JOURNAL OF VIROLOGY, Apr. 1989, p. 1775–1782
0022-538X/89/041775-08$02.00/0

Vol. 63, No. 4

Identification of Bovine Papillomavirus E1 Mutants with Increased Transforming and Transcriptional Activity

JOHN T. SCHILLER,[1]* EVA KLEINER,[2] ELLIOT J. ANDROPHY,[1] DOUGLAS R. LOWY,[1] AND HERBERT PFISTER[2]

Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, Maryland 20892,[1] and Institut fur Klinische und Molekulare Virologie, Friedrich Alexander Universitat, Erlangen-Nurnberg, D-8520, Erlangen, Federal Republic of Germany[2]

Received 12 December 1988/Accepted 4 January 1989

The E1 open reading frame of bovine papillomavirus type 1 (BPV) has been shown previously to encode *trans*-acting functions, M and R, that are involved in extrachromosomal replication of the viral genome. We have determined that several E1 mutants mapping in both the M and R regions and a single mutant of the upstream regulatory region have a higher transforming activity on mouse C127 cells than the wild-type genome does. A representative mutant in M, a mutant in R, and the upstream regulatory region mutant were complemented in *trans* by the wild-type genome, but the two E1 mutants did not complement each other, suggesting that they affect the same inhibitory function. A long terminal repeat-activated clone constructed to express the intact E1 open reading frame reversed the high-transformation phenotype of the mutants. In contrast to the high-copy-number autonomous replication of the wild-type genome, the genomes of the E1 mutants were, as previously described for other E1 mutants, integrated at lower copy numbers in the transformed cells. Relative to the viral genome copy number, both the E1 M and R mutant transformed cells contained an average of 10-fold more BPV-specific transcripts than did the wild-type transformed cells. Cycloheximide treatment of the cells transformed by the E1 mutants did not lead to the rapid 10-fold increase in the accumulation of viral transcripts observed with the wild-type genome. These results suggest either that integration of the BPV genome makes it unresponsive to a labile repressor or that an E1 gene product, containing both M and R sequences, is a repressor of BPV transcription.

In cultured cells transformed by the cloned bovine papillomavirus type 1 (BPV) genome (Fig. 1), the viral DNA is maintained as an autonomous nuclear replicon with a copy number of approximately 100 to 500 (17). The early region of the genome, which contains the open reading frames (ORFs) E1 through E8, is transcribed at low levels in these cells (2, 12, 34). *cis*-Acting signals involved in promotion and enhancement of early gene transcription and genome replication are located in the upstream regulatory region (URR, also called the long control region) that immediately precedes the early genes (Fig. 1). Transcription from the URR can be activated by a gene product of the full-length BPV E2 ORF and inhibited by a product of the carboxy-terminal half of the ORF (16, 33). Other studies have shown that viral gene expression is negatively regulated by a labile factor that represses BPV transcription, since treatment of BPV-transformed cells with the protein synthesis inhibitor cycloheximide leads to a severalfold increase in viral transcriptional activity (2, 14). However, it is not known whether the labile inhibitory factor is encoded by the E2 ORF, another viral ORF, or a cellular gene.

E1 is the largest and most conserved early papillomavirus ORF (nucleotides [nt] 813 through 2663). Two distinct functions encoded by the E1 ORF have been shown to be involved in the replication of the BPV genome. One, designated R, is a positive replication function which is required for autonomous replication, and the other, M, is a negative modulator function that ensures that a steady-state copy number is maintained (4, 21, 26). The R function is encoded by the 3′ portion of the E1 ORF, and the M function appears to be encoded, at least in part, by an exon from the 5′ portion of the E1 ORF. Because M and R mutants complement each other for replication when cotransfected, it is likely that M and R are separate genes. Consistent with this hypothesis, a protein corresponding to the M gene product has recently been reported (35), but no R gene product has been unambiguously identified. To date, no functions attributable to the entire E1 ORF have been described, although this ORF is intact in all papillomavirus genomes that were cloned from virions or autonomously replicating copies. We now report that BPV E1 mutants have two phenotypes, in addition to being replication defective. Mutations throughout the E1 ORF, including the M or R domains, increase the transforming activity of the BPV genome and lead to a derepression of viral transcription.

MATERIALS AND METHODS

Plasmid constructions. The generation of the *Xho*I linker insertion mutants of the full-length BPV clone pdBPV-1 has been previously reported (29). Clone pE1 was constructed by linking the 1.15-kilobase-pair (kb) *Eco*RI-to-*Aat*II fragment of pM15 (containing the Moloney murine sarcoma virus long terminal repeat plus 0.55 kb of downstream sequence including the viral donor splice site) (7) to the 2.2-kb *Aat*II (nt 769)-to-*Xho*I (nt 2970) fragment of the BPV *Xho*I linker mutant 112. This ligation product was inserted into the 3.0-kb *Eco*RI-to-*Xho*I fragment BPV *Xho*I linker mutant 133 which contains the pML2d sequences and the BPV sequences between the linker insert at nt 4040 and the *Bam*HI site at nt 4450 (Fig. 1). Clones pE1-760 and pE1-783 were generated by replacing the *Aat*II-to-*Bst*EII fragment of pE1 (BPV nt 769 through 2405) with the corresponding fragment of *Xho*I linker insertion mutants 760 and 783 (in which the 8-bp linker was inserted between nt 1019 and 1051

* Corresponding author.

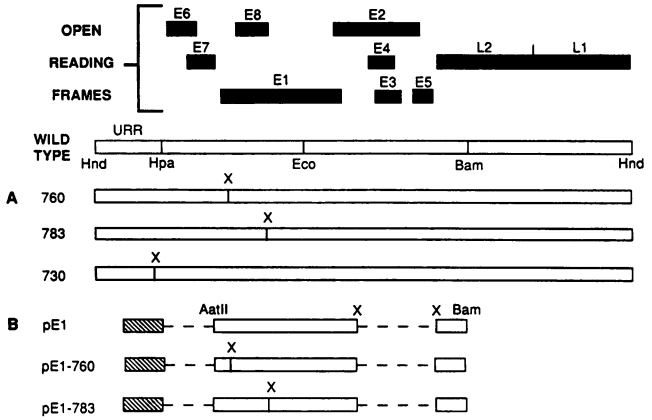

FIG. 1. Full-length BPV-1 *Xho*I linker insertion mutants and subgenomic clones. The genome of BPV-1 linearized at the unique *Hin*dIII site in which it was cloned into the pBR322 derivative pML2d (pd BPV-1 [27]) is shown. The linked pML2d is not shown. The locations of the ORFs are indicated above the genome. The early ORFs are designated E1 through E8, and the late ORFs are designated L1 and L2. □, BPV sequence included in the clone; ----, deleted sequence; ×, site of *Xho*I linker insertion; Bam, *Bam*HI recognition site; Hnd, *Hin*dIII recognition site; Hpa, *Hpa*I recognition site; Eco, *Eco*RI recognition sites. (A) Linker insertion mutants of the full-length genome. (B) E1 expression vectors.

and between nt 1610 and 1626, respectively). Both mutations introduce frameshifts into the E1 ORF.

Cells and DNA transfection assay. C127 cells and the calcium chloride transfection procedure have been described previously (18). Except where noted, 0.5 μg of intact plasmid DNA was used per dish. In cotransfection experiments with the E1 expression vectors, 0.5 μg of the full-length BPV genome and 0.5 μg of the expression vector were used. In transfection involving selection for G418 resistance, 0.1 μg of the plasmid pGV16 (13), which expresses the bacterial neomycin resistance gene from a retroviral long terminal repeat, was included and the cells were grown for 10 days in 1 mg of G418 per ml. To assay growth in soft agar, cells from transfected cultures were harvested on day 9 and 10^5 cells were plated in 0.4% Noble agar (Difco Laboratories, Detroit, Mich.).

Southern blot analysis. Total cellular DNA was prepared by the protocol of Ebeling et al. (9) with slight modifications. DNA samples of 10 μg were digested with restriction enzymes (Boehringer Mannheim Biochemicals, Indianapolis, Ind., and Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and electrophoresed on 1% agarose gels. Blotting analysis was performed by the protocol of Southern (32). Hybridization to ^{32}P-nick-translated full-length pdBPV1 DNA was carried out as described previously (10).

Northern (RNA) blot analysis. Cells from confluent monolayers were collected by trypsinization, and total RNA was prepared by the guanidium thiocyanate method (6). RNA samples (30 μg) were denatured in 50% formamide–2.2 M formaldehyde in MOPS (morpholinepropanesulfonic acid) buffer (400 mM MOPS [pH 7.0], 100 mM sodium acetate, 10 mM EDTA). After heating for 10 min at 65°C, samples were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde in MOPS buffer. Transfer of RNA to nitrocellulose filters was performed in 20× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate). Filters were stained with methylene blue (22) to quantitate the amount of total RNA bound to the nitrocellulose. Filter-bound RNA was hybridized with ^{32}P-labeled pdBPV1 probes (5×10^4 cpm/cm^2) for 48 h in the presence of 50% formamide–5× SSC–50 mM PIPES [piperazine-*N*-*N*′-bis(2-ethanesulfonic acid)] (pH 6.5)–5× Denhardt solution–0.1 mg of yeast RNA per ml. Autoradiography was on Kodak X-AR5 film at −70°C, with intensifying screens. Viral mRNAs were quantitated by scanning the autoradiographs in a DD2 densitometer joined to a BD5 micrograph (Kipp and Zonen Delft BV, Delft, The Netherlands).

In vitro transcription in isolated nuclei. Nuclear run-on transcription assays were performed basically as described by Greenberg and Ziff (11). Cells from four confluent 150-cm^2 culture flasks were harvested by trypsinization. After centrifugation at 500 × *g* for 5 min, the cell pellet (4×10^7 cells) was suspended in 5 ml of Nonidet P-40 lysis buffer (10 mM Tris hydrochloride [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% [vol/vol] Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride), incubated for 5 min on ice, and centrifuged at 500 × *g* for 5 min. The nuclear pellet was washed once with 5 ml of Nonidet P-40 lysis buffer and centrifuged again. The nuclei were suspended in 50 mM Tris hydrochloride (pH 8.3)–40% (vol/vol) glycerol–5 mM $MgCl_2$–0.1 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride at a concentration of 10^7 nuclei per 100 μl and frozen in liquid N_2. For the run-on transcription assay, 400 μl of the nuclear suspension were thawed and mixed with 50 μl of 10× run-on buffer (50 mM Tris hydrochloride [pH 8.0], 25 mM $MgCl_2$, 1.5 M KCl, 2.5 mM ATP, GTP, and CTP) and 50 μl of ^{32}P-labeled UTP (500 μCi, 3,000 Ci/mmol). After incubation for 30 min at 30°C, the nuclei were immediately lysed by pipetting with a 10-ml pipette after the addition of 6 ml of guanidium thiocyanate buffer (5 M guanidium thiocyanate, 50 mM lithium citrate, 0.1% lithium lauryl sulfate, 0.1 M β-mercaptoethanol) and RNA was purified as described by Chirgwin et al. (6). The run-on products were hybridized to plasmid DNA immobilized on nitrocellulose by the method of Marzluff and Huang (23) with slight modifications; the prehybridization and hybridization buffer consisted of 50% deionized formamide, 5× SSC, 1× Denhardt solution, 0.1% sodium dodecyl sulfate, 50 mM PIPES (pH 7.0), and 250 μg of *Escherichia coli* RNA per ml. After hybridization, the filters were washed twice for 15 min in 0.1% sodium dodecyl sulfate–0.1× SSC at 52°C. Filters were exposed to X-Omat film (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

Identification of BPV mutants with elevated transforming activity. To begin an analysis of the transforming activity of BPV, we generated a series of mutants of the cloned full-length BPV genome, each of which contained a single random 8-base-pair (bp) *Xho*I linker insertion (Fig. 1 and Table 1). The transforming activity of 40 mutants was tested in a focus formation assay by transfection of the mutant DNAs onto mouse C127 cells. When compared with the wild-type clone, nine of the mutants had increased transforming activity in that they induced more foci per microgram of DNA and the foci appeared sooner (Table 1) (other mutants, which had unaltered or decreased transforming activity, have been discussed previously [29]). Eight of the mutants with the high transformation phenotype mapped to the E1 ORF, and the ninth mutant mapped to a segment of the URR that immediately proceeds the early-region genes. We do not believe that the relatively high transforming activity of these mutants was due to a defect in our wild-type clone or in the preparation of its DNA, since similar results

TABLE 1. Focal transformation of C127 cells by BPV insertion mutants

Mutant	Interrupted ORF	Location (nt) of linker	Foci/0.5 μg of DNA on day:		
			9	13	21
WT[a]			9	24	81
760	E1	1019–1051[b]	153	189	309
135	E1	1350	130	163	229
137	E1	1400	125	150	206
105	E1	1400	121	164	222
347	E1	1450	100	127	193
783	E1	1610–1626[b]	99	119	167
743	E1	1700–1850	88	129	171
181	E1	1950	64	79	174
761	L2	4150	9	18	53
702	L2	4400	12	15	59
177	L1	7100	10	17	30
120	URR	7600	22	30	66
730	URR	7889–7918[b]	94	133	208

[a] WT, Wild type.
[b] Location determined by DNA sequencing. The other inserts were mapped by the relative mobility of restriction fragments after gel electrophoresis.

were obtained with several DNA preparations (data not shown), and mutants that had linker insertions in the late region of the genome (Table 1, mutants 761, 702, and 177), which is not transcriptionally active in cultured cells (12, 34), had transforming activities that were similar to that of the wild-type clone. The activating mutations in E1 are located in both the R and M domains of the ORF. A representative E1 M and E1 R mutant, 760 and 783, respectively, and the URR mutant 730 (Fig. 1A) were also tested by a more stringent transformation assay, anchorage-independent growth. As predicted from their higher focus-forming efficiency, the three mutants produced more and larger colonies in soft agar than did the wild-type clone (Fig. 2A through D).

Cotransfection experiments. When the three high-transforming mutants 760, 783, and 730 were individually cotransfected with the wild-type genome onto C127 cells, the lower-level transforming activity of the wild-type genome was observed (Table 2). These results make it unlikely that the mutant genomes synthesize altered E1 gene products that activate transformation. Rather, they suggest that the wild-type genome produces a diffusible inhibitory factor(s) that is inactivated by these mutations. When the 760 E1 M and 783 E1 R mutants were cotransfected, the high-transforming phenotype was observed (Table 2), indicating that the two mutants did not functionally complement each other in the transformation assay. Similarly, 760 did not complement the 730 URR mutation. Anchorage-independent growth assays confirmed the focal transformation results (representative results are shown in Fig. 2E through G).

E1 expression vector. To confirm that the transforming activity of the BPV genome could be inhibited by a *trans*-acting product of the E1 ORF, a long terminal repeat-activated expression vector, pE1, was constructed (Fig. 1B). In this clone, E1 and the small E8 ORF, which is entirely contained within E1, were the only intact ORFs, and the first ATG start codon of E1 was the first ATG in the cloned BPV fragment. As controls for pE1, pE1-760 and pE1-783 were constructed by introducing the 760 and 783 frameshift mutations (which do not interrupt the E8 ORF) into pE1 (Fig. 1B). When cotransfected with the full-length BPV mutants 760, 783, or 730, pE1 inhibited the transforming activity of the mutants (Table 3). We believe that this inhibition was due to the activity of an E1 protein product, since the frameshift mutations in pE1-760 and pE1-783 greatly reduced or abolished the ability of pE1 to inhibit transformation induced by 760, 783, or 730 (Table 3). Plasmid pE1 did not inhibit the transforming activity of the wild-type genome. These differences in focus formation were confirmed in assays of the cotransfected cells for growth in soft agar (the results for 760 are shown in Fig. 2H through J).

Since pE1 contains PMS-2 (19), which can function as an origin of replication when BPV replication factors are provided in *trans*, but only one of the *cis*-acting elements (the negative controllers of replication) (26) which appear to be required for the control of replication of the wild-type genome, we were concerned that pE1 might be inhibiting the transforming activity of the mutant genomes by acting as an uncontrolled replicon in the cotransfected cells and thereby killing them. To test this possibility, we examined the ability of pE1 to inhibit the formation of G418-resistant colonies by the bacterial neomycin resistance gene. When compared to the neomycin resistance plasmid alone, neither pE1 nor the two derivatives, either alone or with the BPV mutants, significantly reduced the number of G418 colonies generated in the transfection experiments (Table 3, experiment 3). From these results, we conclude that the pE1-induced inhibition of the high transforming activity of the BPV mutants is not due to a cytotoxic activity of pE1.

BPV transcription in E1 mutant-transformed cells. One possible way that E1 mutations could increase BPV transforming activity is by inducing an increase in viral transcription. To begin an examination of the relationship between E1 expression and BPV transcription, transformed foci were isolated after transfection of C127 cells with either wild-type BPV DNA or the E1 M mutant 760 or the E1 R mutant 783. The foci were expanded and analyzed for BPV DNA and RNA content. Eight 760 foci (760-2 to 760-9) and three 783 foci (783-1, 783-4, and 783-8) were compared with five wild-type BPV-induced foci (W1 through W5). Whereas W1 through W5 cells harbored approximately 500 autonomously replicating copies of the BPV genome, the lines derived from 760 and 783 foci and three mass cultures of 783-transformed cells (783-I, -II, and -III) contained viral DNA which was integrated into the host chromosome (representative results are shown in Fig. 3). The copy number of the 760 genome varied between 5 and 200, and that of the 783 varied between 50 and 140 (Table 4). RNA was extracted from the E1 mutant and wild-type transformed lines and analyzed by Northern blot hybridization with radiolabeled BPV DNA as a probe (Fig. 4). In comparison with the wild-type transformed cells, the ratio of viral RNA to viral DNA was 7.5- to 12.5-fold higher in the 760 mutant-transformed cells and 3.0- to 20-fold higher in the 783-transformed cells (Table 4). The patterns of the viral transcripts in mutant and wild-type transformed cells were similar, except for some high-molecular-weight viral RNA bands that were consistently observed in the 760 and 783 cells.

To determine whether the higher steady-state ratio of viral RNA to viral DNA detected in the E1 mutant-transformed cells was due to higher rates of viral transcription or increased stability of the viral mRNAs, nuclear run-on transcription assays were performed. As measured by this assay, the rates of transcription of the mutant genomes in lines 760-7, 783-4, and 783-III were significantly higher than the transcription rate of the wild-type genome (Fig. 5), despite the fact that they had lower numbers of BPV genomes (Table 4). Consistent with the conclusion that the higher ratio of

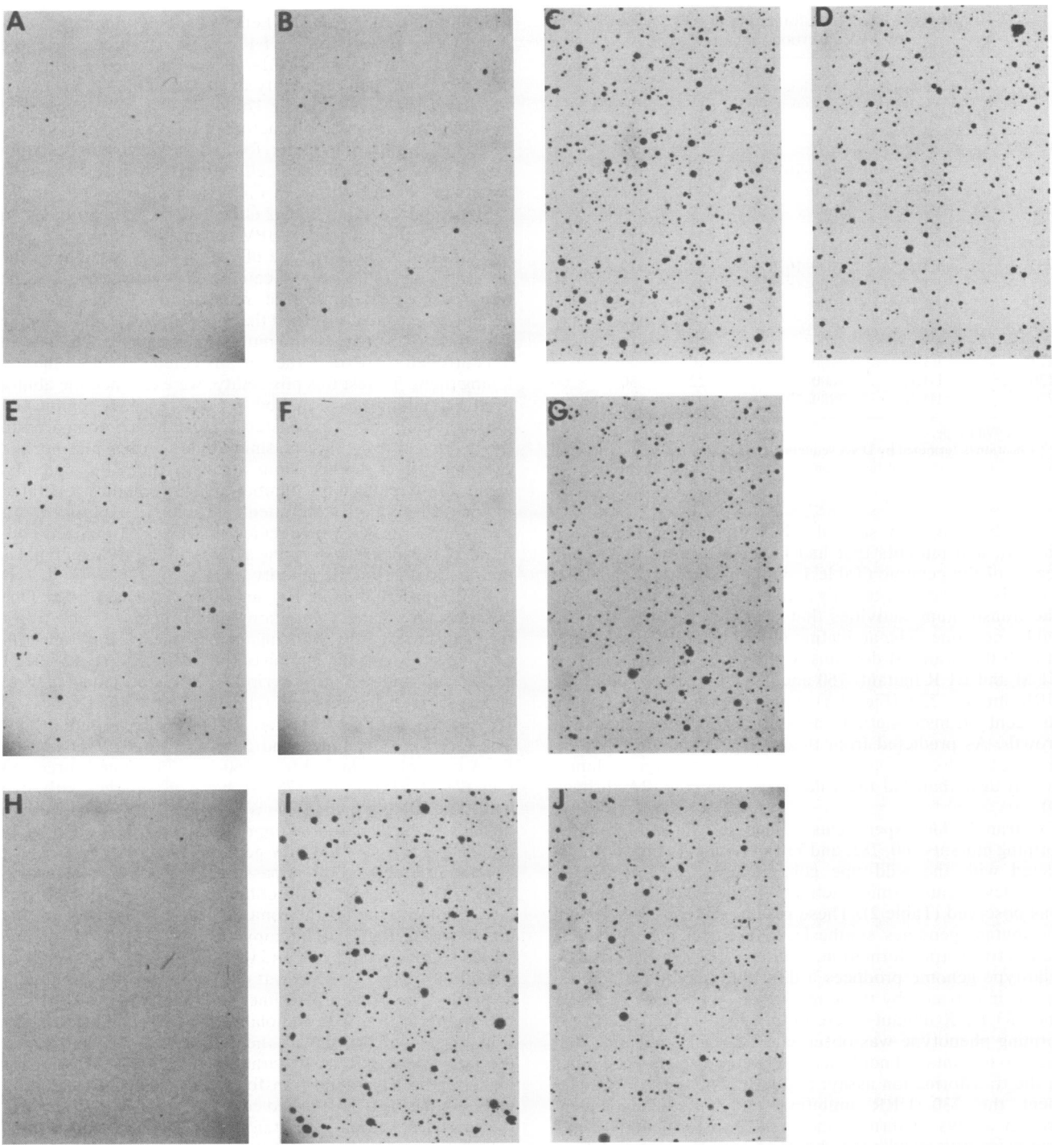

FIG. 2. Growth of transfected C127 cells in agar. Cells were photographed 2 weeks after suspension in agar. Cells: A, C127 controls; B, wild-type BPV DNA transfected; C, 760; D, 783; E, wild-type and 760 cotransfected; F, wild-type and 783; G, 760 and 783; H, 760 and pE1; I, 760 and pE1-760; J, 760 and pE1-783.

BPV mRNA to DNA in the mutant transformed cells is due to an increase in the rate of transcription, the half-lives of the BPV RNAs in the 760 and 783 lines did not differ significantly from those of the BPV RNAs in wild-type lines (data not shown).

Effect of cycloheximide treatment of E1 mutant-transformed cells. Inhibition of protein synthesis by cycloheximide stimulates an increase in BPV-specific transcription in wild-type BPV-transformed C127 cells, implying that BPV transcription is controlled in part by a labile repressor of transcription (14). Since the rate of viral transcription was higher in the lines transformed by the E1 mutants, it was of interest to determine whether the E1 mutant genomes were already derepressed or whether their transcription could still be activated by treatment with cycloheximide. Total RNA was isolated from treated and untreated cells and analyzed

TABLE 2. Complementation: wild-type (WT) and high-transformation mutants

BPV DNA[a]	Foci/0.5 μg of DNA in expt:	
	1	2
WT	38	14
760	171	68
783		60
730	236	
WT + 760	27	35
WT + 783		10
WT + 730	43	
760 + 783		143
760 + 730	138	

[a] When single DNA samples were transfected, 0.5 μg of BPV DNA was used. When two DNA samples were cotransfected, 0.25-μg samples of each DNA were mixed together in experiment 1 and 0.5-μg samples of each DNA were mixed together in experiment 2. Experiment 1 was counted 11 days and experiment 2 was counted 21 days after transfection.

by Northern blot hybridization. The levels of virus-specific RNA in mutant-transformed cell lines were only one- to threefold increased after 2 h of cycloheximide treatment, at which time W1 already showed the maximal 10-fold effect (Fig. 4 and Table 4). Continued cycloheximide treatment of clone 760-5 led to a delayed accumulation of viral RNA, generating an eightfold-increased level in 4 h. Since this pattern of induction was not seen in the other clones transformed by 760 and the kinetics of viral RNA induction differed significantly from that of wild-type BPV, this phenomenon was not investigated further.

To determine whether the modest increase in the levels of BPV RNA seen in the mutant cell lines after cycloheximide treatment was due to an increase in BPV-specific transcription or to stabilization of the mRNA, nuclear run-on experiments were performed on lines 760-7, 783-4, and 783-III. No stimulation in the rate of BPV transcription was detected after the addition of cycloheximide (Fig. 5). Therefore, the E1 mutant genomes do not appear to be under the control of a labile repressor of transcription. The observed accumulation of viral RNA in the mutant-transformed cells is consistent with the twofold RNA stabilization effect of cycloheximide described previously (14).

TABLE 3. Activity of E1 expression vector

BPV DNA	Foci/0.5 μg of DNA in expt:			No. of G418[R] isolates in expt 3
	1	2	3	
WT[a]	22	14	9	196
WT + pE1	30	12	7	106
WT + pE1-760	12	17	18	94
WT + pE1-783		32	9	144
760	330	68	189	320
760 + pE1	63	2	56	208
760 + pE1-760	366	74	177	282
760 + pE1-783		20	264	346
783		60	106	418
783 + E1		4	14	222
783 + pE1-760		21	83	234
783 + pE1-783		28	106	262
730	489			
730 + pE1	50			
730 + pE1-760	416			
pE1	0	0	0	198
pE1-760	0	0	0	234
pE1-783	0	0	0	458
Neo[R] alone	0	0	0	230

[a] WT, Wild type.

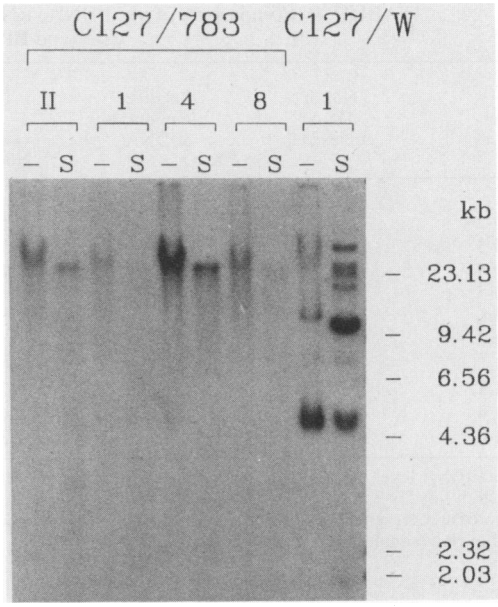

FIG. 3. Analysis of BPV genomes in stable cell lines. Southern blot analysis of total cellular DNA from four 783 lines (II, 1, 4, and 8) and a wild-type-transformed cell line (W1) is shown. –, Uncut DNA; S, *Sac*I-digested DNA (does not cleave BPV genome); linear molecular size markers are indicated on the right.

DISCUSSION

In this study, we have determined that expression of an E1 gene product inhibits the transforming activity of the BPV genome and that the ratio of viral transcripts to genome copy number in the E1 mutant-transformed cells was higher than in cells transformed by the wild-type genome. On the basis of these observations, Lambert and Howley (15) have conducted a more precise mapping of the transcripts in E1 mutants and have recently reported that, in both M and R mutants, the transcription from the P_{89} promoter, which controls the expression of the E6 transforming gene, is increased at least 15-fold and the transcription from the P_{2443} promoter, which controls the expression of the E5 transforming and the E2 *trans*-activating genes, is also increased but to a lesser extent.

At least two distinct mechanisms could account for the increased transcriptional activity of the E1 mutants. One possibility is that autonomously replicating BPV genomes are transcriptionally less active than integrated copies. The reduced transcription could be due to the differential activity of the labile repressor, detected in the cycloheximide induction experiment, on autonomously replicating as opposed to integrated genomes. If this were the case, M and R E1 mutants would be expected to have the same phenotype with respect to transformation and transcription because the genomes of both classes of mutants are integrated in stably transfected cells (21). Clone pE1 would complement the E1 mutants by providing the necessary replication factor(s) in

TABLE 4. Comparison of viral DNA, RNA, and cycloheximide response of C127 cells transformed by wild-type BPV 1 or 760 or 783 mutant DNA

Cell line	No. of DNA copies/cell	Relative amount of DNA	Relative amount of mRNA after cycloheximide treatment[a]:			DNA/RNA (without cycloheximide)	Cycloheximide-induced increase (fold) in mRNA[b]	
			None	2 h	4 h		2 h	4 h
C127 W1	500	1	1	10	10	1:1	10	10
C127 760-2	20	0.04	0.5	1	1	1:12.5	2	2
C127 760-3	5	0.01	0.1	0.1	0.2	1:10		2
C127 760-4	10	0.02	0.15	0.3	0.3	1:7.5	2	2
C127 760-5	150	0.3	2	5	16	1:8.6	2–3	8
C127 760-6	100	0.2	2.5	6.5	8.5	1:12.5	2–3	3
C127 760-7	200	0.4	4	6	12	1:10	2	3
C127 783-II	50	0.1	2.0	2.4	2.4	1:20	1–2	1–2
C127 783-1	35	0.07	0.7	0.8	0.8	1:10	1–2	1–2
C127 783-4	140	0.3	1.6	2.2	3.2	1:6	1–2	2
C127 783-8	50	0.1	0.3	0.4	0.4	1:3	1–2	1–2

[a] The viral RNA level in untreated C127 W1 cells is arbitrarily set to 1. RNA levels of all cell lines, with and without cycloheximide treatment, have been compared with this level.

[b] The increase represents the increment viral RNA level after 2 and 4 h of cycloheximide treatment, compared with the viral RNA level of the cell line in the absence of cycloheximide.

trans. According to this model, pE1 has no effect on the transforming activity of the wild-type genome because the wild-type genome encodes all the functions required for autonomous replication.

We have previously reported that an amino-terminal E2 mutant of the full-length genome exhibited the wild-type increase in transcriptional activity (10-fold) after cycloheximide induction even though it was integrated into cellular DNA (14). These results could suggest that integration is not sufficient to make the BPV genome unresponsive to the labile repressor. However, the uninduced transcriptional activity of this mutant was reduced 10-fold relative to the wild-type genome, presumably because of the absence of the E2 *trans* activator. We can not rule out the possibility that this difference in basal transcriptional activity or that effects E2 might exert on cellular transcription might account for

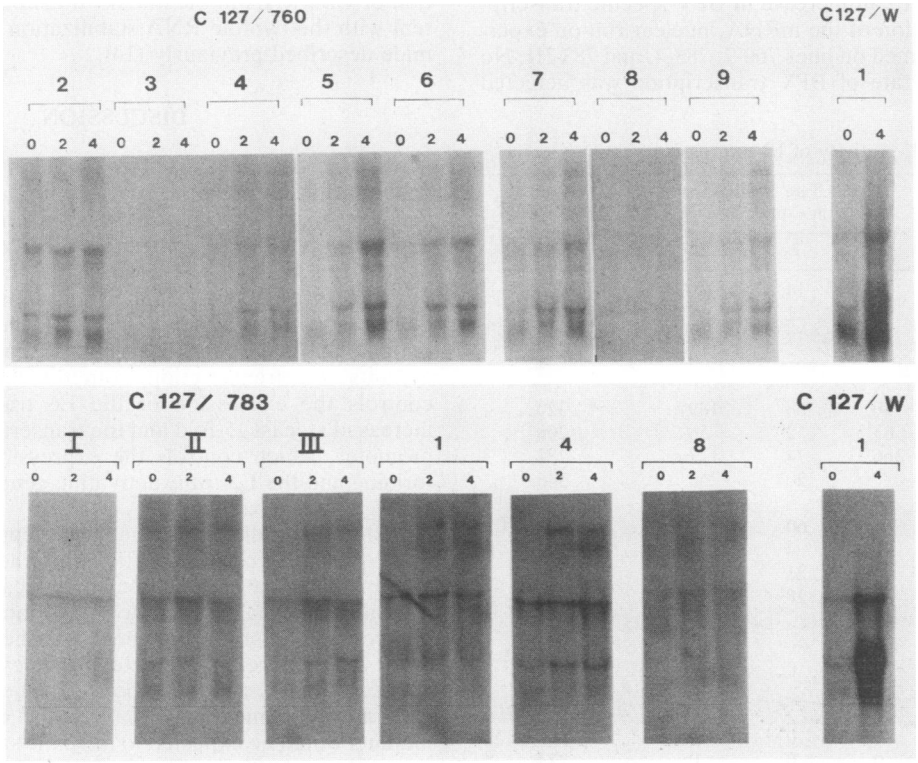

FIG. 4. Analysis of BPV-specific transcription in stable cell lines. Northern blot analysis of total cellular RNA from eight 760-transfected cell lines (760-2 to 760-9), six 783 transformed lines (I, II, III, 1, 4, and 8), and a wild-type-transformed cell line (W) is shown. RNA was extracted from untreated cells (lanes 0), after 2 (lanes 2) and 4 (lanes 4) h of cycloheximide treatment. Upon longer exposure, transcripts from clones 3, 4, and 8 were clearly visible.

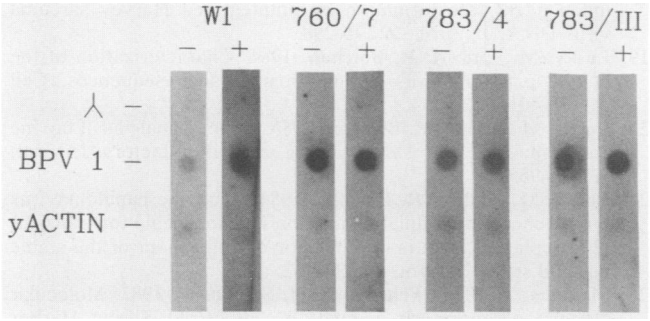

FIG. 5. Nuclear run-on transcription assay. In vitro transcription was performed with isolated nuclei from lines W1, 760-7, 783-4, and 783-III without cycloheximide (−) or after 4 h of incubation in cycloheximide (+). The ^{32}P-labeled RNA was immediately extracted and hybridized with lambda (λ), gamma-actin (γACTIN), or pdBPV-1 (BPV 1) plasmid DNA that was immobilized on nitrocellulose filters.

the different responses of integrated E2 and E1 mutants to cycloheximide.

A second possible explanation for the higher transcriptional activity of E1 mutants is that the E1 ORF encodes a third function that is the repressor, or the inducer of the repressor, of BPV transcription detected in the cycloheximide induction experiment. The observations that mutations in the M and R domains have the same effect on BPV transcription and that no complementation between the M and R mutants was detected in the transformation assays could suggest that a product containing both domains is responsible for repression. The existence of this third E1-encoded function would also offer a potential explanation for the conservation of an intact E1 ORF in all authentic full-length papillomavirus genomes that have been examined.

The results presented here do not rule out either the direct or indirect mechanism of E1-induced repression. Since vectors that allow for the autonomous replication of the BPV genome in the absence of E1 functions have not been developed, we can not determine whether autonomous replication per se is sufficient for downregulation of viral transcription. Preliminary attempts to demonstrate repression of transcription of specific BPV promoters by pE1 in transient transfection assays have been unsuccessful, so we have no evidence that an E1 gene product can directly repress viral transcription.

The only high-transforming mutation not in E1 (730) interrupted the URR, the region of the genome that is involved in the expression of the early-region genes. We initially thought that it might interrupt a site at which the E1 product exerts its inhibitory effect. However, the observation that the wild-type genome is dominant when cotransfected with 730 makes this hypothesis unlikely. We therefore suggest that this mutation may reduce the expression of the E1 gene product responsible for transformation inhibition (presumably without inhibiting the expression of the E6- or E5-transforming genes). This mutation deletes sequences between the TATA box and the transcription start site of the P_{7940} promoter, including a binding site for the BPV E2 *trans*-activating protein (1) (Fig. 6). This raises the possibility that the mRNA that encodes this E1 function may be initiated from the P_{7940} promoter and, because of the close proximity of the binding site and the TATA box, may be negatively regulated by E2.

BPV E1 mutants generated by other investigators were,

```
   CCAAT                TATA
GAGCCAATCAAAATGCAGCATTATATTTTAAGCTCACCGAAACCGGTAAGTAAAGACTAT
7860

              **   **          CCAAT
GTATTTTTTCCCAGTGAATAATTGTTGTTAACAATAATCACACCATCACCGTTTTTTCAA
7920

                          TATA                              *
GCGGGAAAAAATAGCCACGTAACTATAAAAAGCTGCTGACAGACCCCGGTTTTCACATGG
35                                                          94
```

FIG. 6. Location of the 730 insertion-deletion mutation relative to the P_{7940} and P_{89} promoters. The sequence of BPV between nt 7860 and 94 is shown. Sequences similar to the CCAAT and TATA box consensus sequences are indicated. *, Transcription start site (2). The sequences deleted in the 730 mutation are underlined, and the broken line above the sequence indicates the consensus E2 protein-binding site.

with the exception of one R mutant, not reported to have increased transforming activity (20, 28). In fact, M mutants had a reduced transforming activity (21). The mutations in many of these mutants are interspersed with those in our mutants, so it is likely that at least some of them interrupt the same E1 function(s). We believe that it is possible that differences in transfection procedures or growth conditions may account for the differences in results. Using our protocol, we have also found some differences in the activities of other BPV genes. In contrast to other laboratories, we have found in our assays that mutations in the E2 ORF have little effect on the transforming activity of the full-length genome and that E6, rather than E5, is the major contributor to transformation of C127 cells (J. T. Schiller and D. R. Lowy, unpublished observations). Using an independently generated E1 mutant, Lambert and Howley (15) have also observed increased transforming activity relative to the wild-type genome.

Given the conservation of the papillomavirus E1 ORF, the observation that BPV E1 mutants possess increased transcriptional and transforming activity may be relevant to some aspects of genital infections in humans by human papillomaviruses (HPVs). In benign papillomas, the viral genomes are usually maintained as high-copy-number autonomous replicons, while the papillomavirus genomes in most genital carcinomas and carcinoma-derived cell lines are integrated into the chromosomal DNA (3, 5, 8, 25). This pattern may arise because as the tumors progress they become nonpermissive for autonomous replication of the viral genome. However, this explanation would not account for the observation that the E1 ORF is frequently interrupted in the carcinoma cells (24, 30, 31). Our results suggest that E1 may be preferentially interrupted in HPV-induced tumors because the loss of E1 function promotes their progression by derepressing the expression of the HPV genome. Consistent with this hypothesis is the observation that HPV transcription in carcinoma cell lines cannot be induced by cycloheximide (14). It will be interesting to determine whether an HPV E1-expressing clone can inhibit HPV transcription and/or tumorigenicity when introduced into established human cell lines that contain integrated HPV genomes in which this ORF has been disrupted.

ACKNOWLEDGMENTS

We thank William C. Vass for excellent technical assistance. This work was supported in part by Wilhelm Sander Stistung.

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