-$D_b$, lane d contains DNA from colony n-$D_d$, lane e contains DNA from colony n-$D_e$, and lane f contains DNA from colony n-$D_f$.

TABLE 1. Properties of G418$^r$ clonal populations

| DNA | G418$^r$ clone | Viral DNA copy number[a] | No. of soft agar colonies[b] |
|---|---|---|---|
| n-BPV-1$^{E2TR, E8/E2TR}$ | n-A | 6 | 1 |
| | n-B | 13 | 2 |
| | n-C | 270 | 51 |
| | n-D | 70 | 26 |
| o-BPV-1$^{E2TR, E8/E2TR}$ | o-A | 3 | 4 |
| | o-B | 10 | 0 |
| | o-C | 90 | 1 |
| | o-F | 120 | 84 |

[a] Total viral DNA copy number present in each G418$^r$ clonal population. Quantitation was done by using Phosphoimager analysis of Southern blots with known copy number standards. By comparison, all G418$^r$ clonal populations harboring wild-type BPV-1 had a viral DNA copy number ranging from 50 to 100 (data not shown).

[b] The transformation ability of the G418$^r$ expanded clonal populations was assessed by placing $10^5$ cells in soft agar. After a period of 4 weeks, colonies were scored. Colony counts of fewer than five colonies per $10^5$ cells reflect background levels of soft agar colony formation for C127 cells. By comparison, all G418$^r$ clonal populations harboring wild-type BPV-1 gave rise efficiently to soft agar colonies (data not shown).

**Transformation competence correlates with genetic reversion.** In order to understand why a subset of rescued BPV-1 DNAs were transformation competent, these viral DNAs were further characterized. Diagnostic restriction enzyme digestion analyses carried out with the DNAs isolated from the soft agar colonies that were transformation competent in the focus assay (Table 2) indicated that there were no large deletions or rearrangements present in the rescued DNAs (data not shown). The E2TR mutation at BPV-1 nt 3092, present in both the original and the new E2 double-repressor mutants, was found to be intact in these rescued DNAs on the basis of restriction enzyme analysis (data not shown). DNA sequence analyses of these rescued, transformation-competent plasmids, however, indicated that reversions had occurred at the E8/E2TR mutation (Fig. 3 and Table 2). No other nucleotide changes were detected in the region surrounding the original mutations. These results demonstrate that transformation-competent plasmid clones rescued from the soft agar colonies carry reversions at the sequences encoding the minor E2 repressor species E8/E2TR. Furthermore, we have determined that these specific reversions are both necessary and sufficient to confer the transformation competence displayed by these rescued plasmids (data not shown).

**Reversion is detectable in viral DNA isolated directly from soft agar colonies but not from parental G418$^r$ clonal populations.** To rule out the possibility that the genetic reversions could be attributed to passaging the rescued DNAs through bacteria, BPV-1 viral DNA isolated directly from the soft agar colonies was characterized. Reversions of the E8/E2TR mutations were detected by PCR-assisted DNA sequence analyses in the DNA extracted directly from a fraction of the expanded soft agar clones arising from transfection with either the original or the new E2 double-repressor mutant (Table 2). Therefore, the revertant genotype was not an artifact of the bacterial rescue protocol.

Another possible artifact that could have given rise to the presence of BPV-1 replicons carrying intact E8/E2TR genes in cells transfected with the E2 double-repressor mutants is the presence of contaminant BPV-1$^{E2TR}$ single-repressor mutant plasmid in the transfections. This is unlikely, since the acquired transformation phenotype of the double-repressor mutants was reproducibly seen in many independent experiments with multiple preparations of viral recombinant DNAs. Nevertheless, to address this potential caveat, we looked for the presence of E8/E2TR$^+$ DNA in the early passage G418$^r$ clonal populations from which the soft agar clonal populations were derived. Since these G418$^r$ cell populations were each originally derived from a single transfected cell, any contaminant DNA introduced at the time of transfection should be easily detectable in the expanded clonal populations. We were unable to detect the presence of the revertant genotype in total cellular DNA extracted from the early passage G418$^r$ cell populations (data not shown). This result strongly suggests that the presence of E8/E2TR$^+$ is not a consequence of contaminant plasmids in the transfection but rather is the result of the

TABLE 2. Properties of soft agar clonal populations

| DNA transfected | Soft agar colony | | Properties of bacterially rescued DNA | | | Presence of E8/E2TR reversion | |
|---|---|---|---|---|---|---|---|
| | Designation[a] | Viral DNA copy number[b] | No. of rescued plasmids[c] | Plasmid structure[d] | Transformation phenotype[e] | Rescued DNAs[f] | Soft agar DNA[f] |
| o-BPV-1$^{E8/E2TR, E2TR}$ | o-$F_b$ | 75 | 4 | All int | + | + | − |
| | o-$F_d$ | 1 | 12 | 10 int, 2 del (5 kb) | Int, +; del, − | Int, +; del, − | + |
| | o-$F_e$ | 1 | 0 | | | | NT |
| | o-$F_f$ | 2 | 13 | All int | NT | + | − |
| n-BPV-1$^{E8/E2TR, E2TR}$ | n-$C_a$ | 45 | 3 | All int | NT | + | + |
| | n-$C_c$ | 8 | 0 | | | | + |
| | n-$C_e$ | 1 | 1 | Int | NT | + | + |
| | n-$C_f$ | 31 | 0 | | | | + |
| | n-$D_b$ | 241 | 0 | | | | NT |
| | n-$D_d$ | 176 | 6 | All int | + | + | − |
| | n-$D_e$ | 83 | 6 | 3 int, 3 del (9.6 kb) | Int, +; del, − | Int, NT; del, + | − |
| | n-$D_f$ | 375 | 9 | All int | NT | + | − |

[a] The first two letters in the colony designation refer to the G418$^r$ parental population described in Table 1 from which the soft agar colony arose.

[b] Total viral DNA copy number present in each soft agar colony. Quantitation was done by Phosphoimager analysis of Southern blots with known copy number standards.

[c] The number refers to number of ampicillin-resistant colonies obtained from bacterial transformation.

[d] Abbreviations: int, intact 10.6-kb plasmid; del, deleted form of viral plasmid (plasmid size is given in parentheses).

[e] + or −, presence or absence, respectively, of focal transformation of C127 cells by plasmid in transfection experiment.

[f] +, presence of reversion on the basis of sequence analysis; −, absence of reversion detectable by sequence analysis; NT, not tested.

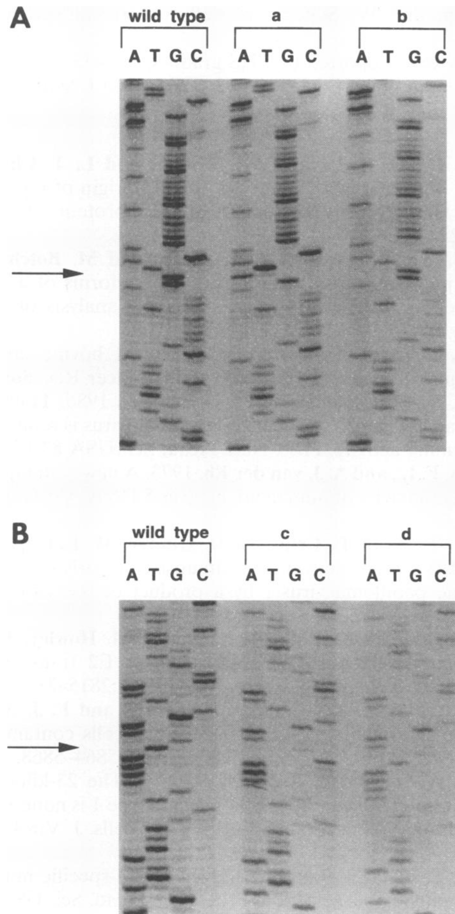


FIG. 3. Genetic analyses of the transformation-competent DNA rescued from long-term soft agar colonies. (A) Sanger sequence analysis of wild-type BPV-1 DNA, the o-BPV-$1^{E8/E2TR,\ E2TR}$ DNA (bracket a), and a transformation-competent, bacterially rescued o-BPV-$1^{E2TR,\ E8/E2TR}$ clone (bracket b). An arrow indicates the position of BPV-1 nt 1236, which is a G residue in wild-type BPV-1 and an A residue in the o-BPV-$1^{E2TR,\ E8/E2TR}$ DNA mutant. Note the wild-type sequence at nt 1236 in the transformation-competent, bacterially rescued clone (bracket b). At the top of each lane is indicated the dideoxynucleotide present in each sequence reaction. (B) Sanger sequence analysis of wild-type BPV-1 DNA, the n-BPV-$1^{E2TR,\ E8/E2TR}$ DNA (bracket c), and a transformation-competent, bacterially rescued n-BPV-$1^{E2TR,\ E8/E2TR}$ clone (bracket d). An arrow indicates the position of BPV-1 nt 1205, which is a T residue in wild-type BPV-1 and a C residue in the n-BPV-$1^{E2TR,\ E8/E2TR}$ DNA mutant. Note the wild-type sequence at nt 1205 in the transformation-competent, bacterially rescued clone (bracket d). At the top of each lane is indicated the dideoxynucleotide present in each sequence reaction.

generation of a revertant plasmid species during the outgrowth of the transfected cells.

**Mutations in the E1 ORF rescue the transformation defect of E2 double-repressor mutants.** The E2 proteins modulate the transforming capacity of BPV-1, at least in part, through their regulation of the viral promoters $P_{89}$ and $P_{2443}$ (7, 27), which direct the expression of the transforming proteins E5, E6, and E7 (32). Consistent with the known capacity of the E2 repressors to inhibit transactivation by the E2TA protein (2, 14, 15), cells harboring an E2 double-repressor mutant exhibit higher levels of E2 transactivation than do cells harboring

TABLE 3. Phenotypic analysis of BPV-1 E1, E2 triple-repressor mutants

| DNA | Transformation[a] | | RNA/DNA ratio[b] |
|---|---|---|---|
| | No. of foci | No. of soft agar colonies | |
| BPV-$1^{WT}$ | 45 | 70 | 1 |
| o-BPV-$1^{E2TR,\ E8/E2TR}$ | 1 | 6 | 2 |
| o-BPV-$1^{E2TR,\ E8/E2TR,\ E1^{TTL\ @\ nt\ 1811}}$ | 45 | 552 | 16 |
| o-BPV-$1^{E2TR,\ E8/E2TR,\ E1^{TTL\ @\ nt\ 2113}}$ | 48 | 530 | 29 |
| BPV-$1^{E1^{TTL\ @\ nt\ 1811}}$ | 42 | 176 | 7 |

[a] Transformation assays were performed as described in the text, except that transfections were done by the calcium phosphate precipitation protocol (5). For each transfection, each 6-cm plate of subconfluent C127 cells received 1.0 μg of the designated BPV-1 plasmid DNAs. Foci were stained and counted after 2 weeks, and the soft agar colonies were stained and counted after 4 weeks.

[b] C127 cells were transfected by the calcium phosphate precipitation protocol with 0.5 μg of p302-3 and 1 μg of the designated BPV-1 plasmid DNA. G418$^r$ colonies were pooled after 3 weeks of selection and expanded, and RNA and DNA were extracted by standard protocols described in references 27 and 23, respectively. RNA was quantitated by slot blot hybridization, and DNA was quantitated by Southern analysis as detailed in reference 12. The RNA/DNA ratio for cells harboring wild-type BPV-1 was set at 1.

wild-type BPV-1 (14). Yet, the same cells contain extremely low steady-state levels of viral mRNA compared with cells harboring wild-type BPV-1, and this correlates with the absence of cellular transformation (14). We therefore questioned whether, in cells harboring the E2 double-repressor mutants, another transcriptional repressor acts to suppress E2TA-dependent transcription of the viral transforming genes. One possible candidate for this repressor is the full-length E1 gene product. Mutations that disrupt the full-length E1 gene are known to lead to approximately 20- and 4-fold increases in the steady-state levels of viral transcripts arising from $P_{89}$ and $P_{2443}$, respectively, per viral genome copy (12). This altered viral transcription pattern correlates with increases in viral transformation of C127 cells by BPV-$1^{E1}$ mutants (12, 25), particularly in soft agar transformation assays, which predominantly measure the activity of the E6 protein, a BPV-1 oncogene expressed from the $P_{89}$ promoter. Recently, the E1 protein has been argued to suppress E2 transactivation of the $P_{89}$ promoter (22). To determine if E1 might be responsible for suppressing transformation by the E2 double-repressor mutants, two plasmids were constructed that contain translational termination linkers (TTLs) inserted into different positions in the E1 ORF, within the context of the original E2 double-repressor mutant plasmid, o-BPV-$1^{E8/E2TR,\ E2TR}$. These BPV-1 E1, E2 triple-repressor mutants, o-BPV-$1^{E8/E2TR,\ E2TR,\ E1^{TTL@1811}}$ and o-BPV-$1^{E8/E2TR,\ E2TR,\ E1^{TTL@2113}}$, were constructed by ligating the 935-bp *Bst*BI (BPV-1 nt 1470)-to-*Bst*EII (BPV-1 nt 2405) restriction fragments from p1136 and p1137, respectively (15), to the 9,645-bp *Bst*BI-to-*Bst*EII restriction fragment of o-BPV- $1^{E8/E2TR,\ E2TR}$.

These plasmids were characterized for transformation in the two acute transformation assays. Disruption of the full-length E1 gene was found to rescue the transformation defect of the E2 double-repressor mutant in both the focus and the soft agar colony assays (Table 3). The high level of transformation in the soft agar assay (Table 3) suggests that E6 oncoprotein levels are elevated in the background of BPV-1 deficient in the expression of both E1 and E2 repressor species. It is likely that E1 also affects levels of the E5 protein, given that steady-state levels of $P_{2443}$ promoter-specific RNAs are elevated in cells harboring E1 mutants (12). As was originally seen with BPV-$1^{E1^{TTL}}$ mutants (12), the high transformation efficiency dis-

played by the o-BPV-1$^{E8/E2TR,\ E2TR,\ E1^{TTL}}$ mutants correlated with a 16-to-29-fold increase in the ratio of BPV-1 RNA to BPV-1 DNA present in these cells compared with that in cells harboring wild-type BPV-1 (Table 3). These data suggest that high levels of the E1 protein's transcriptional repressor activity may be induced in the E2 double-repressor mutants and that this is responsible for the tight suppression of viral transcription observed in these mutants.

**Role of E1 and the E2 repressors in modulating transformation.** In the context of our cumulative data, we propose a model to explain the complex transformation phenotype of the E2 double mutants in which (i) in the absence of any E2 repressor activity, E1 protein is a potent inhibitor of E2TA function at viral promoters, especially $P_{89}$, and this inhibition leads to the strong down regulation of viral transcription and consequently viral transformation; and (ii) a threshold level of E2 repressor activity supplied by the reversion of the E8/E2TR mutation is sufficient to counterbalance the potent negative effects of the E1 protein on E2TA and thereby lead to an acquired transformation phenotype. This model leads to several predictions. The first of these is that the activity level of the E1 protein should be high in the E2 double-repressor mutant. Consistent with this, we have found that the E2 double-repressor mutant replicates more efficiently than wild-type BPV-1 (33) in transient assays known to be dependent upon E1 protein activity (30). In addition, the disruption of E1 in the context of the E2 double-repressor mutant led to an 8- to 15-fold increase in viral RNA/DNA ratios in cells compared with viral RNA/DNA ratios in cells harboring the E2 double-repressor mutant (Table 3). A second prediction is that the E8/E2TR reversion event would cause an increase in the expression of the viral transforming proteins in these cells. In support of this assertion, high levels of E5 and E6 proteins were detected in late passage morphologically transformed cells transfected with the original E2 double-repressor mutant (11, 14).

It is necessary to consider the antagonistic consequences of E1 and E2 repressor activities on viral DNA replication in the context of our proposed model. While E1 protein is required for viral DNA replication (30), the E2 repressors inhibit viral DNA replication presumably via their suppression of E2TA replication activity (1). This leads to the prediction that the E2 double-repressor mutants should replicate very efficiently. While this prediction is true in transient replication assays (33), the E2 double-repressor mutants are maintained at varying copy numbers relative to wild-type BPV-1 in clonal population of stably transfected cells. A possible reason for this complex replication phenotype is that, in the absence of E2 repressors, the E1 protein so efficiently represses transcription that it lowers expression of itself and other viral proteins to levels that are insufficient to support high levels of viral DNA replication in the long term. This prediction is currently being tested. Another possibility that must be considered is that the initial high-level replication of the double-repressor mutant could be cytotoxic to cells and that the cells that outgrow and continue to harbor the viral DNA necessarily have acquired a mutation that suppresses uncontrolled viral DNA replication. It is interesting in this regard that E2 repressor activity, a consequence of reversion of the E8/E2TR mutation, is predicted to lead to modulation of viral DNA replication.

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