Efficient transactivation and morphologic transformation by bovine papillomavirus genes expressed from a bovine papillomavirus/simian virus 40 recombinant virus

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ABSTRACT To efficiently introduce bovine papillomavirus type 1 genes into cultured cells, we constructed a hybrid viral genome in which the simian virus 40 early region is replaced with a segment of the bovine papillomavirus type 1 transforming region. High-titer stocks of simian virus 40 virions containing the recombinant genome were produced in monkey cells that express simian virus 40 large tumor antigen. Cells infected with this virus efficiently expressed the bovine papillomavirus type 1 E2 and E5 genes. Expression of the E2 gene caused transactivation of genes linked to the bovine papillomavirus type 1 control region, resulting in up to a 1000-fold induction. At high multiplicity of infection of a cell line containing an integrated reporter gene, most cells were infected and responded to transactivation. Within 48 hr of infection with wild-type virus but not with an open reading frame E5 mutant, mouse C127 cells displayed dramatic changes in morphology and growth characteristics similar to those seen in tumorigenic transformation. This system can be used to determine the acute cellular response to introduction of bovine papillomavirus type 1 transforming and regulatory genes; it can also be used to induce foreign genes stably incorporated into cultured mammalian cells.

Bovine papillomavirus type 1 (BPV1) and other papillomaviruses can induce tumorigenic transformation of established rodent cells growing in culture (1, 2). Cell transformation by the papillomaviruses is a topic of considerable interest because of the strong association between human papillomavirus infection and some human squamous cell carcinomas, but little is known about the molecular mechanisms of papillomavirus transformation. This deficiency is due in large part to the lack of a cell culture system that allows papillomavirus propagation. Moreover, it is cumbersome to isolate wild-type virus from cow warts, and constructed viral mutants cannot be packaged into virus particles.

Therefore, genetic analysis of transformation by these viruses has been largely restricted to determining the activity of transfected viral genes and mutants in stable transformation assays (3). In these assays, open reading frames (ORFs) E5 and E6 have been identified as the major BPV1 transforming genes (4–9). The 44-amino acid ORF E5 protein is required for efficient focus formation in the established line of mouse C127 cells (4, 10–12). The efficiency of transformation is also influenced by expression of ORF E2. The full-length E2 protein transactivates promoters linked to the BPV1 long control region (LCR), whereas separate proteins encoded by the 3' end of ORF E2 antagonize transactivation (5, 6, 13–18).

Because stable transformation is the result of relatively infrequent events and probably represents the endpoint of a multistep process, other assays must be developed to identify the virus-induced biochemical events that lead to transformation. To generate virus particles that efficiently infect cells and express BPV1 transforming and regulatory genes, we constructed a BPV1/simian virus 40 (SV40) recombinant viral genome that replicates and is packaged into SV40 virions in permissive monkey cells. Cells infected with this virus efficiently express the BPV1 E2 and E5 genes, and this expression results in transactivation and acute morphologic transformation.

MATERIALS AND METHODS

Structure of SV40/BPV Recombinant Plasmids. Standard procedures were used to construct pPava-1 from cloned SV40 and BPV1 DNA [pCC2 (19) and pBPV-142-6 (20), respectively]. pBPV-1 consists of the BstEII [nucleotides (nt) 2405]–BamHI (nt 4450) small fragment of BPV1 DNA inserted in place of the HindIII (nt 5171)–Bcl I (nt 2770) large tumor (T) antigen-coding fragment of SV40 DNA. These viral sequences are cloned into pBR322 at the unique EcoRI site in the SV40 late region. The HindIII, BamHI, and Bcl I sites at the junctions of the DNA fragments were lost, and a Xho I linker was inserted at the junction upstream of the BPV1 DNA. To generate pPava-E2am1 the BstEII–BstXI fragment of pPava-1 was replaced with the corresponding fragment of pBPV-E2am9 (17). To generate pPava-E5d29, pPava-1 was digested with Spe I and BstXI, and the ends were ligated after they were made blunt enzymatically. This mutant contains a 29-base-pair (bp) deletion (nt 3888–3916).

Preparation of Virus Particles. To generate virus particles, the EcoRI fragments of pPava-1 and its mutants were circularized by ligation at a DNA concentration of 5 μg/ml and transfected into CMT4 cells (21) by using calcium phosphate. After 4–6 hr, the cells were subjected to glycerol shock as described (18) and then incubated for 5 days at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 1 μM CdSO4, and 100 μM ZnCl2. Primary virus stocks were prepared by freeze/thawing the cells, and the clarified supernatant was used to infect fresh induced CMT4 cells. After an additional 6- to 8-day incubation, virus was harvested, concentrated by 3-hr centrifugation at 100,000 × g, and resuspended in a small volume of medium. Typical titers of virus preparations were ~106 infectious units per ml, as determined by diluting a virus stock, amplifying each dilution on CMT4 cells, and determining the maximum dilution that resulted in detectable transactivation (see below). Experiments involving virus were done under P2 containment.

Transactivation Assays. To measure transactivation of chloramphenicol acetyltransferase (CAT) expression, CV1

Abbreviations: BPV, bovine papillomavirus; SV40, simian virus 40; T antigen, large tumor antigen; CAT, chloramphenicol acetyltransferase; LCR, long control region; ORF, open reading frame.

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cells in 60-mm plates were transfected with 1 μg of p407-1. In addition, some plates received cotransfected DNA, and others were infected immediately after the glycerol shock by incubation with 1 ml of crude virus stock or mock lysate for 15 hr. Cells were then incubated with medium containing 5 mM sodium butyrate for 24 hr, incubated in normal medium for 24 hr, and harvested and assayed for CAT activity according to standard protocols (22). To measure transactivation of the β-galactosidase gene, NL-3D cells in 60-mm dishes were infected with dilutions of a concentrated virus stock, exposed to 5 mM sodium butyrate for 24 hr, and incubated for an additional 24 hr in normal medium. Protein extracts were prepared by freeze/thawing the cell pellet in 10 mM Tris, pH 7.5/1 mM EDTA. Extract from 2 × 10^2 cells was incubated in 50 mM KPO4, pH 7.8/10 mM MgCl2, and 5 mM chlorophenol red-β-d-galactopyranoside (Boehringer Mannheim) for 15 min at 37°C, conditions under which substrate conversion is proportional to amount of added enzyme. Enzyme activity was quantitated by spectrophotometry at 574 nm.

**Establishing the NL-3D Cell Line.** Plasmid p407-lac was constructed by replacing the HindIII-to-HpaI fragment containing the CAT gene-coding sequences from plasmid p407-1 (13) with the BamH1-to-HpaI fragment containing the lacZ coding sequences from plasmid pCH110 (Pharmacia). CV1 cells were cotransfected with plasmid pKOneo and a 5-fold molar excess of p407-lac by the calcium phosphate method. Individual G418-resistant colonies were screened for inducible lacZ expression after infection with Pava-1 by monitoring the color of the culture medium after the addition of 5 mM chlorophenol red-β-d-galactopyranoside 48 hr after infection. NL-3D cells were cloned from a colony exhibiting high-level induction.

**Immunofluorescence of β-Galactosidase.** NL-3D cells on glass slides were infected at a multiplicity of ~1,000, exposed to 5 mM sodium butyrate for 24 hr, and incubated in normal medium for an additional 24 hr. Indirect immunofluorescence for β-galactosidase was performed after fixing the cells in methanol for 10 min at −20°C followed by acetone for 1 min at −20°C. Cells were washed three times with phosphate-buffered saline (PBS) and then incubated with a 1:10 dilution of a monoclonal antibody against β-galactosidase (Promega Biotec, Madison, WI) for 45 min at 37°C. Cells were then washed three times with PBS and incubated with a 1:40 dilution of fluorescein-conjugated goat anti-mouse IgG for 45 min at 37°C. Cells were washed three times with PBS, mounted in 50% (vol/vol) glycerol in PBS, and viewed with a fluorescence microscope.

**Detection of the E5 Protein.** Dishes (10 cm) of CMT4 cells were infected at 80% confluence with 1 ml of crude virus lysate, and after 2 hr of virus adsorption, medium containing 1 μM CdSO4 and 100 μM ZnCl2 was added. Forty-eight hours after infection the medium was replaced with 2 ml of medium containing 0.5 mM Cl− (1 Ci = 37 GBq) each of [35S]methionine and [35S]cysteine. After 5 hr of labeling, total protein was extracted and immunoprecipitated with an anti-E5 antiserum as described by Schlegel et al. (11). The immunoprecipitated products were analyzed by NaDodSO4/PAGE and fluorography.

**RESULTS**

**Construction of the Recombinant Virus.** To construct the BPV1/SV40 hybrid plasmid, the T antigen-coding sequences in a plasmid containing the wild-type SV40 genome were replaced with a fragment of BPV1 DNA containing ORFs E2, E3, E4, and E5 (Fig. 1). The resulting plasmid, pPava-1, contains the intact SV40 late region and origin of replication, the BPV1 sequences linked in the correct orientation to the SV40 early promoter, and the complete pBR322 genome. Transfected pPava-1 induces the formation of stably transformed foci in mouse C127 cells (data not shown). To generate virus particles from pPava-1, the viral sequences were released by digestion with EcoRI, circularized in vitro, and transfected into CMT4 cells, a line of monkey cells that expresses SV40 large T antigen in response to zinc and cadmium (21). Large T antigen allowed high-level DNA replication from the SV40 replication origin, efficient expression of the SV40 capsid genes, and virion assembly. Crude stocks of the virus (designated Pava-1) were isolated from transfected cells, and high-titer virus stocks were generated by repassage on fresh induced CMT4 cells. Pava-1-infected CMT4 cells contained a substantial amount of replicated viral DNA, and restriction mapping indicated that the DNA had not undergone any obvious rearrangement (data not shown). We also constructed two mutants of Pava-1. pPava-E2am1 contains a premature termination codon in BPV1 ORF E2.

**Fig. 1.** Structure of Pava-1. pPava-1 contains the BPV1 sequences encoding the E2, E3, E4, and E5 ORFs (heavy line) linked in the correct orientation to be transcribed from the SV40 early promoter. Arrows indicate direction of transcription. The plasmid also contains the intact SV40 late region and origin of replication. Viral DNA is inserted into pBR322 as indicated.

**Fig. 2.** Transactivation of CAT expression in Pava-infected cells. CV1 cells were transfected with p407-1, which contains an ORF E2-responsive CAT gene. In addition, cells received the following treatments: A, cotransfected calf thymus DNA; B, 5 μg of cotransfected c59 DNA (which expresses ORF E2); C, mock infection; D, Pava-1 infection; E, Pava-E2am1 infection. Forty-eight hours later, extracts were prepared, and CAT activity was measured in an in vitro reaction followed by thin-layer chromatography, which is shown above.
that does not affect any of the other ORFs and causes a substantial defect in E2-mediated transactivation (17, 23). pPava-E5d29 contains a frameshift in BPV1 ORF E5, the gene required for efficient C127 cell focus formation. High-titer virus stocks were prepared from each of these mutant plasmids, and restriction mapping of viral DNA isolated from infected CMT4 cells confirmed the presence of the mutation and the absence of other alterations (data not shown).

Expression of the E2 Gene After Pava-1 Infection. To demonstrate the expression of a biologically active BPV1 E2 gene product in Pava-infected cells, we performed a transactivation assay using the plasmid p407-lac, which contains the BPV-1 LCR linked to the bacterial CAT gene (13). Expression of the CAT gene from p407-lac requires ORF E2 activity (Fig. 2, columns A and B) (13). CV1 cells were transfected with p407-1 and then infected with stocks of Pava-1 or Pava-E2am1. After 48 hr extracts were prepared, and CAT activity was measured in vitro (Fig. 2, columns C, D, and E). Mock-infected cells did not express detectable levels of CAT activity. Cells infected with Pava-1 exhibited high levels of CAT activity, indicating that the E2 gene is efficiently expressed in infected cells, resulting in transactivation of the BPV1 LCR. The level of induction in the experiment shown was ~1000-fold as determined by scintillation counting of the reaction products separated by thin-layer chromatography. Pava-E2am1-infected cells failed to express CAT activity, confirming that the virus-induced transactivation is ORF E2-dependent. The amount of Pava-1 and Pava-E2am1 virus used in these infections was judged to be roughly equivalent because comparable amounts of replicated viral DNA were obtained from CMT4 cells infected with each at low multiplicity.

Transactivation of a Stable Cell Line by Pava Infection. To further characterize the ORF E2-dependent transactivation in virus-infected cells we developed a stable cell line in which transactivation could be easily measured. We first constructed the plasmid p407-lac, which contains the Escherichia coli lacZ gene linked to the BPV1 LCR. Cotransfection experiments demonstrated that expression of the β-galactosidase gene in p407-lac requires transactivation by ORF E2 (data not shown). To generate stable cell lines containing the BPV1 LCR-linked β-galactosidase gene, p407-lac DNA was introduced into CV-1 cells by cotransfection with a plasmid conferring G418 resistance. Individual G418-resistant colonies were isolated and expanded into cell lines that were tested for their ability to express β-galactosidase after infection with Pava-1. One of the resulting cell lines, NL 3D, expressed low levels of β-galactosidase activity in the absence of infection, whereas infection resulted in great induction of activity. As expected, Southern blotting analysis using a β-galactosidase gene probe demonstrated the presence of integrated sequences in NL 3D cell DNA, with no evidence of extrachromosomal DNA (data not shown).

To measure lacZ expression in NL 3D cells, an extract was prepared from cells 48 hr after they were mock-infected or infected with stocks of Pava-1 or its derivatives, and β-galactosidase activity was assayed in an in vitro reaction (Fig. 3). Enzyme activity was very low in the absence of infection but was dramatically induced by Pava-1 infection. At low multiplicities of infection, the activity was directly propor-

![Fig. 3](image-url)

![Fig. 4](image-url)
Cells were either mock infected or infected with Pava-1 (WT), Pava-E2am1 (E2\(^{\pm}\)), or Pava-E5d29 (E5\(^{\pm}\)), as indicated. Labeling and immunoprecipitation with an anti-E5 antiserum were done as detailed in text. An autoradiogram of the samples after electrophoresis is shown. Numbers at left indicate protein size in kDa.

Morphologic Transformation After Pava Infection. We also tested the effect of Pava-1 infection on C127 cells, a standard host for BPV1-mediated transformation. Within 24–48 hr of infection, cells underwent a dramatic morphologic change, in which they became more spindle-shaped and refractile, whereas mock-infected cells remained unchanged (Fig. 6A and B). The infected cells displayed a number of other characteristics of cells stably transformed by BPV1, including the ability to overgrow the monolayer (reaching a saturation density several-fold higher than mock-infected cells), disruption of actin filaments, and rapid acidification of the culture medium; moreover, infection stimulated cellular DNA synthesis in cells made quiescent by serum starvation and contact inhibition (data not shown). Transforming activity banded at the density of authentic SV40 virions upon centrifugation in CsCl and was abolished by incubation of Pava-1 with SV40 neutralizing antibody but not by incubation with preimmune serum (data not shown). We have not seen the development of stable transformed foci after repassage of cells acutely transformed by Pava-1 infection. To identify the BPV1 gene(s) required for acute transformation, we tested the two viral mutants. Cells infected with Pava-E5d29 remained flat and were morphologically indistinguishable from mock-infected cells (Fig. 6D). Thus, the rapid morphologic change caused by Pava-1 infection requires expression of the E5 gene product. Cells infected with Pava-E2am1 initially exhibited morphological transformation comparable to that indicative of E2-mediated transactivation of the lacZ gene in these cells (Fig. 4D). Thus, Pava-1 infection results in the introduction of biologically active BPV1 genes in the majority of cells in the target population.

Expression of the E5 Protein in Pava-Infected Cells. To test whether the BPV1 E5 gene is expressed in Pava-infected cells, induced CMT4 cells were infected, metabolically labeled, and subjected to immunoprecipitation with an anti-E5 peptide antiserum (11). Immunoprecipitated proteins were visualized by NaDodSO\(_4\)/PAGE and fluorography (Fig. 5). Mock-infected CMT4 cells and cells infected with Pava-E5d29 expressed no E5 protein, whereas Pava-1 and Pava-E2am1-infected cells expressed large amounts of the 7-kDa E5 protein. The amount of E5 protein expressed in Pava-infected CMT4 cells is far greater than that seen in C127 mouse cell lines stably transformed by BPV1.

Fig. 5. Detection of the E5 protein in Pava-infected cells. CMT4 cells were either mock infected or infected with Pava-1 (WT), Pava-E2am1 (E2\(^{\pm}\)), or Pava-E5d29 (E5\(^{\pm}\)), as indicated. Labeling and immunoprecipitation with an anti-E5 antiserum were done as detailed in text. An autoradiogram of the samples after electrophoresis is shown. Numbers at left indicate protein size in kDa.

Fig. 6. Morphologic transformation of C127 cells after Pava infection. C127 cells at 90% confluence on glass slides were either mock-infected (A) or infected with Pava-1 (B), Pava-E2am1 (C), or Pava-E5d29 (D) at a multiplicity of ~1000. Forty-eight hours later the cells were photographed using phase-contrast microscopy. (×150.)
seen in Pava-1-infected cells (Fig. 6C); however, after several days they appeared to be significantly less transformed than Pava-1-infected cells.

**DISCUSSION**

To circumvent the block to papillomavirus production in tissue culture, we have developed Pava-1, a recombinant virus that contains BPV1 E2 and E5 genes. Similar approaches have been used to encapsidate a variety of cellular and viral genes (24). After infection of cultured cells with this virus, the BPV1 genes are efficiently expressed, and their protein products are biologically active. Pava-1 infection of C127 cells causes acute morphologic changes and altered growth characteristics similar to those seen with tumorigenic cells. The E5 gene, which is required for efficient focus formation by transfected BPV1 DNA, is also required for this acute response. ORF E6, the other BPV1 gene known to release cells from contact inhibition, is not present in Pava-1 and is therefore not required in this acute transformation system. The rapid transformation of the vast majority of infected C127 cells indicates that rare cellular events are not required for transformation by the E5 protein. However, cells acutely transformed by Pava-1 infection rarely if ever progress to stable transfectants, suggesting that there are additional requirements for stable transformation. Such abortive transfections are commonly seen in other viral transformation systems and is thought to result from unsustained expression or inefficient integration of the viral DNA (25). The ability of Pava-1 DNA isolated from infected CMT4 cells to induce focus formation (unpublished results) demonstrates that Pava-1 has not acquired a mutation that prevents stable transformation. We have recently constructed numerous E5 missense mutants and established their activity in stable transformation assays (26). By testing these mutants in the acute assay, we can determine whether acute transformation and stable transformation require the same structural features of the E5 protein.

ORF E2 normally controls BPV1 genes maintained on a plasmid (27). However, our results show that the E2 gene product can transactivate a responsive element that is integrated into cellular DNA, implying that it has the potential to affect expression of endogenous cellular genes. Therefore, ORF E2 mutations may block transactivation of both cellular genes and viral genes, and both effects may contribute to the transformation defect caused by these mutations.

The Pava-1 system eliminates the major disadvantage of infection with authentic BPV1 virions—namely the inability to construct and test viral mutants. In addition, the simplicity and reproducibility of preparing virus from infected cells in culture has obvious advantages over harvesting virus from cow warts. This system also has significant advantages over transfection with viral DNA as a method of introducing papillomavirus genes into cells. Gene transfer by virus infection is far more efficient and less toxic than transfection. Because infection with high-titer stocks of the Pava viruses generates relatively homogeneous populations of cells responding to the BPV1 transforming and regulatory genes, the acute cellular responses to these genes can be analyzed biochemically and subjected to genetic dissection. For example, biochemical comparison of C127 cells infected by Pava-1 and its transformation-defective derivatives may identify the pathways involved in growth control that are affected by the E5 protein. Infection with a recombinant retrovirus has also been used to introduce BPV1 genes into cultured cells (28). Although stable transformed foci are induced by infection with this virus, it has only been possible to generate low-titer virus stocks, and unpredictable rearrangements have occurred during passage in cells.

This system allows the transient induction of any foreign gene linked to the BPV1 regulatory region. After introducing such a gene into cells susceptible to SV40 infection, the expression of the gene can be specifically induced by infection with Pava-1. Such a system may be useful for inducing the expression of proteins that would otherwise be toxic to host cells. Delicate regulation of the level of expression of the target gene may also be possible because expression is minimal in the absence of infection and the extent of induction is proportional to the multiplicity of infection. Because essentially all cells in a culture can be infected and respond to transactivation, the effect of the induced gene on the cells can easily be monitored. The efficiency and ease of infection and the high-level induction that can be obtained indicate that this may be a useful system for manipulating gene expression in cultured mammalian cells.

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