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The 23-Kilodalton E1 Phosphoprotein of Bovine Papillomavirus Type 1 Is Nonessential for Stable Plasmid Replication in Murine C127 Cells

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The 23-kDa protein encoded by the 5′ segment of the E1 open reading frame of bovine papillomavirus type 1 (BPV1) was previously ascribed a negative regulatory function for the replication of viral plasmid DNA. However, results from recent functional and biochemical studies do not readily support this genetic assignment. Therefore, we have reassessed the role of this protein in papillomavirus DNA replication by using a mutant of BPV1 which is unable to express this E1 protein. This mutant viral DNA was found to replicate extrachromosomally with stability and copy number per cell similar to those of wild-type plasmid DNA. Thus, the absence of expression of the 23-kDa E1 protein did not lead to deregulated viral plasmid replication. We conclude that the 23-kDa E1 protein is nonessential for stable plasmid replication.

Bovine papillomavirus type 1 (BPV1) is a small tumor virus with a double-stranded DNA genome of 7,946 bp which causes benign fibropapillomas in cattle. The capacity for BPV1 to transform rodent cells in vitro has led to its use as a prototype for the analysis of papillomavirus DNA replication and its regulation (reviewed in reference 13). When the genetics of BPV1 plasmid replication was first analyzed by stable transfection of mouse C127 cells, it was found that the E1 translational open reading frame (ORF) is required for plasmid replication (9, 21, 24). In these studies, small deletions or inserted translational termination linkers throughout the E1 ORF led to the integration of the BPV1 genome in stably transfected cells. In contrast, when BPV1 DNAs with frameshift (fs) mutations in the E1 ORF were analyzed in transient and stable replication assays, two complementation groups were identified (18, 20) (Fig. 1). The 5′-E1 ORF complementation group, E1-M, was assigned a modulatory function for viral DNA replication, since fs mutants in the 5′-E1 ORF could replicate transiently, but were found to be cytotoxic to cells in long-term replication assays (19). The 3′-E1 ORF complementation group, E1-R, was assigned a positive replication function because no viral plasmid replication was observed in short- and long-term assays, and only integrated viral DNA could be found in stably selected cell populations. The identification of these two complementation groups contributed to the proposal of a biphasic model of viral plasmid replication in which E1-R and E1-M functions played central roles in the establishment and maintenance of cell cycle-controlled replication of the papillomavirus genome (2, 3).

Recently, two E1 phosphoproteins were identified biochemically, a 23-kDa protein encoded by the 5′ third of the E1 ORF, which was assigned to be the E1-M gene product (27), and a 68- to 72-kDa protein encoded by the entire E1 ORF (22, 26) (Fig. 1). No biochemical evidence has been found for an E1-R-like protein derived from the 3′-E1 ORF. The full-length 68- to 72-kDa E1 protein, together with the full-length 48-kDa E2TA protein, was found to be necessary and sufficient for BPV1 plasmid replication when analyzed in a transient assay (28). From the results of these recent biochemical and functional studies, it seems likely that the positive replication function of E1 is encoded by the full-length ORF. This reasoning leads to a quandary in the assessment of the phenotype of fs mutants in the 5′-E1 ORF, since such BPV1 DNAs must be defective for the expression of not only the 23-kDa E1 protein but also the full-length E1 ORF gene product now demonstrated to be required for plasmid DNA replication. Therefore, the genetic assignment of a replication-modulatory function to the 23-kDa E1 protein must be reconsidered.

To reevaluate the role of the 23-kDa E1 protein in viral plasmid DNA replication, we have analyzed a mutant BPV1 DNA, pBM8 (previously referred to as p1471-1 [15]), which was predicted to be defective for the expression of the 23-kDa E1 protein while retaining the capacity to produce a full-length E1 protein. pBM8 contains a point mutation at nucleotide (nt) 1236 in the background of the entire BPV1 genome (p142-6 [23]). This mutation alters the 5′ splice site consensus sequence of the intron from GT to AT (Fig. 1) and is therefore expected to prevent 1235^3225 mRNA splicing. In a previous study, pBM8 was found to be defective for the expression of the E8/E2TR fusion protein, a gene product that is expressed from a 1235^3225-spliced mRNA (14). Since this splicing event is also required for expression of the 23-kDa E1 protein (27), pBM8 was expected to be deficient in its expression as well. Viral transforming efficiency, level of viral transcription, and plasmid copy numbers for wild-type (wt) BPV1 (p142-6) and pBM8 were analyzed previously and found to be similar (15). In this study, we have further characterized pBM8 to substantiate the absence of 23-kDa E1 protein expression from this viral genome and to assess more completely its replication phenotype.

Assessment of cytotoxicity by cotransfection and selection for resistance to G418. E1-M mutant BPV1 DNAs were reported to be cytotoxic to C127 cells on the basis of the reduction of the number of G418-resistant colonies in cotransfection experiments with a neomycin-resistant (Nm^r) marker plasmid and increasing amounts of BPV1 plasmid DNAs (19). To determine whether the 1235^3225 splice

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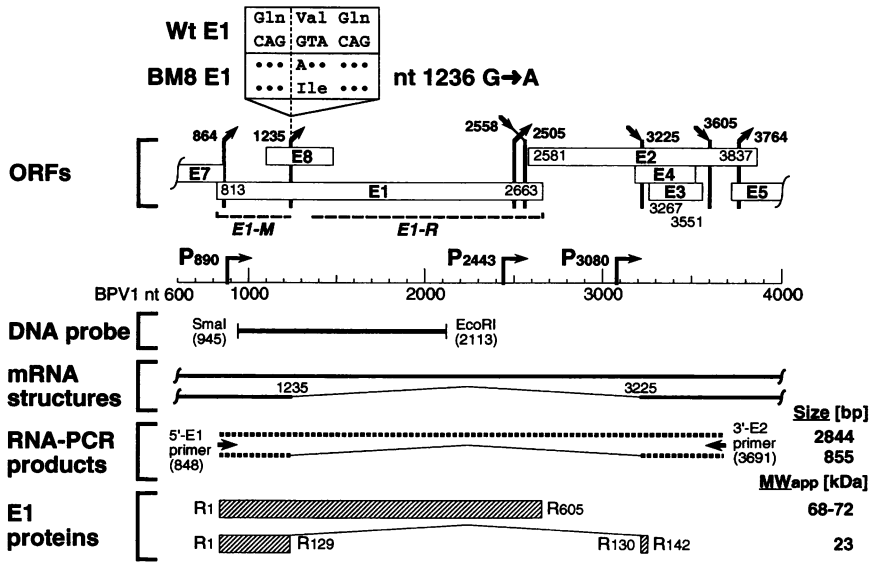

FIG. 1. Map of the early region of BPV1. The position of the nt 1236 G→A mutation of pBM8 is indicated at the top. The locations of 5′ and 3′ splice sites are denoted by diagonal arrows above the ORFs (open boxes), and their nucleotide positions are indicated. The size of the subgenomic DNA probe for RNA-PCR product analysis (in Fig. 2) is indicated below the ORFs. The depicted mRNA structures (solid lines) reflect splicing behavior observed in C127 cells (1). The expected lengths of the PCR products (dashed lines) from amplified cDNA with the primer set 5′-E1 and 3′-E2 are shown at the right. Amino acid residue numbers and apparent molecular masses are indicated for the E1 proteins (hatched boxes).

mutant pBM8 is also cytotoxic, low-passage subconfluent C127 cells were cotransfected with viral DNA and the Nm^r plasmid p302-3 (17) and selected for resistance to G418 (geneticin), and drug-resistant colonies were analyzed as described previously (16). Transfections were carried out with 1.0 μg of the Nm^r vector and 0.125 to 2.0 μg of *Bam*HI-digested p142-6 or pBM8 DNA. No reductions in G418-resistant colony counts were observed with increasing amounts of wt BPV1 or pBM8 DNA (11). G418-resistant colonies from cotransfections with 1.0 μg of viral DNA were expanded into mass culture and single-colony-derived cell lines and continually subcultured for over 25 passages. After performing BPV1-specific Southern hybridization of total cellular DNA, we found that extrachromosomal pBM8 DNA was maintained in both types of cultures (11). We conclude from these observations that the mutant pBM8 DNA is not cytotoxic and that C127 cell populations which stably harbor pBM8 DNA can be established. For comparison, we also analyzed E1-Sma (2), one of the original 5′-E1 fs mutant BPV1 DNAs that was used to define the E1-M-deficient phenotype. In our hands, E1-Sma did not appear to be cytotoxic in cotransfection experiments with a Nm^r marker plasmid. E1-Sma DNA was found integrated at a low copy number when G418-resistant cell populations were analyzed for viral DNA content (12). These experiments were carried out under conditions identical to those performed in the original study with different sources of C127 cells, including cells obtained from the authors of the original study (19). Thus, we could not reproduce the cytotoxic phenotype of E1-M-deficient BPV1 DNAs in our genetic analyses of the 1235^3225 splice mutant pBM8 and the 5′-E1 fs mutant E1-Sma. The absence of cytotoxicity and the ability to establish pBM8-harboring cell lines allowed us to characterize further the replication phenotype of pBM8.

Analysis of viral RNAs by E1-specific RNA-PCR. To determine whether 1235^3225-spliced mRNAs could be produced from the pBM8 DNA, we used a coupled reaction protocol to detect E1 ORF-derived cDNAs by reverse transcribing cellular RNA and amplifying specific cDNAs with the polymerase chain reaction (RNA-PCR). Figure 1 depicts the structure of the expected PCR products from cDNA amplification with a 5′-E1 sense primer and a downstream 3′-E2 antisense primer, encompassing the coding regions of the 23-kDa E1 protein from nt 849 to 1235 and nt 3225 to 3263. A PCR product generated from the 1235^3225-spliced mRNA species is expected to be 855 bp in size. We carried out RNA-PCR with total cellular RNA samples isolated from wt BPV1- and pBM8-harboring C127 cells between passages 72 and 83 by acidic phenol extraction (6) and analyzed the products by Southern blotting and BPV1-specific probing. Figure 2 shows the presence of 855-bp PCR products generated by 1235^3225-spliced RNAs from wt-transformed C127 cells (lanes 3 and 4) which are absent in pBM8-derived samples (lanes 5 and 6). We estimated the sensitivity of our assay by using mixed RNA samples to reconstruct cellular copy numbers for 1235^3225 mRNA. Standards containing the equivalent of 0.1, 1, 10, and 100 E1-specific RNA copies per cell were prepared with in vitro-transcribed N7-1 (E1-M cDNA [27]) RNA in the background of total RNA isolated from pBM8-transformed cells and analyzed as described above. We were able to detect RNA-PCR products from as little as 60 fg of N7-1 RNA, which is equivalent to 0.1 copy per cell (lanes 7 through 10). To assess the quality of the cellular RNA preparations, samples were analyzed by RNA-PCR with a primer pair to detect BPV1-specific unspliced and 2505^3225-spliced RNAs. wt BPV1- as well as pBM8-derived samples yielded comparable levels of PCR products from both kinds of RNA (11). The correct structure of RNA-PCR products derived from 1235^3225-spliced RNAs with the 5′-E1 and 3′-E2 primer pair was verified by digesting samples with *Xma*I or *Asp*718I, which cleave at unique sites in the BPV1 genome within the putative E1-M mRNA.

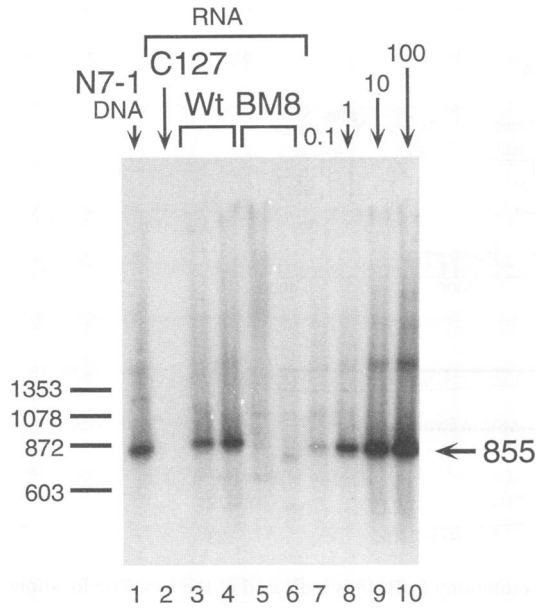

FIG. 2. E1-specific RNA-PCR. Shown is an autoradiograph of a Southern hybridization analysis of RNA-PCR products generated from total cellular RNA isolated from untransformed (lane 2), wt BPV1-transformed (lanes 3 and 4), and pBM8-transformed (lanes 5 and 6) C127 cells. cDNAs were synthesized from 5 μg of total cellular RNA by primer annealing (10 μl with 25 ng of coprecipitated 3′-E2 primer [BPV1 nt 3691 to 3672], 80 mM Tris-HCl [pH 8.3], and 80 mM KCl; 3 min at 90°C, 20 min at 55°C, and cooling to 30°C in 60 min) followed by primer extension (20 μl with 90 mM Tris-HCl [pH 8.3], 115 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 100 μg of actinomycin D per ml, each deoxynucleoside triphosphate at 500 μM, 50 μg of bovine serum albumin per ml, 40 U of RNase inhibitor [Boehringer Mannheim], 25 U of avian myeloblastosis virus reverse transcriptase [Boehringer Mannheim]; 90 min at 42°C). Reactions were stopped (2 μl of 500 mM EDTA), phenol-chloroform extracted, precipitated, and resuspended. The cDNA products were subsequently amplified by PCR (20 μl with each of the 5′-E1 [BPV1 nt 848 to 866] and 3′-E2 primers at 1 μM, each deoxynucleoside triphosphate at 200 μM, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl, 100 μg of gelatin per ml, 2.5 U of *Taq* DNA polymerase [Boehringer Mannheim]; 60 cycles of 92°C for 1 min, 48°C for 2 min, and 72°C for 4 min), resolved on 2% (wt/vol) agarose gels, Southern blotted, and hybridized to a subgenomic BPV1 DNA probe (see Fig. 1) that was uniformly labeled with 5′-[α-^{32}P]-dCTP (Amersham) by random priming (Boehringer Mannheim kit). Lane 1 shows the PCR product generated with the same primers by using pON-N7-1 (E1-M cDNA) plasmid DNA as a template. Lanes 7 through 10 contain RNA-PCR products obtained using by total cellular RNA from pBM8-transformed C127 cells mixed with 60 fg, 600 fg, 6 pg, and 60 pg of in vitro-transcribed N7-1 RNA, respectively. Positions of migration of *Hae*III-digested φX174 replicative-form DNA markers are indicated at the left in nucleotides.

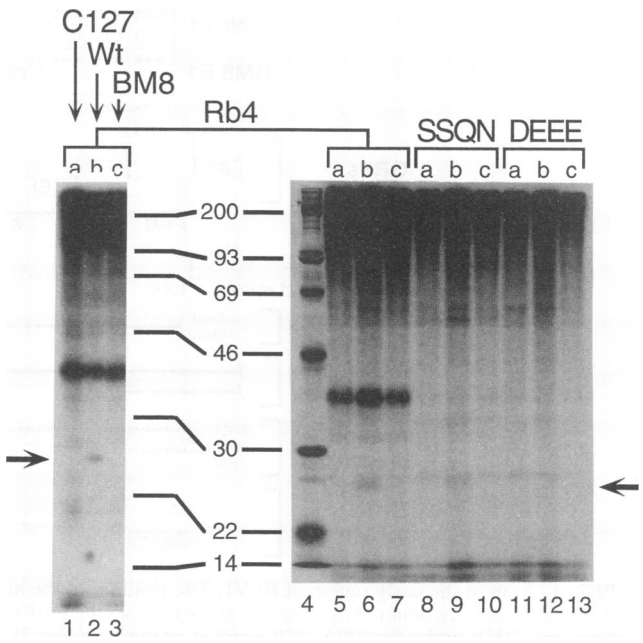

FIG. 3. E1 N-terminus-specific radioimmunoprecipitation. Shown are autoradiographs of SDS-polyacrylamide gels with resolved ^{32}P-radiolabeled proteins after immunoprecipitation from untransformed (a; lanes 1, 5, 8, and 11), wt BPV1-transformed (b; lanes 2, 6, 9, and 12), and pBM8-transformed (c; lanes 3, 7, 10, and 13) C127 cell lysates. Antisera used: Rb4 (27) in lanes 1 to 3 and 5 to 7, SSQN (25) in lanes 8 to 10, and DEEE (25) in lanes 11 to 13. Samples were resolved on an SDS–12% (wt/vol) polyacrylamide gel (lanes 1 to 3) or a 10% gel (lanes 4 to 13). The migration positions of the 23-kDa E1 phosphoprotein are indicated by arrows at both sides. The positions of ^{14}C-labeled protein size markers (lane 4) are indicated in kilodaltons between the panels.

Restriction fragments, indicating the predicted cleavage pattern by these two enzymes, were detected only in wt-derived cDNA samples, not in pBM8-derived ones (11). We conclude from these findings that 1235^3225-spliced mRNAs are clearly present in wt-transformed cells but that their abundance in pBM8-derived samples is less than 0.1 molecule per cell. Importantly, no other RNA-PCR products arising from alternative RNA splicing, with a possible coding capacity for a different E1 N-terminus-containing protein, were observed with pBM8-derived RNA samples. We also verified that the nt 1236 A→G mutation is retained in pBM8-transformed cells of high passage. E1-specific RNA-PCR products were digested with *Bst*NI, for which a site is lost at the location of this mutation. No cleaved products indicative of wt sequences were detected in pBM8-derived samples (11).

Analysis of viral proteins by E1 N-terminus-specific radioimmunoprecipitation. To test whether any 23-kDa E1 phosphoprotein could be detected in BPV1-transformed C127 cell populations, we performed E1-specific immunoprecipitations with ^{32}P-labeled cell lysates essentially as described previously (27). Figure 3 shows the immunoprecipitates from lysates of untransformed, wt BPV1-transformed, and pBM8-transformed C127 cells (lanes a, b, and c, respectively). An E1 phosphoprotein with an apparent molecular mass of 23 kDa could be detected only with the E1 N-terminus-specific antisera Rb4 (27) and SSQN (25) in wt samples (lanes 2, 6, and 9) but not in pBM8 samples (lanes 3, 7, and 10). The 23-kDa E1 protein was not detected by the E1 C-terminus-specific DEEE antiserum (25) (lanes 12 and 13). Longer autoradiographic exposure did not produce a specific band in the pBM8 sample (lane 3) (11). The full-length E1 phosphoprotein could not be detected in these single-step immunoprecipitations because of the high background radioactivity. Consistent with our RNA-PCR results, no alternative E1 N-terminus-containing proteins of a size comparable to the 23-kDa protein were detected with antisera specific for this protein region.

Analysis of viral DNA from BPV1-transformed C127 cells subcultured over time by Southern hybridization. Finally, we

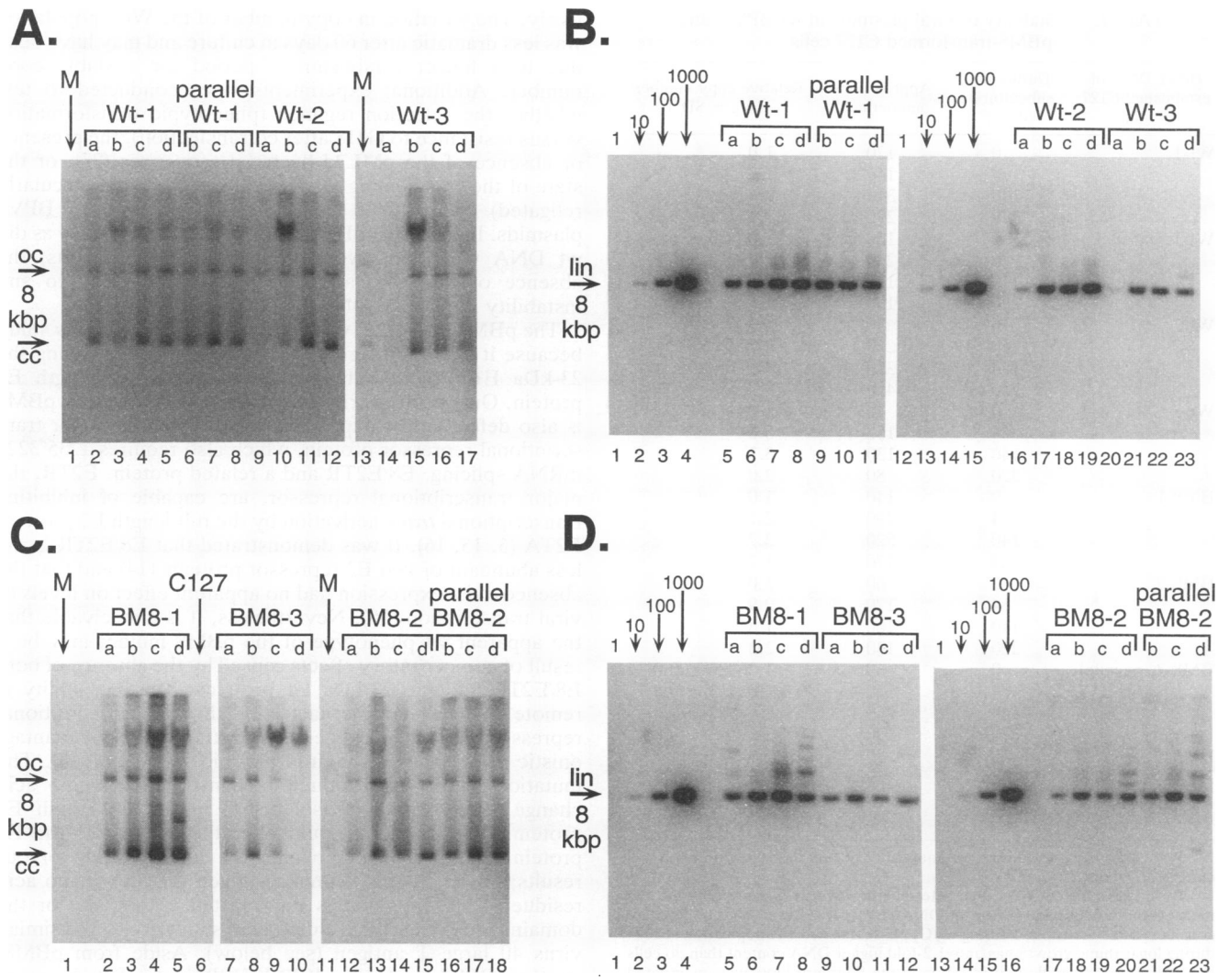

FIG. 4. Analysis of viral DNAs. Shown are autoradiographs from Southern hybridization analyses of total cellular DNA isolated from BPV1-transformed cells after 0 days (a lanes), 64 days (b lanes; 60 days for Wt-1), 140 days (c lanes), and 220 days (d lanes) in subculture. Samples (5 μg) of sheared or digested DNA were resolved on 0.8% (wt/vol) agarose gels and analyzed by Southern blotting and hybridization to a full-length BPV1 DNA probe as described for RNA-PCR. (A and C) Sheared DNAs from wt BPV1-transformed and pBM8-transformed C127 cell lines, respectively. The marker lanes (M) contain plasmid DNA similar in migration to BPV1 monomers. The positions of 8-kbp plasmid DNA monomers are indicated at the left by arrows denoting open circles (oc) and closed circles (cc). A negative control for nonspecific hybridization to DNA from untransformed C127 cells is shown in lane 6 of panel C. (B and D) *Eco*RI-digested wt BPV1- or pBM8-derived DNAs. For copy number quantitation, reconstruction series were prepared by combining p142-6 plasmid DNA with total DNA from untransformed C127 cells and digesting with *Bam*HI. The designated lanes contain 1, 10, 100, and 1,000 viral copies per cell, equivalent to 2.6 pg through 2.6 ng of 8-kbp BPV1 DNA per 5 μg of cellular DNA, respectively. The position of linearized (lin) 8-kbp plasmid DNA monomers is indicated at the left.

sought to determine the stability of viral plasmids over time by Southern hybridization analysis of cellular DNA from focal populations of wt BPV1- and pBM8-transformed C127 cells. Transfection and selection for morphological transformation were performed as described previously (16) except that 1.0 μg of *Bam*HI-digested and unimolecularly religated BPV1 DNA (8 kbp) was used. Established cell lines (Wt-1 [line 24.5.1], Wt-1 parallel [24.5.2], Wt-2 [24.5.4], Wt-3 [24.5.6], BM8-1 [24.6.2], BM8-2 [24.6.5.1], BM8-2 parallel [24.6.5.2], and BM8-3 [24.6.6]) were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 7.5% fetal bovine serum and antibiotics and then passaged at confluence every 4 days with a split ratio of 1:25 (up to passage 26), 1:33 (up to passage 59), and 1:50 (for higher passages). Total cellular DNA was isolated after 0, 64, 140, and 220 days by a sodium dodecyl sulfate (SDS) lysis protocol (23). Figure 4 depicts the state of the viral DNA in wt BPV1- (Fig. 4A) and pBM8-derived (Fig. 4C) samples of sheared total cellular DNA. In the majority of both wt- and pBM8-derived samples, the viral DNA is replicated extrachromosomally over time, as reflected by the prominent closed circular and open circular bands of the 8-kbp plasmid monomers. The low-level hybridization signal from the diffuse smear of high-molecular-weight DNA above the open circular band indicates that some viral DNA is also replicated as plasmid multimers or that it is integrated into the host cell

TABLE 1. Stability of viral plasmids in wt-BPV1- and pBM8-transformed C127 cells

BPV1 DNA of established C127 cell line	Duration of subculturing (days)[a]	Approx copy number/cell[b]	Relative copy number[c] (avg ± SD[d])
Wt-1	0	120	1.0
	64	150	1.3
	140	310	2.6
	220	290	2.4
Wt-1, parallel	0	120	1.0 (1.0 ± 0.0)
	60	240	2.0 (1.7 ± 0.5)
	140	180	1.5 (2.1 ± 0.6)
	220	190	1.6 (2.0 ± 0.4)
Wt-2	0	30	1.0
	64	260	8.7
	140	310	10.3
	220	450	15.0
Wt-3	0	40	1.0
	64	150	3.8
	140	130	3.3
	220	80	2.0
BM8-1	0	140	1.0
	64	290	2.1
	140	520	3.7
	220	170	1.2
BM8-2	0	60	1.0
	64	170	2.9
	140	100	1.8
	220	130	2.3
BM8-2, parallel	0	60	1.0 (1.0 ± 0.0)
	64	120	2.0 (2.5 ± 0.5)
	140	260	4.6 (3.2 ± 1.4)
	220	130	2.3 (2.3 ± 0.0)
BM8-3	0	80	1.0
	64	130	1.5
	140	60	0.7
	220	80	1.0

[a] Four days of subculturing are equivalent to one passage with ≈4.5 cell culture doublings.

[b] Parameters for copy number determination are as follows. The haploid mouse genome contains 3 Gbp of DNA. BPV1-transformed C127 cells are considered to be tetraploid during G_0/G_1 phase. An average population of cells during logarithmic growth has an ≈1.2-fold higher DNA content than do cells in G_0/G_1 phase. Specific hybridization in the Southern blots was quantitated by BetaGen scanning.

[c] Normalized to values at 0 days of subculturing for each cell line.

[d] Determined separately for the Wt-1 and BM8-2 populations maintained in parallel at each time point.

genome. To determine the BPV1 DNA copy number per cell, total cellular DNAs were digested with *Eco*RI, which cuts the viral genome at a unique site, and then analyzed by Southern hybridization. We included a reconstruction series equivalent to 1, 10, 100, and 1,000 BPV1 molecules per cell on each Southern blot to permit accurate copy number estimation by using a Betagen scanner. The relative changes over time in the viral copy numbers per cell were normalized to the starting copy number of each independently maintained cell population; the results are summarized in Table 1. With the exception of the Wt-2 cell line, the relative copy numbers for both wt BPV1 and pBM8 varied within a factor of 5 during the 220 days of subculturing. To estimate the variability of the assay over time, parallel cultures of the Wt-1 and BM8-2 cell lines were set up after the zero time point and maintained independently throughout the experiment. Per time point, the standard deviation in the relative copy numbers, as determined for parallel cultures, did not exceed 0.6 and 1.4 for cell lines Wt-1 and BM8-2, respectively. The variation in copy number of the Wt-2 population was less dramatic after 60 days in culture and may have been due to a longer establishment period for a stable copy number. Additional experiments were conducted to test whether the selection regimen (phenotypic transformation versus resistance to G418 after cotransfection), the presence or absence of the pML2d bacterial sequences (23), or the state of the transfecting DNA (linearized or unimolecularly religated) could affect the replication behavior of BPV1 plasmids. In all cases, pBM8 DNA replicated as stably as did wt DNA when the two were compared (11). Thus, the absence of the 23-kDa E1 protein did not lead to any instability in plasmid DNA replication in C127 cells.

The pBM8 DNA (15) was chosen for analysis in this study because it was predicted to be deficient for expressing the 23-kDa E1 protein but able to produce a full-length E1 protein. Our results verify this prediction. However, pBM8 is also defective for expression of E8/E2TR, a minor transcriptional repressor protein, which also requires 1235^3225 mRNA splicing. E8/E2TR and a related protein, E2TR, the major transcriptional repressor, are capable of inhibiting transcriptional *trans* activation by the full-length E2 protein, E2TA (5, 15, 16). It was demonstrated that E8/E2TR is the less abundant of two E2 repressor proteins (14) and that the absence of its expression had no apparent effect on levels of viral transcription (15). Nevertheless, it is conceivable that the apparent wt phenotype of the pBM8 mutant may be a result of compensatory effects caused by the absence of both E8/E2TR and the 23-kDa E1 proteins. This possibility is remote in view of the fact that the E2 transcriptional repressor proteins have been demonstrated to play an antagonistic role in papillomavirus plasmid replication (4). The mutation in pBM8 introduces a conservative amino acid change, from valine to isoleucine, in the full-length E1 protein. This mutation could alter the activity of the E1 protein and thereby complicate the interpretation of our results; however, this is unlikely given that the amino acid residue at this position is not located within any of the domains in E1 that have amino acid similarity to the simian virus 40 large T antigen (see below). Aside from pBM8, another BPV1 mutant (p1039-1 [10]) is predicted not to express the 23-kDa E1 protein but to be capable of expressing an intact full-length E1 protein. In this mutant, 1235^3225 mRNA splicing is abrogated by an altered consensus sequence at the nt 3225 3′ splice site. However, we chose not to include this mutant in our analysis because it not only is similarly deficient in the expression of E8/E2TR (14) but also is altered in its transformational and transcriptional properties. These phenotypic changes are presumably caused by the disruption of many additional BPV1 mRNA splicing events that utilize the 3225 3′ splice site.

Our experiments did not produce evidence for a modulatory function of the 23-kDa E1 protein in BPV1 plasmid replication. Still, the possibility remains that this protein plays a role in viral DNA replication, since it is colinear at the N terminus with the full-length E1 ORF product, known to be required for plasmid replication (28). This shared N-terminal region harbors a putative nuclear localization signal and potential phosphorylation sites, as predicted by amino acid homology with simian virus 40 large T antigen (7; reviewed in reference 8). Consistent with these predictions, both E1 proteins were found to be nuclear phosphoproteins (26, 27). While a function for the 23-kDa E1 protein in latent viral plasmid replication in BPV1-transformed C127 cells was not observed (this study), this protein may be involved in the amplification of viral DNA during the vegetative phase

of BPV1 infection. Experiments to investigate this possibility are currently in progress.

To summarize, our genetic and biochemical analyses of pBM8 demonstrate that this mutant BPV1 DNA is not cytotoxic to C127 cells and does not produce detectable amounts of an mRNA species required for the expression the 23-kDa E1 protein. Similarly, no ^{32}P-labeled 23-kDa phosphoprotein could be detected in pBM8-transformed C127 cells. The replication phenotype of pBM8 with respect to plasmid copy number and stability is comparable to that of wt BPV1. We conclude from these findings that the 23-kDa E1 protein is not essential in stable plasmid replication.

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