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Bovine Papillomavirus Type 1 E1 Replication-Defective Mutants Are Altered in Their Transcriptional Regulation

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Bovine papillomavirus type 1 (BPV-1) is capable of replicating as a stable, high-copy-number plasmid in transformed rodent cells. The BPV-1 E1 open reading frame (ORF) encodes multiple functions involved in viral DNA replication. Mutations which disrupt the translational integrity of the E1 ORF disable the viral genome from replicating as a stable plasmid and result in the integration of the viral genome into the host chromosome generally at a low copy number. Despite the low copy number of the integrated genomes, BPV-1 E1 mutants transform rodent cells to anchorage independence very efficiently, at levels equal to or greater than that of wild-type (wt) BPV-1. Studies were performed to provide insight into why these low-copy-number, replication-defective mutants are capable of expressing an equal or greater transformation potential than wt BPV-1. Analysis of viral RNA revealed higher rates of transcription per viral genome in cells harboring E1 mutated BPV-1 DNA than in cells containing wt BPV-1 DNA. Furthermore, the levels of viral RNA mapping the P_{89} promoter were found to be 15- to 35-fold higher in cells transformed by E1 mutated DNAs compared with wt BPV-1 transformants. This promoter controls expression of the BPV-1 E6 transforming gene and is regulated by the viral E2 gene products. The studies presented in this report determined that the E1 mutants were perturbed in their E2 transcriptional regulation, suggesting a possible explanation for the observed P_{89} induction. Mutations throughout the E1 ORF, in either of the two regions previously identified as encoding distinct replication functions, were altered in viral transcription.

The bovine papillomavirus type 1 (BPV-1) DNA genome persists as a stable multicopy plasmid in virally transformed mouse cells (13). This property is thought to reflect the quasi-latent state of infection in the cells of the basal epithelium and in the dermal fibroblasts of a BPV-1 fibropapilloma (10). These transformed rodent cells have been instrumental in characterizing the BPV-1 replication functions in tissue culture. The BPV-1 E1 open reading frame (ORF) encodes at least two replication functions, the 5′ end of the E1 ORF encodes a modulator (E1-M) function and the 3′ two-thirds of the E1 ORF encodes a replication (E1-R) function (Fig. 1) (14, 16). The E1-M protein has been recently identified as a 23-kilodalton phosphoprotein in BPV-1-infected cells (28). Mutation of either of these genes results in replication-defective BPV-1 DNAs which are integrated usually at low copy number into the host chromosomes of cells stably transformed by these DNAs (15, 19, 21). In addition to their DNA replication defects, E1 mutants are also altered in their ability to transform rodent cells. Initial studies, using a focus transformation assay, determined that E1 mutants were capable of transforming C127 cells at relatively high efficiencies (19, 21), indicating that the efficiency of transformation by E1 BPV-1 mutants does not correlate with the viral gene copy number. Using an anchorage-independence assay, these same E1 mutants have transformation efficiencies greater than that of wild-type (wt) BPV-1 DNA. Furthermore, cells harboring an E1 mutant BPV-1 DNA have recently been found to contain steady-state concentrations of viral RNA which were higher per viral genome copy than that found in wt BPV-1-transformed cells (E. Kleiner, D. Lowy, H. Pfister, and J. Schiller, submitted for publication). This result suggests that the observed high efficiency of transformation may be the consequence of an increased rate of viral gene expression.

In this study, we have measured the transcriptional activity of E1 mutated BPV-1 genomes and have examined the mechanism by which such mutations lead to altered viral gene expression. We have found that BPV-1 DNAs containing E1 ORF mutations, regardless of the location of the mutation, exhibited increased rates of viral transcription initiation, which correlated with increased efficiencies of cellular transformation. Analysis of specific viral promoter utilization indicated that this increased transcription was manifest most dramatically at the P_{89} promoter. Furthermore, we found increased levels of E2 transactivation in cells transformed by the E1 mutants. These data indicate that the replication-defective E1 mutants have specific alterations in their viral transcription patterns which correspond to a perturbation in the E2 transcriptional regulatory circuit. The significance of these findings with respect to the coordination of viral gene expression and viral DNA replication is discussed.

MATERIALS AND METHODS

Plasmid DNAs. The plasmid, p142-6, containing the full-length wt BPV-1 genome cloned into the unique *Bam*HI site of pML2d has been previously described and served as the parental DNA into which all the mutations described below (and shown in Fig. 1) were introduced (2, 20). The previously described BPV-1 mutants, p743-23, p745-1, and p771-2, contain an oligonucleotide translational termination linker (TTL) containing termination codons in each of the three potential translation frames inserted at BPV-1 nucleotide (nt) 1299, 945, or 2878, respectively (19). The plasmids,p1306-1 and p1307-1, contain a TTL inserted at BPV-1 nt 1515 and 1811, respectively, and were constructed by replacing the 2,410-base-pair *Xma*I-*Kpn*I fragment obtained from either p1135-1 or p1136-1 (12) into p142-6. The double mutant, p1308-1, contains TTLs at both nt 1299 and nt 2878 and was constructed by replacing the 1,168-base-pair *Xma*I-*Eco*RI

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fragment from p743-23 into p771-2. The plasmid, pCW1-28, is a cDNA which contains the contiguous BPV-1 sequences from nts 845 to 4203 and expresses the E2 transcriptional repressor function (12). The plasmid, pMMTneo, contains the bacterial tn5 neomycin resistance gene cloned downstream of the mouse metallothionein gene promoter. The plasmids utilized for chloramphenical acetyltransferase (CAT) assays have been previously described (5, 23, 25).

Cell culture and transfection protocols. Low passage mouse C127 cells were maintained at 10% CO_2 in complete medium composed of Dulbecco modified medium supplemented with 10% heat-inactivated fetal calf serum, glutamine, and antibiotics. Calcium phosphate transfections were performed essentially as previously described (6). Briefly, a total of 10 μg of DNA, consisting of high-molecular-weight salmon sperm DNA and various amounts of plasmid DNA, as indicated, was used in forming the precipitate which was added to subconfluent 6-cm-diameter dishes of C127 cells. After 4 h of incubation, the precipitate was removed; the cells were treated with 15% glycerol in phosphate-buffered saline solution for 1 min and incubated for 12 h in complete medium containing 5 mM sodium butyrate. For transfection with pMMTneo, cells were split 1:6 at 48 h posttransfection and maintained in complete medium supplemented with 500 μg of G418 per ml for 2 weeks. For assaying efficiency of growth in soft agar, cells were trypsinized 72 h posttransfection and dispersed in 0.35% molten Noble agar which was prepared with complete medium and poured over a previously solidified 0.6% Noble agar–complete medium underlay. The agar plates were incubated for the indicated times with periodic feedings, and colonies were stained by incubation overnight in 0.05% iodonitrotetrazolium chloride.

BPV-1 DNA and RNA quantitation methods. Total cellular DNA was isolated from cells as previously described (19), and Southern analysis was performed by using nick-translated BPV-1 DNA. Total cellular RNA was isolated by the procedure described by Phelps and Howley (18). RNA slot blot analysis was performed by transferring indicated amounts of RNA to nitrocellulose as previously described, and hybridizing with nick-translated BPV-1 DNA (27). Nuclear runon analysis was performed as previously described (7), and the resulting radiolabeled RNA was used to probe linearized BPV-1 plasmid DNA immobilized on nitrocellulose. Primer extension analysis was performed on 10 μg of total cellular RNA by using ^{32}P-end-labeled oligonucleotides, complementary to viral mRNAs, as previously described (1). Radiolabeled primer extension products were resolved by electrophoresis on 6% polyacrylamide–7.5 M urea gels. All quantitation was performed on a Zieneh laser densitometric scanner, and autoradiographs were generated by exposure of the film in the absence of an intensifying screen.

RESULTS

BPV-1 DNAs mutated in the E1 ORF exhibit increased efficiency of transformation when assayed for anchorage independence. The transformation capacity of BPV-1 DNAs containing TTL insertions in the E1 ORF were assayed for anchorage independence in soft agar. The specific mutated DNAs used in this analysis are listed in Fig. 1. Briefly, each mutated DNA contains a TTL insertion at a single site in the full-length viral genome cloned in pML2d as described in Materials and Methods. When transfected into C127 cells, the E1 mutated DNAs resulted in greater numbers of anchorage-independent colonies than did wt BPV-1 (Table 1).

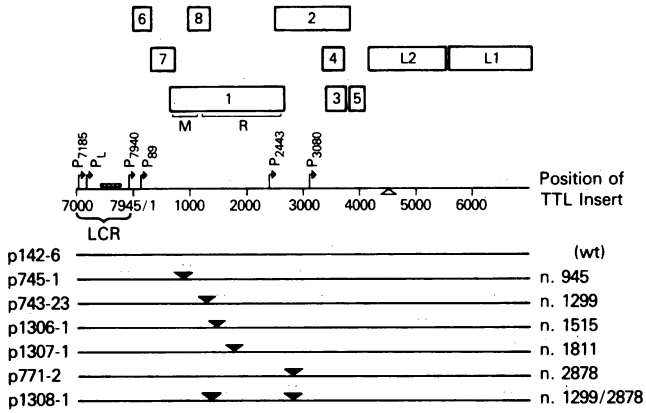

FIG. 1. BPV-1 genome. Illustrated at the top is a line drawing representing the physical map of the circular BPV-1 genome, linearized at nt 7000 for means of presentation. Arrows indicate positions of known promoters as described in the text. The position of the LCR is indicated. The position of the bacterial vector, pML2d, into which the BPV-1 genome has been cloned at the *Bam*HI site, is indicated by the open triangle. Above the line drawing are boxes which indicate the positions of the translational ORFs. L1 and L2 represent the late, structural gene products while all other numbered boxes represent the early ORFs which are contained within the region of the viral genome expressed in transformed rodent cells (2). The E1-M (M) and E1-R (R) complementation groups are delineated by brackets beneath the E1 ORF. The solid triangles indicate the positions of TTLs in the various BPV-1 mutants. The TTLs at nts 945, 1299, 1515, and 1811 affect the E1 ORF, and the TTL at nt 2878 affects the E2 ORF.

The colonies observed for the E1 mutated DNAs appeared earlier during the assay and grew larger than did those for wt BPV-1 DNA (Table 2). Each of the E1 mutated DNAs tested was found to exhibit this phenotype regardless of whether the mutation was located in the region of the E1 ORF corresponding to the E1-M or to the E1-R complementation group. These data are similar to those described by Kleiner et al. (submitted), who have also demonstrated that BPV-1 E1 mutated DNAs are enhanced in their transformation capacity compared with wt BPV-1 DNAs.

Elevated steady-state levels of BPV-1 RNA in E1 mutants correlate with increased rates of transcriptional initiation. The augmented transformation phenotype of the replication-defective BPV-1 E1 mutants raised the possibility that these mutants could be altered in their transcriptional regulation. Levels of viral RNA were therefore compared in cells transfected with wt and E1 mutant BPV-1 DNAs. Full-length clones of wt and E1 mutated BPV-1 genomic DNAs were cotransfected with pMMTneo, using mouse C127 cells. Pooled G418-resistant colonies were expanded, and total cellular DNA and RNA were analyzed. Based upon South-

TABLE 1. Anchorage-independent growth of BPV-1 E1 mutants

Plasmid(s)	Mutation	No. of colonies/ 0.5 μg DNA
p142-6	None	94
p745-1	TTL at nt 945	295
p743-23	TTL at nt 1299	293
p1306-1	TTL at nt 1515	271
p1307-1	TTL at nt 1811	178
No DNA		3

TABLE 2. Time course of colony growth in soft agar

Plasmid	Incubation period (days)	No. of colonies in indicated size range (nm)[a]		
		78–88	89–109	>107
p142-6	14	3	3	1
	21	17	11	7
p743-23	14	15	11	10
	21	29	26	29

[a] No colonies in these size ranges were detected on control transfection plates which did not receive BPV-1 DNA.

ern blot analysis of cellular DNAs cleaved with a no-cut enzyme for the transfected BPV-1 plasmids, the E1 mutated BPV-1 DNAs were found to be integrated (data not shown) and in low copy number, in agreement with previous studies (8, 15, 19, 21). This result contrasts with the extrachromosomal state and high copy number of wt BPV-1. The differences in the ratio of the DNA copy number among wt BPV-1 DNA and the E1 mutated DNAs ranged from 14- to 34-fold in these pooled cell populations (Fig. 2A). BPV-1 RNA was quantitated by slot blot analysis (Fig. 2B). The levels of BPV-1 RNA did not correlate with the DNA copy number. The cells harboring the E1 mutated viral genomes contained three- to ninefold greater amounts of BPV-1 RNA per viral genome than did wt BPV-1 DNA transfected cells (Fig. 2C). Cells were also selected on the basis of their transformed phenotype, and the viral RNA levels were found to be on average fivefold higher per genome for the E1 mutant BPV-1 transformants when compared with levels for wt transformants (data not shown). The increased level of BPV-1 RNA was seen with each of the E1 mutated DNAs regardless of the location of the linker insertion (Fig. 2C). The increased transforming capacities of the individual E1-M and E1-R mutants, therefore, did not correlate with the viral DNA copy number, but rather with the steady-state concentrations of viral RNA.

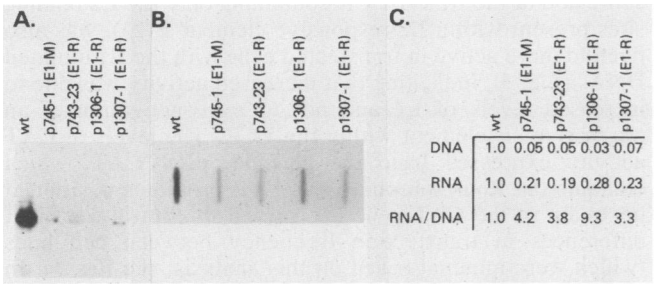

FIG. 2. BPV-1 DNA and RNA quantitation. (A) Autoradiograph of a Southern analysis in which 10 μg of total cellular DNA from each source was restricted with *Bam*HI, electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and probed with nick-translated BPV-1 DNA. The lanes are labeled to indicate the source of total cellular DNA from pooled G418-resistant cells cotransfected with wt or E1 mutated BPV-1 plasmids. (B) Autoradiograph of a slot blot in which 5 μg of total cellular RNA was immobilized onto nitrocellulose and probed with nick-translated BPV-1 DNA. The transfected DNAs are indicated at the top. (C) Summary of the quantitation of BPV-1 DNA and RNA from panels A and B. In each column, the integrated peak area obtained for the wt BPV-1 was given the value of 1.0. The integrated peak area for the E1 mutated DNAs was then calculated as the fraction of the wt peak area. The RNA/DNA ratio for each E1 mutant was then determined.

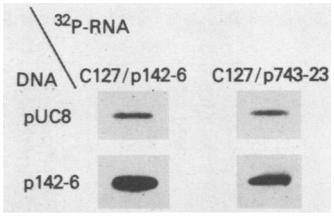

FIG. 3. Autoradiograph of hybridization by radiolabeled RNA obtained by nuclear runons to specific DNAs. Above the autoradiograph is indicated the BPV-1 plasmid harbored in the cells from which the radiolabeled nuclear runon RNA product was isolated. To the left are shown the DNA plasmids which were immobilized on nitrocellulose.

To determine whether the increased steady-state level of BPV-1 RNA seen in E1 mutant-transfected cells was the consequence of an increase in the rate of viral transcription initiation, transcriptional runon experiments were performed on nuclei isolated from cells harboring wt or E1 mutant BPV-1 genomes (Fig. 3). BPV-1 specific runon transcripts were quantitated on the basis of the extent of hybridization of the radiolabeled runon RNA to fixed amounts of immobilized BPV-1 DNA (Fig. 3). The relative level of BPV-1 RNA synthesis in the E1 mutant-transfected cells (30% of the viral specific hybridization measured in wt BPV-1-transfected cells) was normalized to the genome copy number. The cells transfected with the E1 mutated DNAs were found to synthesize sixfold more BPV-1-specific RNA per genome than the wt BPV-1 DNA-transfected cells. This result suggested that the increased levels of steady-state BPV-1 RNA observed by the RNA slot blot analysis was the result of increased rates of transcription initiation.

Analysis of viral promoter utilization in E1 mutants. These initial observations led us to look at the relative abundance of RNAs mapping to specific BPV-1 promoters. Total cellular RNAs from wt BPV-1 and E1 mutant BPV-1-transfected cells (G418 pooled colonies described above) were analyzed by quantitative primer extension analysis (Fig. 4), using oligonucleotides complementary to the BPV-1 mRNAs at locations downstream from previously mapped BPV-1 transcriptional promoters. The positions of these promoters on the physical map of BPV-1 are indicated in Fig. 1. The sites of RNA initiation were the same for the individual promoters in the cells harboring E1 mutants and in wt-transfected cells and compared favorably with previously published primer extension analysis of these promoters (1a); however, the relative abundance of the RNAs was clearly different (Fig. 4). The absolute amount of RNA mapping to the P_{89} promoter in the E1 mutant cells was equal to or greater than the amount found in the wt BPV-1 cells, even though the E1 mutant-transfected cells contained 14- to 34-fold less viral DNA than did wt BPV-1-transfected cells. In contrast, there was much less RNA mapping to the P_{2443} and P_{3080} promoters; an amount more reflective of the BPV-1 DNA copy number in the E1 mutant-transfected cells. The primer extension products for each of these BPV-1 promoters were quantitated and normalized to the BPV-1 DNA copy number in the various cell lines (Table 3). On the basis of this quantitation, there was 15- to 35-fold more P_{89} RNA per genome copy in cells harboring E1 mutated DNAs compared with those containing wt BPV-1 DNA. A smaller but reproducible, two- to sixfold, increase in the relative abundance of P_{2443} RNA was observed in the E1 mutants compared with that of wt BPV-1, whereas, there was no detectable change

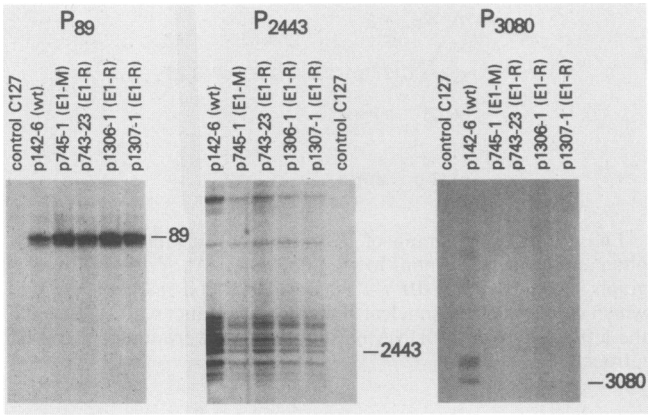

FIG. 4. Primer extension analysis performed as described in Materials and Methods, using BPV-1-specific oligonucleotides. The three panels are autoradiographs showing the virus-specific primer extension products which map to the P_{89}, P_{2443}, and P_{3080} promoters, respectively. The major 5′ end for each set of primer extension products is indicated. Lanes are labeled to indicate the BPV-1 plasmid harbored in the C127 cell populations from which total cellular RNA was isolated. The lanes labeled control C127 contain RNA from G418-resistant C127 populations which were transfected with pMMTneo only.

in the relative abundance of P_{3080} RNA for the E1 mutants. The levels of RNA mapping to the P_{7185} and P_{7940} promoters were too low to quantitate accurately (data not shown).

On the basis of the primer extension analysis, all of the E1 mutated BPV-1 DNAs were found to have increased steady-state levels of RNA initiating from the P_{89} promoter. This specific induction in viral RNA levels could explain the increased transformation efficiency of E1 mutants in anchorage-independence assays. BPV-1-specific mRNA and cDNA analyses have previously established that P_{89} is responsible for the transcription of the 5′ E6 and E7 ORFs (30). The E6 ORF has been shown to encode a transforming gene (22, 30), and mutational analysis has shown that expression of this region is required for anchorage independence and efficient tumorigenicity of C127 cells (21; D. DiMaio, personal communication). Thus, one plausible explanation is that the induction of the P_{89} promoter in E1 mutants leads to increased expression of the E6 transforming gene and this results in high-efficiency growth in soft agar.

E1 mutants exhibit increased E2 transactivation. The P_{89} promoter is the major E2-responsive promoter in BPV-1. In addition, the P_{2443} promoter has also been shown to be an E2-responsive promoter (9a). The E2 ORF encodes three gene products which are expressed in BPV-1-transformed cells (10a). On the basis of recent genetic analysis (P. F. Lambert, N. Hubbert, E. Androphy, P. M. Howley, and J. Schiller, manuscript in preparation), these proteins have been identified as the full-length E2 transactivator (25, 31), the E2 repressor (12), and the E8/E2 ORF fusion product which represents a second repressor (11; M. Botchan, personal communication; P. F. Lambert and P. M. Howley, manuscript in preparation). Activation of P_{89} by the E2 transactivator is mediated through the BPV-1 E2-responsive enhancer elements, E2RE, which are located just upstream of the P_{7940} and P_{89} promoters within the long control region (LCR) (9, 24). E2 transactivation is mediated through the direct binding of the E2 transactivator to specific DNA sequences located within the E2-responsive element (1, 17a). The E2 repressor inhibits transactivation, presumably by competing for binding at the E2 DNA-binding sites within the enhancer (17) or by formation of mixed heterodimers with the E2 transactivator (A. McBride, J. Bryne, and P. M. Howley, submitted for publication). Thus, the relative concentration of the E2 transactivator and repressor proteins determine, at least in part, the activity of the responsive promoters. The induction of P_{89}, and to a lesser extent P_{2443}, in cells transfected wit E1 mutated BPV-1 DNAs raised the question of whether the E2 transcriptional regulatory circuit is perturbed in cells harboring the E1 mutated DNAs.

E2 transactivation was measured in the same pooled G418-resistant C127 cells which were used for the DNA and RNA quantitative studies described above (Fig. 1 to 4). These cells were transiently transfected with various plasmids carrying the bacterial reporter gene, CAT, under eucaryotic transcriptional control. The expression of CAT was measured by using p407-1, a CAT expression plasmid containing the BPV-1 LCR positioned upstream of the enhancer-deleted simian virus 40 SV40 early promoter (25). Transcription of the CAT gene initiating from the SV40 promoter in p407-1 is dependent on the BPV-1 E2 transactivator protein (25) and can be repressed by the E2 repressor protein (12). The E1 mutant cell lines expressed fivefold higher E2-dependent CAT activities than did the wt BPV-1 cell line when transfected with the p407-1 (Fig. 5). A plasmid, $pE2RE_1i\Delta3$, similar to p407-1 but containing a subregion of the BPV-1 LCR containing only the E2 binding sites present within E2-responsive element 1 (23), was also fivefold more active in transfected cells with the E1 mutated DNAs (Fig. 5), indicating that increased activity was due to increased levels of E2 and not to *trans*-activation of an alternative *cis* element within the LCR. The levels of CAT activity expressed from the plasmid, pRSVCAT, which contains the Rous sarcoma virus LTR promoter positioned upstream of the CAT gene (5), served to control for slight differences in transfection frequency between cell lines (which were minimal based on this analysis, see Fig. 5). In addition to this analysis, similar increases in E2 transactivation were observed with clonal cell lines derived from single foci transfected with E1 mutant BPV-1 compared with wt BPV-1 clones (data not shown). Thus, the increased levels of RNA mapping to the E2-inducible promoters correlated with increased levels of E2 transactivation in E1 mutant-transformed cell lines.

To determine whether the E2 proteins were mediating the transcriptional defect observed with the E1 mutated genomes, we examined the effect of altering the level of E2 transactivation on the viral promoter activity. This was tested by two approaches. First, a double mutant (p1308-1; shown in Fig. 1) containing a TTL insertion in the E2 ORF as well as in the E1 ORF was tested for efficiency of growth in

TABLE 3. Relative abundance of specific BPV-1 promoter RNAs per viral genome copy in transfected C127 cells

DNA(s) transfected	Amt of RNA mapping to indicated promoter[a]		
	P_{89}	P_{2443}	P_{3080}
p142-6	1.0	1.0	1.0
p745-1	22	3.3	1.0
p743-23	15	5.1	0.63
p1306-1	35	2.5	1.7
p1307-1	15	6.0	0.9

[a] Values represent the relative integrated peak area obtained by densitometric scanning of autoradiographs shown in Fig. 4, after correction for differences in copy number for cell populations as provided in Fig. 2C.

Plasmid	Structure	% acetylated CAM		
		pMMTneo	pMMTneo + p142-6	pMMTneo + p743-23
pRSVCAT	RSV CAT	51	64	72
pA$_{10}$CAT	SV40 CAT	1.1	0.53	2.6
p407-1	BPV-1 SV40 CAT	3.5	13 (0.20)	69 (0.96)
pE2RE$_1$iΔ3	BPV-1 SV40 CAT	2.2	7	41

FIG. 5. CAT activity. Schematics represent the genetic elements present in each of the three indicated CAT plasmids. The box labeled CAT represents the position of the bacterial CAT gene. The box labeled RSV indicates the position of the Rous sarcoma virus LTR upstream of the CAT gene. The SV40 box represents the position of the SV40 early promoter which has been deleted for its enhancer, while the BPV-1 box in p407-1 represents the position of the BPV-1 LCR containing the BPV-1 sequences from nts 6958 to 7945. In pE2RE$_1$iΔ3, the BPV-1 box represents the ACCN$_6$GGT motifs present in E2RE$_1$ and includes BPV-1 sequences from nts 7611 to 7646 and nts 7753 to 7806 (23). The columns to the right display the level of CAT activity obtained from extracts of G418-resistant colonies obtained from transfection with the indicated DNAs. The fraction of the activity of p407-1 to that of pRSVCAT is indicated in parentheses.

soft agar and for levels of BPV-1 RNA. Cells transfected with this doubly mutated DNA were very inefficient for growth in soft agar (Table 4) and contained very low concentrations of viral RNA (data not shown). These results are similar to those observed for the E2 mutant itself (p772-1) (Table 4) (19). Thus, the E1 mutation does not result in high levels of E2-independent activation of the viral promoters. Second, an E1 mutant, p743-23, was cotransfected with the cDNA, pCW1-28 which expresses the E2 repressor under heterologous control (12), in an attempt to alter the ratios of E2 regulatory proteins in the E1 mutant cells. This led to decreased efficiency of growth in soft agar (Table 4) and to decreased levels of P_{89} RNA (Fig. 6). Thus, abolishment or perturbation of the E2 transcriptional regulatory circuit altered the transformation and transcription phenotype of the E1 mutated genomes. These results are consistent with the premise that the E1 mutant phenotypes are mediated through changes in the E2 regulatory circuit.

DISCUSSION

Replication-defective BPV-1 E1 mutants, with TTL linkers inserted at different positions throughout the E1 ORF, all exhibit the same phenotype, with high transformation efficiencies and increased levels of viral transcription per genome. Transcriptional runon experiments demonstrated an

TABLE 4. Role of E2 regulation on E1 mutant phenotype: anchorage independence

Plasmid(s)	Mutation(s)	ORF affected	No. of colonies/0.5 μg DNA
p772-1	TTL at nt 2878	E2	15
p1308-1	TTL at nt 1299/2878	E1/E2	19
p743-23	TTL at nt 1299	E1	293
p743-23 + pCW1-28	TTL at nt 1299	E1	32
No DNA			3

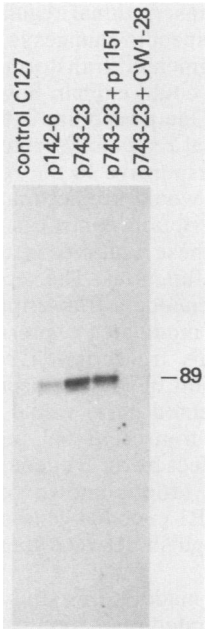

FIG. 6. Primer extension analysis of total cellular RNA from various cell populations for P_{89}-specific viral RNAs. Lanes are labeled for the plasmids present in cells which were cotransfected with pMMTneo. The plasmid p1151 is analogous to pCW1-28 cDNA except that the cDNA insert has been deleted.

increased rate of viral RNA synthesis which accounted for the increased level of steady-state RNA seen in cells harboring these E1 mutated DNAs. On the basis of primer extension analysis, a 15- to 35-fold increased level of viral RNA from the P_{89} promoter was detected for the E2 mutant genomes. This promoter is a major E2-responsive promoter and is responsible for the transcription of the 5′ transforming function mapping to E6 and E7. Induction of expression of the 5′ transforming function could be sufficient to explain the increased efficiency of growth in soft agar by the E1 mutants. Correlation between growth in soft agar and E6 and E7 expression has been recently established (D. DiMaio, personal communication). An increased level of RNA initiating from a second E2-responsive promoter, P_{2443}, was also detected. This promoter is believed to be responsible for the expression of the E2 transactivator (31) and is likely to be the principle promoter for the E5 oncoprotein (D. DiMaio, personal communication). An increase in E5 protein, as predicted by P_{2443} induction, could account for the efficient focus induction by E1 mutants, an assay which has been recently shown to reflect E5 protein levels (D. DiMaio, personal communication). Thus, there is evidence at the mRNA level that expression of both E6 and E5 transforming protein may be increased in E1 mutants. Increased levels of E2 transactivation in cells harboring the E1 mutants correlated with increased ratios of P_{2443} to P_{3080} RNAs. On the basis of the utilization of these promoters for the expression of the E2 transactivator and E2 repressor, respectively, this change in promoter use would be predicted to change the relative abundance of the E2 proteins in favor of increased transactivation. Disruption of the E2 transcriptional regulatory circuit was dominant over the E1 transcriptional and transformation phenotypes, suggesting that viral transcription in E1 mutants was still dependent on the E2 transcriptional regulatory proteins. From these results, we concluded that the E1 replication-defective mutants studied were al-

tered in their E2 transcriptional regulatory circuit and that this correlated with specific changes in viral gene expression which led to the augmented transformation phenotype.

Two mechanisms could explain how E1 mutations affect viral transcription. One possibility is that the expression of the E2 transcriptional regulatory proteins or their activities is itself somehow responsive to the replicative state of the virus genome. This would predict that the effect of the E1 mutations on transcription control is indirect and that the primary defect in these mutants is the disruption of E1-encoded replication functions. The second possibility is that the E1 ORF could encode a transcriptional repressor function which directly modulates expression of the viral promoters. Loss of this transcription repressor would then result in the induction of viral transcription, which, based upon the data presented here, would have to result in the perturbation in E2 transcriptional regulation. This model proposes a direct effect by an E1 gene product on transcription. Unfortunately, little is known yet about the structure and function of the E1-encoded gene products, and we can, therefore, not distinguish between these two possibilities at this time.

The observations made in this study may provide further evidence for coordinated regulation between viral transcription and viral replication. Previous studies have implicated a role for the E2 transcriptional control circuit in viral DNA plasmid replication by demonstrating that BPV-1 E2 mutants are replication defective (3, 4, 8, 19). A mechanistic link between E2 transcriptional regulation and viral DNA replication was provided by the mapping of an E2-responsive element, $E2RE_2$, to the region containing the P_{7185} and P_L promoters (24). These promoters are located in the vicinity of the origin of DNA replication (29) and a plasmid maintenance sequence (13). Furthermore, P_{7185} has been postulated to be responsible for the expression of E1 functions (26). Thus, stable plasmid replication may be affected by the E2 transcriptional regulatory proteins either directly, possibly through the modulation of DNA replication initiation, or indirectly, through the control of E1 gene expression. In this study, the effects of mutations in the E1 replication genes on viral transcription were studied. Transcription was altered with marked induction of the P_{89} promoter and a lesser induction of the P_{2443} promoter. Altered transcription of these promoters correlated with perturbation of the E2 transcriptional regulatory circuit, suggesting that the replicative state of viral genome could affect viral transcriptional regulation. Thus, the experimental evidence suggests that there may be a replication control of transcription in addition to transcriptional control of replication.

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