Genetic Definition of a New Bovine Papillomavirus Type 1 Open Reading Frame, E5B, That Encodes a Hydrophobic Protein Involved in Altering Host-Cell Protein Processing

M. KERRY O'BANION,^{1,2†} VIRGINIA D. WINN,² JEFFREY SETTLEMAN,^{3‡}
AND DONALD A. YOUNG^{1,2*}

Departments of Medicine¹ and Biochemistry,² University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, and Department of Genetics, Yale University Medical School, New Haven, Connecticut 06510³

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We have previously observed that bovine papillomavirus type 1 (BPV-1) induces the appearance of five cellular proteins in C127 mouse fibroblasts, four of which appear to arise by altered processing of resident endoplasmic reticulum proteins. Studies of various cell lines revealed that expression of the 3' end of the BPV early region was sufficient for induction of these changes. To identify the BPV gene responsible, we have utilized the simian virus 40 (SV40)/BPV-1 recombinant virus Pava-1, which expresses the 3' end of the BPV early region behind an SV40 early promoter. C127 cells infected with Pava-1 for 48 h show the expected BPV-associated alterations, as do cells infected with Pava constructs mutated in the E5 or E2 genes. However, a mutation in the start codon of a previously ignored open reading frame extending from nucleotides 4013 to 4170 (E5B) eliminated the BPV-associated changes. Similar results were obtained with COS cells infected with the Pava mutants and C127 cells transformed by full-length mutated BPV. Despite its influence on the processing of cellular endoplasmic reticulum proteins, this mutation in E5B did not alter BPV-transforming efficiency or the ability of transformants to form colonies in soft agar. The E5B open reading frame encodes a hydrophobic 52-amino-acid polypeptide that shares structural similarities with HPV6 E5A and HPV16 E5. Speculations on a role for E5B in the viral life cycle are discussed.

We have previously utilized ultra-high-resolution "giant" two-dimensional (2-D) gel electrophoresis of metabolically labeled proteins to identify the specific changes in host cell protein phenotype induced by transformation of mouse C127 fibroblasts with bovine papillomavirus type 1 (BPV-1) (15, 16, 23, 24). Comparisons of parental C127 cells and BPV-1-transformed cells revealed several proteins that decreased with transformation and a set of five proteins (pvp1 to 4 and p62) that were coordinately induced (16). pvp1 has been shown to be a highly glycosylated form of the resident endoplasmic reticulum (ER) protein, glucose-regulated protein 100 (grp100) (24). pvp2 is the precursor and pvp3 and 4 are intermediates in the BPV-modified processing (assumed to be proteolytic) of a cellular protein, tentatively identified as calcium-regulated protein 55 (CRP55) (24), that also resides in the ER (17). Analysis of cell lines containing various mutated BPV DNAs and segments of the BPV genome expressed from foreign promoters indicated that the five papillomavirus-associated changes arose from expression of the 3' half of the viral early region (open reading frame [ORF] E2 to the polyadenylation site) even in the absence of transformation (16, 23, 24). These studies also indicated that the changes were not due to expression of the full-length E2, E4, or E5 ORFs. Thus, we hypothesized that expression of E2-C (12, 13) might give rise to the papilloma-virus-associated proteins (23).

The recently developed Pava vector is a modified simian

virus 40 (SV40) with the 3' half of the BPV-1 early region (extending from nucleotides [nt] 2405 to 4450) replacing the T antigen sequence (26). When introduced into cells that provide T in *trans*, virus particles that contain BPV genes under control of the SV40 early promoter are produced in high titers. Mouse C127 cells infected with Pava transiently express high levels of BPV messages in nearly all of the cells (26). This offers a strong advantage for rapid genetic studies of BPV. The vector also allows the introduction of these BPV genes into other cell types. Since Pava harbors our region of interest, we predicted that it might elicit the appearance of pvp1 to 4 and p62. Indeed, 2-D gel analysis of C127 cells infected with Pava demonstrated induction of all the papillomavirus-associated proteins. Surprisingly, infection with Pava constructs mutated in E-2C still induced these proteins whereas mutation of a previously unidentified ORF (E5B), with the capacity to encode a short, hydrophobic molecule, abolishes the BPV effect on host cell proteins.

MATERIALS AND METHODS

Cell culture, cell labeling, and 2-D gel analysis. All cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS; Hyclone) without antibiotics. Cells were monitored for mycoplasma contamination by Hoechst 33258 staining (3). The C127 mouse fibroblast line and BPV-transformed C127 cells (ID13) (14) were obtained from Peter Howley. CMT4 cells, supplied by Dan DiMaio, are derived from monkey kidney cells that contain a metallothionein-promoted SV40 T antigen (8). COS A.2 cells were obtained from Louise Chow.

Cells were metabolically labeled for 1 h with Trans-³⁵S-

* Corresponding author.

† Present address: Department of Neurology, University of Rochester Medical Center, Rochester, NY 14642.

‡ Present address: Whitehead Institute for Biomedical Research, Cambridge, MA 02142.

FIG. 1. Induction of papillomavirus-associated proteins by Pava. Proteins from C127, ID13, or C127 cells infected with wild-type Pava for 48 h were metabolically labeled for 1 h with ^{35}S -methionine and subjected to giant 2-D gel electrophoresis. (a) Portions of autoradiographs containing pvps 1, 2, 3, and 4. The locations of these proteins are indicated in the middle panel, as are the locations of glucose-regulated proteins 78 and 100 (grp78, grp100) and protein B, which is the final processed form of pvps 2, 3, and 4. pvp2 is the spot prepared for sequencing. (b) Portions of autoradiographs showing induction of p62 and pvp6 by BPV and Pava.

FIG. 2. Pava mutants examined in this study. The portion of the BPV genome that is present downstream from the SV40 early promoter in the Pava vector is shown with numbering corresponding to the BPV-1 sequence. The locations of the open reading frames (boxes) and initiator methionine codons (dashed lines within the boxes) are indicated as is the normal BPV polyadenylation site at nt 4203. The extent of the E2 and E5 deletion mutations are illustrated by cross-hatched boxes below the sequence, and the locations of the two point mutations are indicated by arrows.

label (200 μ Ci/ml; ICN) in DMEM–10% FCS without methionine. Cells were rinsed twice with ice-cold phosphate-buffered saline and then directly lysed in 2-D gel lysis buffer (9.5 M urea, 2% [wt/vol] Nonidet P-40, 2% [wt/vol] ampholines [LKB, 1.6% pH range 5 to 8, 0.4% pH range 3.5 to 10], 5% [wt/vol] 2-mercaptoethanol). Proteins were separated by giant 2-D gel electrophoresis as described previously (30) and visualized by autoradiography with Kodak XAR film.

Mutants. The original Pava vector (pPava-1), the E5 ORF deletion mutant (pPava-E5d29), and the E2 ORF *Bcl*I deletion mutant (pPava-BMV) have been previously described (26, 27). To aid in manipulation and sequencing, the Pava virus (SV40/BPV) was recloned into pGEM7 (Promega) from its original pBR322 vector. The E2M and E5B mutations were introduced by site-directed mutagenesis using a uracil-rich phagemid template (11) (kit from BioRad). Mutations were confirmed by dideoxy termination sequencing of double-strand template DNA (5) with T7 DNA polymerase (Pharmacia) and oligonucleotide primers adjacent to the mutagenesis sites. These mutations were introduced into wild-type BPV-1 in the vector pML-2 (pdBPV-1 [25]) by using the viral *Spe*I and *Bam*HI sites.

Pava virus propagation and infection. The original Pava virus and its derivatives were propagated as described (26) with some modifications. Briefly, stocks of DNA prepared in bacteria were digested with *Eco*RI to separate the bacterial vector from Pava and then treated with T4 DNA ligase in a dilute solution to favor the formation of circular Pava molecules. The equivalent of 10 μ g of Pava DNA was used to lipofect subconfluent CMT4 cells growing in 75-cm² flasks following the manufacturer's protocols (lipofections carried out in OPTI-MEM media; Life Science Technologies). Twenty-four hours later, large T antigen was induced in these cells from a metallothionein promoter by addition of 1 volume of DMEM containing 20% FCS, 200 μ M ZnCl₂, and 2 μ M CdCl₂. The cells were incubated for 5 to 7 days until cytopathic effects became apparent. All subsequent CMT4

cultures were done in the presence of the two metals. After three freeze-thaw cycles, supernatants of these cultures were used to infect larger flasks (225 cm²) of CMT4 cells. After 4 to 6 days, cells were scraped, pelleted, and sonicated with a microtip in 1 ml of cold DMEM for 1 min at the lowest setting. Clarified sonicates were used for a third passage in large flasks. In general, cytopathic effects were clearly evident by day 4 or 5 of this third passage and cells were collected and sonicated as described. Clarified sonicates were aliquoted and stored at –70°C until use.

Subconfluent (about 50%) C127 or COS cell cultures were infected with 25 μ l of virus stock per 35-mm plate in a reduced volume of medium (0.7 ml) for 2 h at 37°C. Additional medium was added, and all analyses were carried out 48 h later. Volumes of virus stock were proportionally adjusted for larger or smaller cell cultures. No attempt was made to titer the virus stocks; however, effective infection was judged by the presence of morphologic transformation (when E5 was present [26]) or other markers (described in text).

Total RNA was isolated from 10-cm plates of Pava-infected and control C127 cells by lysis in guanidinium isothiocyanate and centrifugation through a cesium chloride cushion (4). Northern (RNA) blot analysis was performed as previously described (24) with a gel-purified *Spe*I-*Bam*HI BPV-1 fragment labeled with ³²P-dCTP by random priming (6).

Transformation assays. Mutant and wild-type DNAs (10 μ g) were transfected into C127 mouse fibroblasts by a modified calcium phosphate precipitation method (2). Forty-eight hours after transfection, cells were split 1:4 and incubated with a change of medium every 3 to 4 days for 12 to 14 days, at which time foci were clearly evident. Foci were stained with crystal violet for enumeration or individually picked to 24-well plates for clonal propagation. Cloned transformant cell lines were labeled for 2-D gel analysis as described above. Anchorage-independent growth was as-

FIG. 3. A point mutation in E5B abrogates induction of the papillomavirus-associated proteins. C127 cells infected for 48 h with Pava and mutants thereof were metabolically labeled for 30 min and analyzed by giant 2-D gel electrophoresis. Only portions of the autoradiographs containing the pvp2, 3, and 4 proteins (calreticulin) are shown. pvp2 (indicated by arrowhead) is seen in cells harboring wild-type, E2 mutant, and E5 mutant Pava constructs but not in cells infected with the E5B point mutant or in uninfected C127 cells. The locations of pvp3 and 4 are denoted by arrowheads in the Pava wt panel. We have often observed that pvp2, 3, and 4 have variable mobility in the second dimension, as is seen upon comparison of the top and bottom sets of panels, which are derived from two different experiments.

sayed by plating 25,000 cells per 35-mm dish in 1 ml of DMEM containing 10% FCS and 0.35% agar overlaying the same medium containing 0.7% agar. Cells were fed with soft agar and medium twice a week, and colonies were examined at 7 and 14 days.

RESULTS AND DISCUSSION

Pava-1 induces pvp1, pvp2 to 4 (calreticulin), p62, and a new protein (pvp6). Metabolic labeling and 2-D gel analysis of Pava-infected C127 cells revealed induction of pvp1 to 4 and p62 to levels similar to those observed in BPV-transformed cell lines (Fig. 1a and b). Furthermore, we noted a new protein (35.5 kDa, pI 7.7) in Pava-infected cells that we had not previously observed (Fig. 1b). Reexamination of 2-D gel autoradiographs from studies of BPV-containing cell lines confirmed that this protein was present whenever the other changes were observed. Pvp6 clearly represents a host cell protein since it is present in cells harboring mutations (see below) that would eliminate expression of E2C, the only viral protein product in the 3' early region that is of similar

size. Like all the papillomavirus-associated proteins, pvp6 is enriched in low-salt-detergent extracts of nuclei. However, pvp6 shares two features with p62 that distinguish these proteins from the BPV-altered ER proteins (pvp1 to 4): (i) we have identified no precursor proteins for pvp6 or p62 on 2-D gels and (ii) neither protein is induced by calcium ionophore treatment (data not shown).

We had previously suggested that pvp2, 3, and 4 were forms of calcium-regulated protein 55 (CRP55), a resident ER protein also known as calreticulin (7, 17, 24, 28). Indeed, microsequencing of a tryptic peptide fragment of pvp2, derived by giant 2-D gel purification, gave a 9-amino-acid sequence that was identical to a predicted tryptic fragment of mouse calreticulin extending from amino acid 112 to 120 (LFPSGLDQK [28]). Calreticulin is believed to be involved in the sequestration of calcium within the ER and smooth-muscle sarcoplasmic reticulum (22).

The E5B ORF is responsible for induction of the papillomavirus-associated proteins. Having established that the papillomavirus-associated proteins were induced in C127 cells by Pava infection, we next tested two previously described

FIG. 4. Pvp2 is induced in COS cells infected with Pava but not by the E5B mutant Pava virus. Proteins were metabolically labeled for 1 h in COS cells infected for 48 h with the indicated Pava derivatives and separated by giant 2-D gel electrophoresis. The upper set of panels shows portions of autoradiographs that include calreticulin. Note that pvp2 (top arrowhead) is strongly expressed in cells infected with wild-type Pava and the E2 point mutant. The locations of pvp3 and 4 are indicated by other arrowheads. All Pava-infected cells showed induction of a very abundant 44-kDa protein (labeled with an "s"; lower panels).

Pava mutants for their ability to induce the pvps and p62. These mutations are illustrated in Fig. 2. The E2 deletion mutant lacks the carboxy-terminal 33 amino acids of E2, giving rise to a molecule incapable of dimerizing or specifically binding to DNA (18, 20). The other mutation is a 29-bp deletion in the E5 ORF. As shown in Fig. 3, neither mutation had an effect on the appearance of the pvps (note that Fig. 3 and 4 show only the region of the gel containing pvp2, 3, and 4; in C127 cells, inductions of pvp1 and 6 and p62 also occurred; data not shown). The appearance of the changes with the E5 mutant is consistent with our previous examination of E5⁻ cell lines; however, the ability of the E2 deletion mutant to induce pvp1 to 6 and p62 was unexpected. Together with our previous work, these results indicate that the papillomavirus-associated changes arise either from an as-yet-undefined function of the E2 molecule residing in the so-called "hinge" region (which except for its length is dispensable for the known biologic functions of E2 [9, 18, 19]) or from expression of a previously unrecognized BPV ORF.

In addition to the previously recognized ORFs, there are two other ORFs in the 3' early region of BPV with methionine initiation codons that are longer than 40 amino acids. One, starting at nt 2972, contains three acg codons for threonine and one ccg codon for proline; neither of these is commonly used in the recognized BPV gene products

(summed for all BPV ORFs except E4, acg is only used for 6% of the threonines and ccg is used for 4% of the prolines). The other ORF, starting at nt 4013, showed no unusual codon preferences. Expression of the papillomavirus-associated proteins by three cell lines transformed with an *NcoI-NcoI* deletion (nt 2878 to 3089) mutant of BPV-1 (NIL-1, 4, and 5) (16) eliminates the possibility of the ORF starting at nt 2972 being responsible for their appearance. We thus constructed point mutations to determine whether the ORF starting at 4013 (we call it E5B) or the E2 hinge region gives rise to the BPV-induced changes (Fig. 2). E2M consists of a stop codon at nt 3103 that interrupts translation of E2 near the beginning of the hinge region and after any methionines that might serve as potential reinitiation sites. The E5B mutation is an ATG-to-GTG change in the predicted single initiation codon at nt 4013. C127 cells infected with the Pava E2M mutant express the pvps and p62 (Fig. 3), albeit at lower levels than observed in wild-type Pava infected cells. In contrast, no inductions were present in cells infected with the E5B mutation. This failure to observe the pvps is not the result of poor infectivity of the Pava-E5B preparation since morphologic changes, consistent with the expression of E5 (26, 27), occurred in the mutant E5B-infected cells (data not shown). Furthermore, Northern blot analysis of cells infected with the same virus stocks showed

FIG. 5. (a) Deduced amino acid sequence of BPV-1 E5B. (b) Hydrophilicity plots of various papillomavirus E5 proteins. Kyte-Doolittle plots using a length averaging value of 6 amino acids were obtained with Protean software (DNASTar, Inc., Madison, Wis.). Shading below the line indicates hydrophobic regions. All plots are drawn to the same scale, with the amino acid positions of HPV6b E5A indicated at the bottom of the figure.

equivalent levels of BPV RNA expression in the wild-type and E5B mutant-infected cells (data not shown).

To verify these findings, we tested the Pava mutants in another cell system. Pava infection of COS cells leads to induction of pvp2 (Fig. 4) but not of the other proteins observed in C127 cells. Nevertheless, this serves as a very specific marker for BPV action. Furthermore, since COS cell infection is productive, several additional proteins are synthesized. Among these is an abundant 44-kDa protein (pI 6.1) that we believe is the major late protein of SV40 (Fig. 4, labeled "s"). Regardless of its identity, it serves as a strong indicator of efficient Pava virus infection. As in C127 cells, the E2M but not the E5B mutation leads to induction of pvp2 (Fig. 4). The E5B mutant virus is clearly active in COS cells since the level of the 44-kDa protein is similar to that seen in the wild-type infection.

Mutation of E5B does not affect focus formation or growth in soft agar. Tada et al. have reported a correlation between the level of expression of the pvp proteins and the degree of transformation (29). Our earlier results indicated that expression of the papillomavirus-associated proteins is not essential nor sufficient for transformation but did not eliminate the

possibility of some role in enhancing BPV-transforming ability. To test the potential role of E5B in transformation, we introduced the E5B mutation back into full-length BPV. In experiments run in parallel, the mutant and wild-type DNAs transformed C127 cells with equal efficiency (data not shown). Furthermore, no gross differences in either colony size, morphology, or the distribution of colony sizes was discerned (note that in both cases, there were a wide variety of morphologies and sizes). Thus we conclude that E5B plays no obvious function in the ability of the virus to form foci on mouse C127 cells. We also cloned individual foci, expanded the cell populations, and performed 2-D gel analyses of metabolically labeled proteins. These experiments uniformly revealed (in 12 different clones) that cells transformed by DNA containing the E5B mutation did not make any of the pvp proteins. Eight of these E5B mutant transformants and four wild-type BPV transformants were also tested for anchorage-independent growth in soft agar assays. Although the number and size of colonies produced varied from clone to clone, these assays revealed no distinctions in the range of phenotypes observed between the mutant and wild-type transformants (data not shown).

E5B encodes a hydrophobic protein. The abrogation of the papillomavirus-associated changes by our E5B mutation in C127 cells infected with Pava, COS cells infected with Pava, and C127 cells transformed by BPV strongly implies that expression of an E5B-encoded protein product is responsible for their appearance. This conclusion is further strengthened by the fact that mutations in all the other ORFs, including E2, E5, and E4, have no effect on pvp induction. E5B is predicted to encode a 52-amino-acid-long polypeptide that is rich in leucine and other hydrophobic residues (Fig. 5a). Comparisons of the hydrophilicity index plots between different papillomavirus E5 ORFs (Fig. 5b) reveal that several E5 ORFs encode proteins with marked hydrophobic regions of approximately equal size and distribution. As seen in Fig. 5b, BPV E5 and E5B have two such domains and HPV-16 E5 and HPV-6 E5A have three. These structural similarities suggest that the nontransforming (in mouse fibroblasts) BPV-1 E5B may have some as-yet-undefined function in the natural host in common with the transforming E5 proteins. In contrast, the hydrophilicity profile of HPV-6 E5B distinguishes it from this group of proteins.

Resident ER proteins play crucial roles in protein trafficking and assembly, and several of them, including grp100 (pvp1), are induced by expression of malformed foreign proteins (10). Thus, the modification of grp100 and calreticulin by E5B suggests that the presence of E5B disrupts normal ER-mediated protein processing. Such changes might result in altered cellular protein secretion, and we have found differences in the secreted proteins of BPV-transformed and untransformed cells (1). However, we have not been able to correlate those differences in secreted proteins with the induction of the papillomavirus-associated proteins (22a). Although we have determined that a mutation in E5B does not affect BPV's ability to transform C127 mouse fibroblasts or confer anchorage-independent growth, it may conceivably be involved in other aspects of mouse cell transformation such as tumor formation in nude mice. Yet, it seems more likely that the function of E5B will be uncovered in future studies of its action in the natural host cells (bovine epithelium) where the virus completes its life cycle and exerts influences on differentiation. However, if the E5B protein is secreted it may serve functions that only become evident in the whole animal, akin to the strategic role of the hepatitis B e antigen in generating immune tolerance (21).

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