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Genetic Analysis of Bovine Papillomavirus Type 1 *trans*-Acting Replication Factors

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The establishment of bovine papillomavirus type 1 in somatic mammalian cells is mediated by extrachromosomal replication and stable maintenance of the viral genome as a multicopy nuclear plasmid. Previous studies indicated the requirement of viral gene expression for bovine papillomavirus type 1 replication and plasmid maintenance (M. Lusky and M. R. Botchan, Cell 36:391–401, 1984; Turek et al., Proc. Natl. Acad. Sci. U.S.A. 79:7914–7918, 1982). To define the viral genes which are necessary for this process, we constructed a series of specific mutations within the viral genome and assayed the resulting mutants for their ability to replicate extrachromosomally in mouse C127 cells. We report here that the bovine papillomavirus type 1 *trans*-acting replication factors were encoded by at least two distinct viral genes since the mutants fell into two complementation groups, *rep* and *cop*. Mutants (*rep*$^-$) affecting the E1 open reading frame (ORF) failed to replicate bovine papillomavirus type 1 DNA extrachromosomally and would integrate into chromosomal DNA. We suggest that this gene product is one of the factors required to specifically preclude the integration event. Mutants (*cop*$^-$) affecting the E7 ORF were maintained in the extrachromosomal state; however, the copy number of the mutant genomes was reduced 100-fold compared with that of wild-type DNA. Analysis of single-cell subclones showed that each cell contained the mutant genomes at a copy number of one to two, indicating that the *cop*$^-$ phenotype did not reflect a simple segregation defect. We propose that the gene defined by mutations in the E7 ORF played a crucial role in stably maintaining the copy number of the viral plasmid at high levels. Genomes with mutations in the *cop* and *rep* complementation groups, when cotransfected, rescued the wild-type phenotype, extrachromosomal replication with a high, stable copy number for both types of plasmids. Therefore, the gene products acted in *trans*, and the mutations were recessive to the wild-type functions. One specific *rep*$^-$ mutant showed a 30-fold-increased transformation efficiency when compared with that of the wild-type genome. In addition, morphological transformation mediated by the *cop*$^-$ mutants appeared to be unstable. These results imply that either or both of the replication functions played some role in regulating the expression of the viral transforming functions.

When bovine papillomavirus type 1 (BPV-1) or cloned viral DNA induces transformation in mouse cells in culture, the viral genome is stably maintained as a multicopy nuclear plasmid (12, 13, 17, 25, 28). The plasmid replication in these cells is believed to resemble the mode of replication of the viral genome in its natural latent state (31). Recent evidence suggests that the viral plasmid replication is synchronized with chromosomal DNA replication, allowing each plasmid molecule to replicate only once per cell cycle. These results imply that initiation events are coordinated and that each of the multiple viral replicons are independently regulated (J. Reynolds and M. Botchan, unpublished data). In addition, the stable maintenance of the viral plasmid copy number within a given cell line indicates that the viral genomes undergo faithful segregation during cell division. Thus, in a formal sense, it appears that the behavior of the papillomavirus replicon resembles that of a eucaryotic chromosome (1).

The experiments reported here initiate a genetic dissection of the BPV-1 replicon to define the viral genes which are necessary in *trans* for DNA replication and plasmid maintenance. The genome organization of BPV-1 is characterized by several overlapping transcription units (2, 11), all detectable polyadenylated RNA species being transcribed from a single strand. Furthermore, eight open reading frames (ORFs) are located on the same strand (6, 8) within that part of the viral genome (69% transforming fragment) which is expressed in cells containing BPV-1 nuclear plasmids. This region has been shown to contain all of the functions required in *cis* and in *trans* for morphological transformation and extrachromosomal replication (14). However, no BPV-1 gene product encoded by this region of the genome has been identified.

We have previously shown that two separate regions within the BPV-1 genome, PMS1 and PMS2, can in *cis* support extrachromosomal replication of the Tn*5* *neo*^{r} marker gene in C127 cells. These experiments thus defined genetically two *cis*-acting sites within the viral DNA which could allow for plasmid replication. Furthermore, such recombinant molecules are maintained as plasmids only in cells which contain resident viral genomes, indicating the requirement for some *trans*-acting viral factors (17). In addition, the analysis of specific deletion mutants within the viral genome showed that BPV-1 gene products required for morphological transformation (21, 26) are dispensable for viral plasmid maintenance (17).

To further analyze the requirement of viral *trans*-acting replication factors and to define their gene structure, we have taken advantage of a series of mutant genomes constructed in vitro. The mutant DNAs were established in mouse C127 cells by DNA transfection selecting for transformed foci and independently by cotransfection with the *neo*^{r} marker gene followed by selection for G418-resistant colonies. The phenotype of the mutants with respect to replication was analyzed by Southern blot analysis after expansion of these clones into cell lines. The experiments

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described here provided evidence for a virally encoded replication (*rep*) function, defined by mutations in the E1 ORF, and for a copy number (*cop*) function, defined by mutations in the E7 ORF. The *rep* function was required to maintain the viral genome in the extrachromosomal state, whereas the *cop* function appeared to influence the number of plasmid copies per cell. We present evidence that both functions defined different genes.

MATERIALS AND METHODS

Construction of mutations within the BPV-1 genome. The plasmid used for all final constructions was pMLBPV-5, which contains the entire BPV-1 genome inserted at its unique *Bam*HI site into a derivative of pML-1 (16), in an orientation such that direction of viral transcription occurs in the opposite direction of transcription of the plasmid-encoded amp^r gene. For the pML vector, the nucleotides between pML-1 positions 29 and 375 (*Hin*dIII-*Bam*HI) were deleted, and the *Hin*dIII site was converted into a *Bam*HI site. We refer to the pMLBPV-5 plasmid as wild-type (WT) DNA. Simple deletions were created by using pMLBPV-5; for other constructions, BPV-1 subclones were used, and after the specific mutation was obtained, the appropriate WT fragment in pMLBPV-5 was replaced with the mutant one. The positions of all mutations analyzed within the BPV-1 genome are shown in Fig. 1.

The deletion mutant *dl*Ba115 has been described elsewhere (17). The deletion affects the ORFs E2, E3, E4, and E5. *dl*211 was created by hydrolyzing pMLBPV-5 DNA with the endonuclease *Nco*I. The cleaved DNA was used to transform *Escherichia coli* HB101, thus allowing for the formation of a 211-base-pair (bp) deletion within the E2 region of the BPV-1 viral genome between positions 2878 and 3089. Similarly *dl*639 (E1, E2) resulted from a limited digestion of pMLBPV-5 DNA with the endonuclease *Sph*I to delete only the sequences between positions 1978 and 2617 and to leave intact the *Sph*I site in pML-1 at position 565 and, thus, sequences between BPV-1 position 2617 and pML-1 position 565. *dl*562 was obtained by limited *Pst*I digestion of pMLBPV-5 DNA, thus creating a 562-bp deletion (E1, E8) between BPV-1 positions 1299 and 1861.

For the first step in the construction of *dl*306, a derivative of *dl*Ba115 was used. The BPV-1 late region was deleted from *dl*Ba115 (see Fig. 7 in reference 17) by cleavage with *Hin*dIII, thus eliminating the sequences between pML-1 position 29 and BPV-1 position 6958. The resulting plasmid *dl*Ba115-1 was then restricted with the enzymes *Bgl*II and *Bcl*I (after propagating the plasmid DNA in an *E. coli* dam^- strain), thus deleting a 306-bp fragment in E1 between BPV-1 positions 1515 and 1811. Since both enzymes have identical cohesive ends, the DNA, upon cleavage, needed no further treatment before transformation into *E. coli*. The resulting plasmid *dl*Ba115-1Δ*Bgl*II-*Bcl*I was then restricted with the nucleases *Hin*dIII and *Eco*RI, and this fragment containing the 306-bp deletion was inserted into pMLBPV-5 DNA after removal of the WT fragment.

The mutants i1515-1 and i1515-2 were created by using *dl*Ba115-1 (see above) as starting material. At the *Bgl*II site at BPV-1 position 1515, a 237-bp simian virus 40 (SV40) fragment from SV40 positions 2533 (*Bam*HI) to 2770 (*Bcl*) was inserted. Both orientations of the inserted SV40 fragment were obtained. From both plasmids, the *Hin*dIII-*Eco*RI fragments spanning the region containing the insertion were gel purified and inserted into pMLBPV-5 upon removal of the WT *Hin*dIII-*Eco*RI fragment, thus creating the mutants i1515-1 and i1515-2 that both affect the E1 ORF.

For the creation of the E1 mutants i2113-1 and i2113-2, pMLBPV-5 DNA was partially hydrolyzed with *Eco*RI to yield linear plasmid DNA. Mutant i2113-1 was obtained by inserting the 311-bp *Eco*RII-G of SV40 at BPV-1 position 2113, the *Eco*RII sites of this fragment being converted into *Eco*RI sites (20). Mutant i2113-2 resulted by repairing the 5′ protruding ends generated by the enzyme *Eco*RI with DNA polymerase I (Klenow), and the resulting plasmids were screened for resistance to *Eco*RI cleavage at this position. The E1 mutants i2405-5, i2405-12, and i2405-24 were obtained by linearizing pMLBPV-5 DNA at its unique *Bst*EII site (BPV-1 position 2405). The 5′ protruding ends generated were then repaired with DNA polymerase I (Klenow). (The DNA sequence around the position of the three mutations is presented in Fig. 8.)

For the creation of mutants i471, *dl*576, and *dl*54, a derivative of $pMLBPV_{69T}$ (15) was used as starting material. $pMLBPV_{69T}$ was deleted for the sequences between BPV-1 position 838 and pML-1 position 971 by using the endonuclease *Nru*I, thus leaving the sequences between BPV-1 positions 6958 (*Hin*dIII) and 838 (*Nru*I) inserted in pML-1. The resulting plasmid was then subjected to limited cleavage with the enzyme *Pst*I to linearize the DNA. The 3′ protruding ends created upon *Pst*I cleavage were treated with T4 polymerase by using the conditions described by O'Farrell (22). Two different plasmids were recovered which were resistant to cleavage with *Pst*I at either BPV-1 position 471 or 576. In a third plasmid, a 54-bp deletion between the *Pst*I sites at BPV-1 positions 522 and 576 had occurred. Restriction analysis showed that no rearrangements had taken place in either of the three plasmids. From all three types of DNA, the *Hin*dIII-*Nru*I fragments were purified via gel electrophoresis and inserted into pMLBPV-5 DNA after removal of the WT fragment, thus creating the mutants i471 (E6), *dl*576, and *dl*54 (E7). DNA sequence analysis showed the mutation in i471 to be an insertion of 4 bp, whereas in *dl*576 a 4-bp deletion had occurred at this position.

DNA sequence analysis. Sequence analysis of mutant DNAs was done by the method described by Maxam and Gilbert (19) as modified by Bencini et al. (3).

Cells and DNA transfection. Mouse C127 cells (9) were maintained at low cell density in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. DNA transfections were done by the procedure of Wigler et al. (30) as described previously (16). Selection for foci and G418-resistant colonies (7) upon cotransfection with the plasmid pNEO5′ was done as previously described (17).

Isolation and analysis of cellular DNA. Total DNA from morphologically transformed or G418-resistant C127 cells was prepared by the standard modifications (18) of the procedure of Thomas et al. (27). DNA analysis by the Southern blot technique and transformation of *E. coli* HB101 with eucaryotic DNA were as previously described (17 and references therein). For all hybridizations upon Southern blot transfer, nick-translated pMLBPV-5 DNA was used.

RESULTS

Creation of BPV-1 mutants and experimental design. The deletion in mutant *dl*Ba115 encompasses all of BPV-1 coding regions E2, E3, E4, and E5 (see Fig. 1). As this mutant is fully capable of autonomous replication (17), the viral functions required in *trans* for plasmid replication must therefore be encoded within *dl*Ba115 DNA. Thus, our search for complementation groups which would define *trans*-acting genes concentrated on the region of the viral genome which is intact in *dl*Ba115. A series of mutations (deletions and

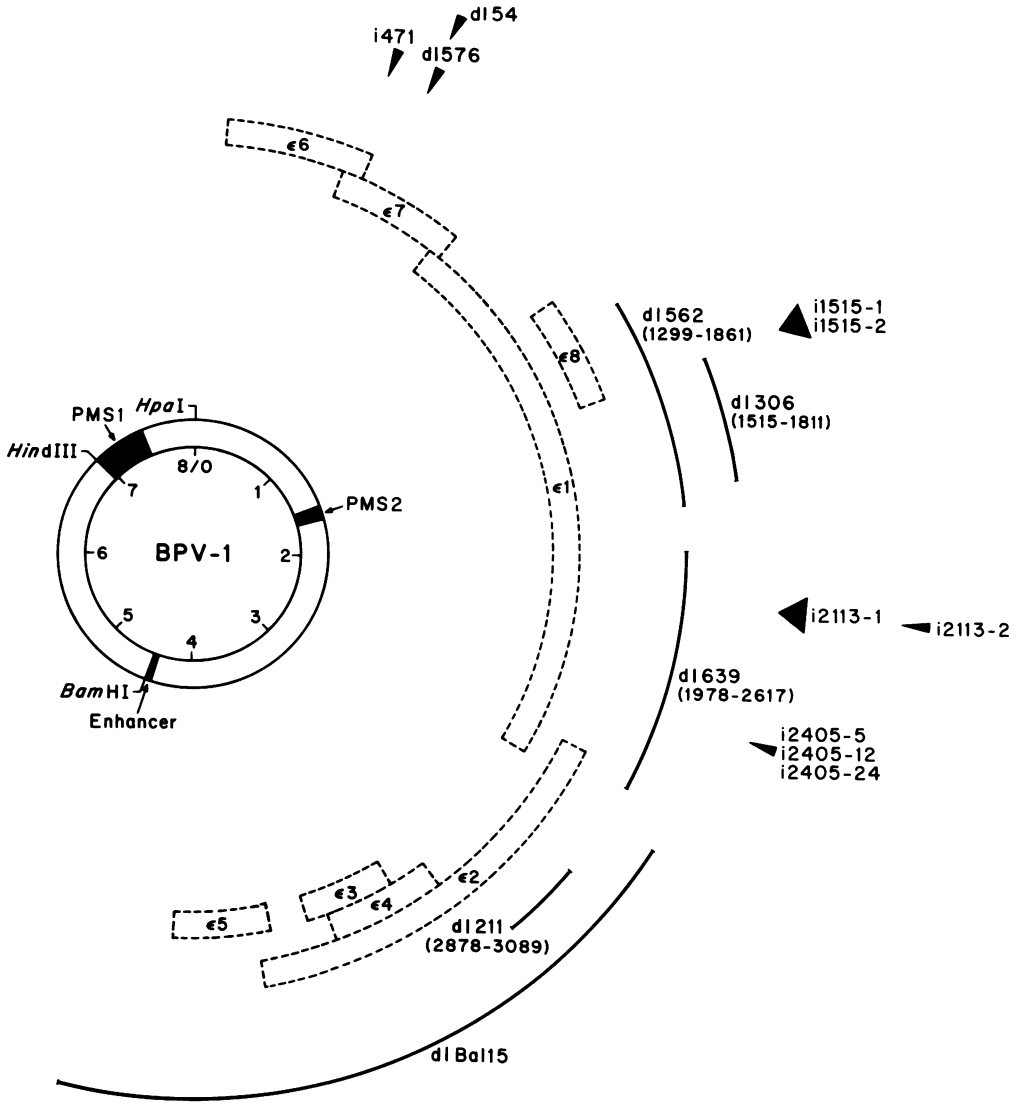

FIG. 1. Genomic organization of BPV-1 DNA and location of mutations in the viral genome. The full-length viral DNA is shown in its circular form. *cis*-acting sequences on the DNA are indicated by boxes. ORFs 1 to 8 within the 69% transforming fragment (*Hin*dIII-*Bam*HI) are indicated by open bars, from the descriptions by Chen et al. (6) and Danos et al. (8) with the corrections by Stenlund and Pettersson (personal communication). The direction of transcription is clockwise. The structure and location of deletions and insertions within the viral DNA are shown by bars and triangles, respectively. For the designation of insertion mutants, the nucleotide position has been used at which the insertion occurred. For the designation of deletion mutants, the size of the deletions has been used. Numbers in parentheses refer to the nucleotide positions marking the endpoints of the deletions.

insertions) was generated at specific positions within the ORFs E1, E6, and E7. Care was taken to generate mutants which had a lesion in only a single one of these regions, based on the hypothesis that mutations covering more than one ORF could affect more than one complementation group. One mutant which had a lesion in the E8 ORF was also generated; however, due to its location it also affected the E1 ORF (see Fig. 1).

We decided in our analysis to first test the effect of mutations upon replication in the context of the entire BPV-1 genome within pML-1 rather than with BPV-1 subclones, for example with *dl*Ba115, for the simple reason that complex interactions between different viral genes might occur. Thus, a given mutation could have a pleiotropic effect, and this would remain undetected if specific subclones of BPV-1 would be used. The construction of all mutants assayed is described in detail above. The location of the mutations within the BPV-1 genome and their nature (deletions, insertions) are shown in Fig. 1. All mutant genomes were introduced into C127 cells by DNA transfection with the focus assay. In independent experiments, C127 cells were cotransfected with the mutant plasmid DNAs, and the *neo*^{r} marker gene on pNEO5′ and G418-resistant colonies were selected. The ability of the mutant plasmids to replicate extrachromosomally could then be tested by Southern blot analysis of the DNAs from morphologically transformed and G418-resistant cell lines expanded from the initial colonies.

Mutations in the BPV-1 E1 ORF result in loss of extrachromosomal replication. The biological properties of all mutants studied are listed in Table 1 and are compared with those of WT pMLBPV-5. All mutations which map within the E1 ORF affect extrachromosomal replication. When

TABLE 1. Replication and transformation of BPV-1 mutants in C127 cells

Plasmid	Mutation	No. of foci per μg of DNA		Plasmid maintenance	Free copies per cell
		Expt 1	Expt 2		
pMLBPV-5	WT	150	135	+	100–150
i471	E6	105	98	+	60
*dl*576	E7	14	11	+	1–5
*dl*54	E7	12	7	+	1–5
i1515-1	E1	97	105	−	0
i1515-2	E1	0	0	−	0
i2113-1	E1	125	115	−	0
i2113-2	E1	109	102	−	0
i2405-5	E1	0	0	−	0
i2405-12	E1	0	0	−	0
i2405-24	E1	120	112	−	0
*dl*562 (12889–1861)	E1, E8	131	95	−	0
*dl*306 (1515–1811)	E1	>2,000	>2,000	+/−	Variable[a]
*dl*639 (1978–2617)	E1, E2	0	0	−	0
*dl*Bal15 (2720–4382)	E2, E4, E5	0	0	+	70
*dl*211 (2878–3089)	E2	1	2	−	0
*dl*211 + pNE05′[b]	E2	145	153	+	30–50

[a] The amount of extrachromosomal *dl*306 DNA in addition to integrated DNA varied between 10 and 30 copies on average from cell line to cell line.

[b] The numbers represent the G418-resistant colonies obtained by cotransfection of *dl*211 and pNE06′ DNA by a molar ratio of 10:1.

DNA obtained from either morphologically transformed cells or G418-resistant colonies was subjected to Southern blot analysis, the following results were obtained. In all cases, with the exception of mutant *dl*306 (see below), the DNA in cell lines harboring these mutant genomes was found to be stably associated with high-molecular-weight (HMW) chromosomal DNA. With each mutant, we routinely screened total DNA from four independent cell lines. Representative results of the DNA analyses are shown in Fig. 2 for mutants i1515–1 and i1515-2 and in Fig. 3 for the mutant i2405-12. When total DNA extracted from C127 cells was analyzed uncut, the hybridizing material migrated with the HMW chromosomal DNA (Fig. 2, lanes A, B, C, D, and E [uncut]; Fig. 3a, lanes A, B, and C [uncut]). In no case could supercoiled DNA be detected which migrated with form I pMLBPV-5 DNA (Fig. 2 and 3a, lanes M [uncut]). This conclusion was supported by analysis of the DNAs upon restriction with endonucleases. The hybridization pattern upon restriction with the enzyme *Hin*dIII, which cleaves once within the plasmid DNAs, is shown in Fig. 2. A characteristic and heterogeneous set of fragments for each cell DNA was obtained (Fig. 2, i1515-2, lanes C, D, and E [*Hin*dIII]). In some cases (Fig. 2, i1515-1, lanes A and B [*Hin*dIII]), the predominant hybridizing fragment comigrated with unit-length plasmid DNA (Fig. 2, lane M [*Hin*dIII]) superimposed on a heterogeneous set of hybridizing fragments. We interpret this result to have been a consequence of integration of more than one plasmid molecule in a tandem fashion. Restriction analysis of the cell DNAs (Fig. 3a) with *Bam*HI, which cleaves the plasmid DNA into its components, pML-1 and BPV-1, is shown in Fig. 3b. All three cell lines (Fig. 3b, lanes A, B, and C) showed a different set of hybridizing *Bam*HI fragments when compared with the marker DNA pMLBPV-5 (lanes M). These experiments indicate that BPV-1 genomes mutated in E1 resulted in a *rep*$^-$ phenotype. They are unable to replicate extrachromosomally and are randomly recombining with chromosomal DNA (23, 24).

An exception to this pattern was observed with mutant *dl*306. Cell lines transformed with this mutant DNA contained its DNA both associated with HMW chromosomal DNA and, in a low-molecular-weight form, as supercoiled plasmid (data not shown). The plasmids were heterogeneous in size, some migrating with the position of the input DNA, some migrating at novel positions. Surprisingly, the extrachromosomal form of *dl*306 DNA could not be detected when DNA was extracted from simple Hirt supernatants, but could only be obtained upon treatment of the Hirt supernatants with either proteinase K or RNase A during the extraction of the DNA (data not shown). In addition, plasmid rescue of E1 mutant genomes from the eucaryotic cells into *E. coli* was only successful with this particular mutant, *dl*306. We viewed the behavior of mutant *dl*306 as leaky with respect to the *rep*$^-$ phenotype.

We concluded from the analysis with all of the E1 mutants described here taken together that the *rep* function was absolutely necessary for BPV-1 plasmid replication and that, in its absence, integration of the viral DNA occurred. Of the

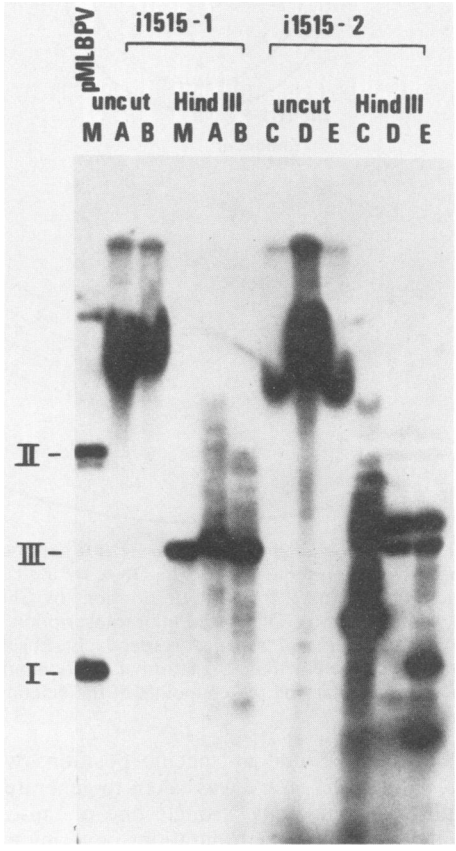

FIG. 2. Southern blot analysis of BPV-1 DNA from C127 cell lines derived upon transfection with mutants i1515-1 and i1515-2 (*rep*$^-$). The analysis for two cell lines containing i1515-1 DNA (lanes A and B) and for three cell lines containing i1515-2 DNA (lanes C, D, and E) is shown. For each lane, 10 μg of total cellular DNA was subjected to agarose gel electrophoresis (0.7%), uncut and upon cleavage with the endonuclease *Hin*dIII. Marker lanes (M) contain 500 pg of pMLBPV-5 DNA prepared from *E. coli*. The positions of the migration of supercoiled (I), nicked-circle (II), and linear (III) DNA are indicated. Hybridization was done as previously described (17) by using 1×10^6 counts of ^{32}P-labeled pMLBPV-5 DNA having a specific activity of 2×10^8 to 3×10^8 dpm/μg of DNA. Exposure of the autoradiogram was for 2 days.

10 E1 mutants described here, 9 failed to replicate the viral genome completely. With respect to mutation *dl*306, it is noteworthy that this mutation was the only in-frame deletion solely within E1, whereas all other mutations led to frameshifts. It could be possible, therefore, that in *dl*306 the E1 function was partially intact, thus leading to the phenotype described above. Complementation with E1 mutants is described below.

Mutations in the E7 ORF lead to low-copy-number plasmid maintenance. The construction of the mutant BPV-1 genomes i471, *dl*576, and *dl*54 is described in detail above. Sequence analysis of the i471 DNA showed that a 4-bp insertion had occurred at the *Pst*I site at BPV-1 position 471, resulting in a frameshift mutation affecting the extreme 3′ part of the E6 ORF. The sequence analysis of *dl*576 DNA showed the mutation to be the result of a 4-bp deletion at BPV-1 position 576, thus creating a frameshift in the E7 ORF. In mutant *dl*54, as characterized by restriction analysis, a 54-bp deletion had occurred between BPV-1 positions 522 and 576.

The phenotype of mutant i471 with respect to extrachromosomal replication and morphological transformation was identical to that of WT pMLBPV-5 (Table 1). The analysis of cDNA clones of transcripts in this region (Berg et al., manuscript in preparation; Y.-C. Yang and P. Howley, personal communication; A. Stenlund and U. Pettersson, personal communication) revealed the presence of a class of transcripts with the potential for translation of a complete E6 gene.

In contrast, the phenotype of mutants *dl*576 and *dl*54 showed a striking difference when compared with that of the WT genome. Analysis of the state of the DNA from four cell

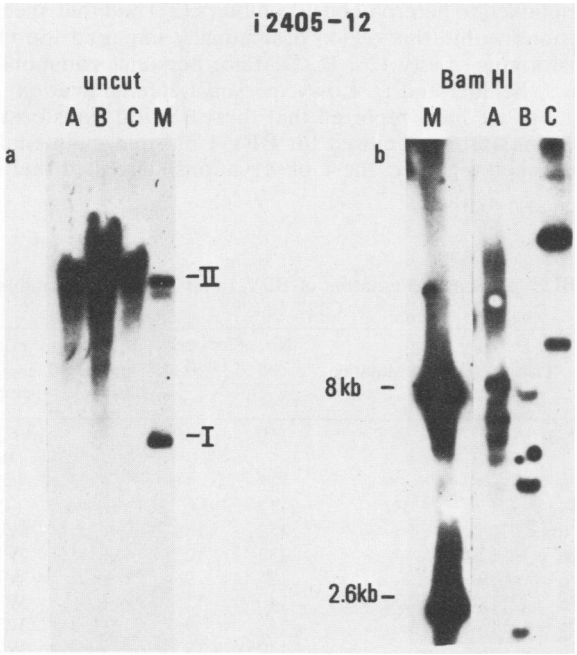

FIG. 3. Southern blot analysis of C127 cells derived upon cotransfection with mutant i2405-12 (*rep*$^-$) and pNEO5′ DNA. The analysis of the DNA extracted from three independent cell lines (lanes A, B, and C) is shown uncut (a) and upon restriction with the endonuclease *Bam*HI (b). Forms I and II DNA and the restriction fragments generated by *Bam*HI cleavage are marked (lane M). Analysis was as described in the legend for Fig. 2. Exposure of the autoradiogram was for 2 days.

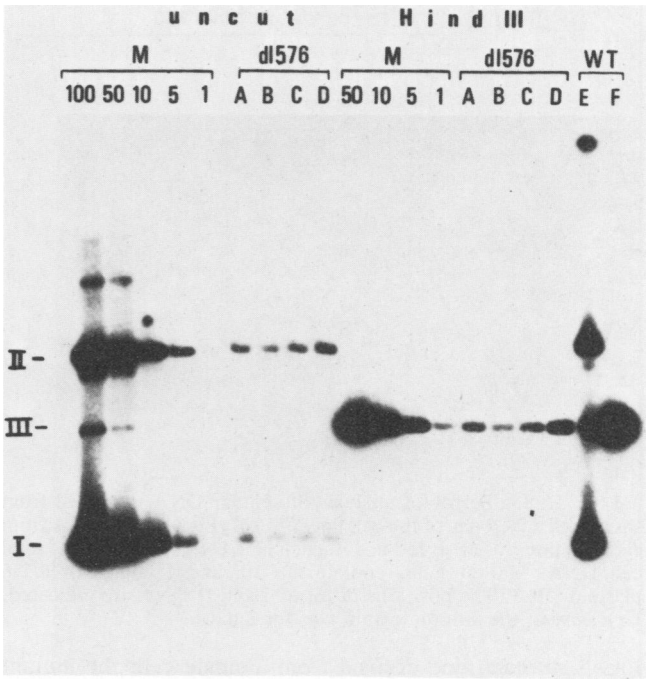

FIG 4. Southern blot analysis of BPV-1 DNA in C127 cell lines derived upon transformation with *dl*576 DNA (*cop*$^-$). Total cellular DNA extracted from four independent cell lines (lanes A, B, C, and D) was analyzed uncut and restricted with *Hin*dIII. The marker lanes (M) contain 100, 50, 10, 5, and 1 copies of bacterially derived *dl*576 plasmid DNA, 1 copy being equivalent to 20 pg of plasmid DNA per 10 μg of total cellular DNA. The positions of forms I, II, and III DNA are indicated. Lanes E and F show the DNA extracted from a C127 cell line derived upon transformation with WT pMLBPV-5 DNA; in lane E the DNA was analyzed uncut; in lane F the DNA was restricted with *Hin*dIII. Exposure of the autoradiogram was for 2 days.

lines (Lanes A, B, C, and D) derived upon transformation with *dl*576 DNA is shown in Fig. 4. The cell DNAs were analyzed uncut and upon restriction with *Hin*dIII, linearizing the plasmid DNAs. Copy number reconstructions with *dl*576 plasmid DNA (100, 50, 10, 5, and 1 copies) are shown in Fig. 4 (lanes M). Lanes E (uncut) and F (*Hin*dIII) show the blot analysis from a C127 cell line containing WT pMLBPV-5 DNA. This particular line contained the WT DNA, with ca. 100 copies per cell. In contrast, the copy number of the mutant plasmids was estimated to range between one and five per cell (lanes A, B, C, and D). The same result was obtained when cell lines derived upon transformation with mutant *dl*54 were investigated (Table 1). From this we conclude that both mutant plasmids *dl*576 and *dl*54 were each maintained exclusively in the extrachromosomal state. However, both mutations led to a *cop*$^-$ phenotype, as the copy number of these plasmids appeared to be very low compared with that of WT DNA. This result led us to ask whether, in fact, every cell in these populations contained the mutant genome with a low copy number or whether the number obtained reflected an average copy number ranging from many copies to zero copies per cell. To address this question, we derived single-cell subclones from each of the four cell lines shown in Fig. 4 and thus expanded individual cells into cell lines, totaling 60 subclones. These were again analyzed for the content and state of the mutant plasmid DNA. Representative results obtained with the subclones from cell line *dl*576-B (see Fig. 4) are shown in

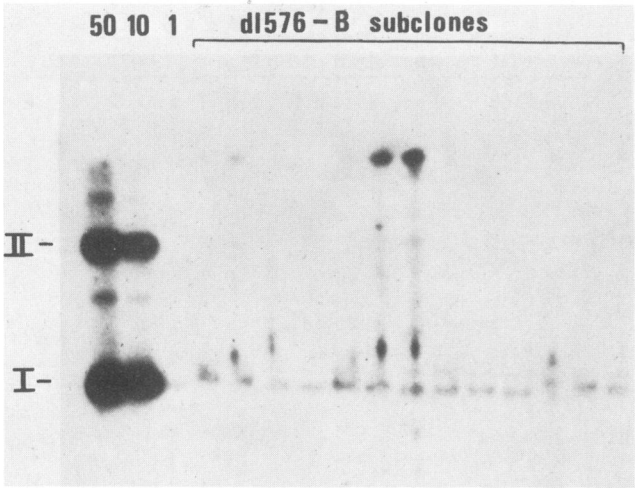

FIG. 5. Southern blot analysis of cellular DNA extracted from single-cell subclones of the cell line *dl*576-B (Fig. 4). The DNA from each subline was analyzed uncut, each lane containing 10 μg of total cell DNA. Marker lanes contain 50, 10, and 1 copies of *dl*576 plasmid DNA. The positions of forms I and II DNA are indicated. Exposure of the autoradiogram was for 2 days.

Fig. 5. In each line derived from a single cell, the mutant DNA was still maintained at a very low copy number, and fluctuations between sister cell lines were not detectable. The cell lines all contained the mutant DNA at a copy number of one to two per cell. We conclude that this phenotype did not reflect a simple segregation defect.

These results indicate that mutations affecting the E7 ORF maintained the ability of the viral genome to replicate autonomously, contrasting the *rep*$^-$ phenotype of E1 mutants. The mutations in the E7 ORF reduced the number of copies that could be stably maintained in the C127 cells, resulting in a *cop*$^-$ phenotype. From our results, it also seemed clear that if there was any integrated DNA present in these cells, the number of integrated genomes must have been less than one copy per cell.

Complementation analysis of *cop*$^-$ and *rep*$^-$ mutants. The mutational analysis of the BPV-1 genome described above showed that at least two regions within the viral genome were important for extrachromosomal replication and stable high-copy-number plasmid maintenance. These two regions were defined by mutations in the ORFs E1 and E7. However, our results did not allow us to decide whether the mutations affected the proper function of *trans*-acting gene products or whether they defined some *cis*-acting regulatory regions necessary for replication. Furthermore, the data did not show whether these putative *trans*-acting factors were indicative of different genes. To address these issues, we performed complementation tests by cotransfecting *cop*$^-$ and *rep*$^-$ mutant DNAs. Mutants belonging to different functional groups should complement each other, whereas mutants belonging to the same class should not do so.

For the cotransfections, the DNA of mutant *dl*576 (*cop*$^-$) was used together with the DNA of several E1 (*rep*$^-$) mutants. Likewise, the DNAs of nonoverlapping E1 mutants were pairwise cotransfected with each other. The molar ratio of the cotransfecting DNAs was in all cases 1:1. The results of these experiments are shown in Table 2 and can be summarized as follows. (i) Cotransfection of *dl*576 DNA with any of the E1 mutant plasmids resulted in morphological transformation with an efficiency comparable to that of WT DNA. (ii) The resulting cell lines harboring both types of mutants maintained both of them stably as high-copy-number plasmids. (iii) In contrast, the E1 mutants could not rescue the phenotype of the WT genome when cotransfected pairwise with each other. A representative Southern blot analysis of total cellular DNA extracted from C127 cells derived upon cotransfection with *dl*576 DNA and *dl*562 DNA, side by side with the analysis from cell lines containing *dl*576 DNA (*cop*$^-$) or *dl*562 DNA (*rep*$^-$), is shown in Fig. 6. The two cell lines (lanes D and E) showed two DNA species migrating with the form I DNA of *dl*576 (lanes A, B, and C) and the form I DNA of *dl*562 (lane M), whereas in the cell lines containing only the mutant genome *dl*562 (lanes F and G), no free replicating plasmid DNA could be detected. That these cell lines indeed contained both types of mutant DNAs, extrachromosomally and with equal high copy numbers, was confirmed by successful rescue of the two types of plasmids from the C127 cells into *E. coli* (data not shown).

The preceding results allow the following conclusions. (i) The mutations mapping to the E1 and E7 ORFs marked functions that acted in *trans* to allow for BPV-1 plasmid replication; they did not affect the function of *cis*-acting sequences. (ii) Mutant *dl*576 (*cop*$^-$) and the E1 mutants (*rep*$^-$) belonged to two different complementation groups since together they brought about the phenotype observed with the WT genome. (iii) All mutations described here mapping to the E1 ORF belonged to the same complementation group. (iv) The cotransfection experiments also showed that the WT functions, *rep*$^+$ and *cop*$^+$, appeared to be dominant over the mutant functions.

Transforming activity of *rep*$^-$ and *cop*$^-$ BPV-1 mutants. Extrachromosomal replication has previously been shown to be dispensable for the expression of the viral oncogenic functions. It has been demonstrated that the 3′ part of the viral early region is sufficient to mediate transformation when linked to heterologous promoters (21) and that specific deletions within this region dramatically impaired the viral transforming ability (26; D. DiMaio, personal communication; J. Schiller and D. Lowy, personal communication). In addition, we have reported that these 3′ viral transforming functions are not required for BPV-1 plasmid maintenance (17). Taken together, these observations suggested that the

TABLE 2. Complementation of BPV-1 *cop*$^-$ and *rep*$^-$ mutants in C127 cells[a]

Plasmid	Mutation	No. of foci per μg of DNA		Plasmid maintenance	Free copies per cell
		Expt 1	Expt 2		
pMLBPV-5	WT	150	135	+	100–150 (WT)
*dl*576	E7	15	11	+	1–5
*dl*54	E7	12	7	+	1–5
*dl*576 + *dl*306		152	198	+	WT
*dl*576 + *dl*562		133	105	+	WT
*dl*576 + *dl*639		125	98	+	WT
*dl*576 + i2113-1	E7 × E1	140	125	+	WT
*dl*576 + i2113-2		113	95	+	WT
*dl*576 + il515-2		112	123	+	WT
*dl*576 + i2405-5		98	113	+	WT
i1515-1 + i2113-1		72	83	−	0
i2113-1 + i2405-5		101	93	−	0
i2113-2 + i2405-12	E1 × E1	105	123	−	0
*dl*562 + i2113-1		128	112	−	0
*dl*639 + i1515-2		0	0	−	0

[a] The molar ratio of the cotransfecting DNAs was, in all cases, 1:1.

viral replication functions and transforming functions were encoded by different parts of the BPV-1 genome. Supporting this notion are the findings reported here that most of our *rep*$^-$ mutants were not impaired in the efficiency of focus formation.

It was not surprising, therefore, to find that mutant *dl*639 (Fig. 1) had lost the ability to induce foci (Table 1). Since this deletion affects the N-terminal part of the E2 ORF as well as the E1 ORF, we assumed that the lesion in E2 was responsible for this phenotype. To test this idea, we created mutant *dl*211 (Fig. 1) which had a 211-bp deletion solely within the E2 ORF. The ability of the mutant DNA to induce foci was, indeed, reduced at least 100-fold when compared with that of WT DNA. Few foci were obtained only when we used at least 10 μg of the mutant DNA for the transfection compared with 0.5 to 1 μg of WT DNA normally used. This was consistent with the results obtained by others (see above; 21, 26; D. DiMaio, personal communication). Analysis of the mutant DNA from flat, contact-inhibited cell lines, derived upon cotransfection with the *neo*^{r} marker gene and *dl*211 DNA showed the DNA maintained in the extrachromosomal state (Fig. 7, lanes A, B, and C). In contrast and unexpectedly, when the DNA from the rare *dl*211-induced transformed foci was subjected to blot analysis, we found the mutant DNA associated with the HMW chromosomal DNA (Fig. 7, lanes D, E, and F). We do not understand, at present, the mechanisms which could lead to the two different phenotypes with respect to replication, as our analysis with mutant *dl*Ba115 showed that the E2 ORF was not required for the autonomous replication (17). It could be possible that the selection for transformation led to rearrangement of this mutant DNA to allow for efficient expression of the viral transforming functions, thereby destroying the viral replication functions.

Mutants i1515-1 and i1515-2 were obtained by inserting a 237-bp SV40 fragment, containing the polyadenylation signals for both SV40 early and late transcription (10), at position 1515 in the BPV-1 genome (see above). Insertion of this fragment such that SV40 early and BPV-1 transcription occurred in the same direction resulted in loss of transfor-

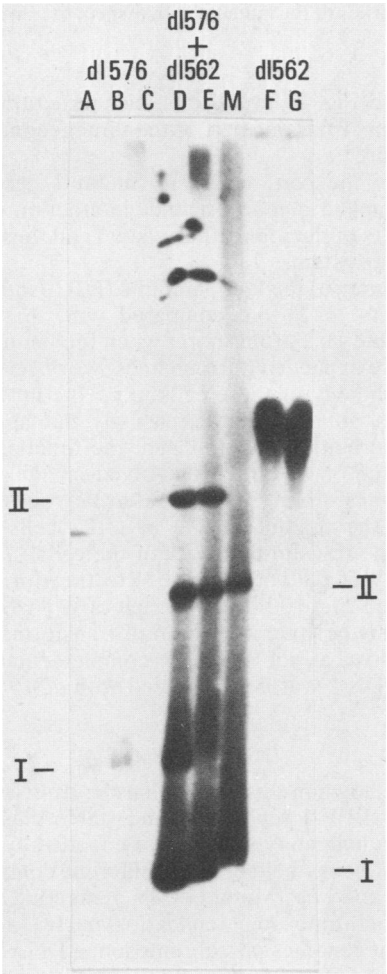

FIG. 6. Complementation of BPV-1 mutants *dl*576 (*cop*$^-$) and *dl*562 (*rep*$^-$) in C127 cells. Total cellular DNA from seven cell lines (lanes A through G) was subjected, unrestricted, to blot analysis. Lanes A, B, and C (see Fig. 4) are cell lines derived upon transfection with *dl*576 DNA (*cop*$^-$); cell lines in lanes F and G were obtained by transfection with *dl*562 DNA (*rep*$^-$); cell lines in lanes D and E were derived upon cotransfection of *dl*576 DNA with *dl*562 DNA (molar ratio, 1:1). Lane M contains 500 pg of bacterially derived *dl*562 plasmid DNA. The positions of forms I and II DNA of plasmids *dl*576 and *dl*562, respectively, are indicated. Exposure of the autoradiogram was for 2 days.

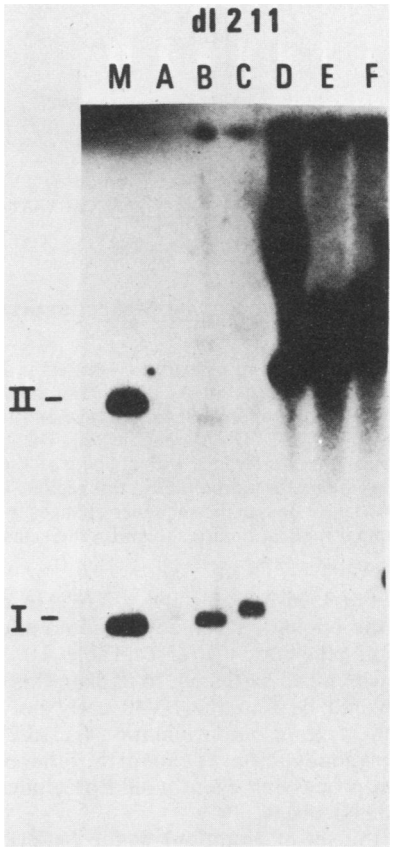

FIG. 7. Southern blot analysis of *dl*211 DNA in C127 cells. Cell lines in lanes A, B, and C were derived upon cotransfection of *dl*211 DNA and pNEO5′ DNA (molar ratio, 10:1) and selection for G418 resistance. The cells were flat and contact inhibited. Cell lines in lanes D, E, and F were expanded from three independently derived foci upon transfection with *dl*211 DNA. The cells showed a fully transformed phenotype. The DNA in each lane was analyzed uncut. Lane M contains 500 pg of *dl*211 plasmid DNA. The positions of forms I and II DNA are indicated. Exposure of the autoradiogram was for 1 day.

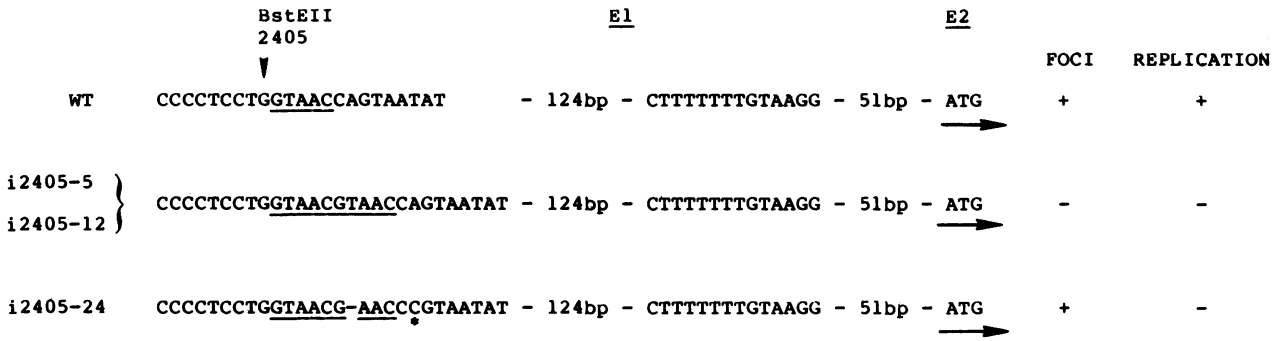

FIG. 8. DNA sequence of insertion mutations at BPV-1 position 2405. The DNA sequence of the three mutants i2405-5, i2405-12, and i2405-24 is compared with that of WT DNA, and the distance of the mutations from the ATG of the E2 region is shown. The sequence of the cohesive ends generated upon *Bst*EII cleavage and the duplication upon repair are underlined. The positions of the 1-bp deletion and the transversion in the i2405-24 DNA are indicated. The phenotype of the mutants could have been the result of effects upon a putative promoter in this region or upon interference with splicing of 3′ exons. For example, the DNA sequences around BPV-1 position 2414 might constitute an effective TATA box for a promoter in this region. Therefore, critical nucleotides 5′ to this putative −30 promoter element may be affected by alterations in space or specific sequence changes in this region. The biological properties of the mutants with respect to transformation and extrachromosomal replication are shown by the signs + and −.

mation activity (i1515-2 [Table 1]), whereas insertion of this fragment in the opposite orientation had no effect upon the transformation efficiency (i1515-1 [Table 1]). One simple hypothesis, which can be tested, to explain this difference in phenotype would be that the SV40 early polyadenylation signal was effective in one orientation (i1515-2), leading to premature polyadenylation, but not in the other one (i1515-1). This aberrant processing event would preclude the splicing together of distal exons.

Similarly, the set of mutations at the *Bst*EII site, BPV-1 position 2405, affected the structural gene for the *rep* function and may also have altered DNA sequences involved in the expression of other viral genes. Mutants i2405-5 and i2405-12 lost the ability to induce foci, whereas mutant i2405-24 did so as efficiently as the WT genome (Table 1). To map the mutation, we performed marker rescue experiments with recombinant DNAs constructed in vitro. A restriction fragment from WT pMLBPV-5 DNA spanning the region from BPV-1 positions 2113 to 2617 (*Eco*RI-*Sph*I) was isolated and inserted into the DNAs of the mutants i2405-5 and i2405-12 after removal of the *Eco*RI-*Sph*I fragments of the mutant genomes. The resulting plasmids were able to transform C127 cells and replicate extrachromosomally as efficiently as the WT genome (data not shown). When this region of the mutant genomes was subjected to DNA sequence analysis, we found the alterations shown in Fig. 8, when compared with WT DNA. We do not know at present how the differences in sequence could have led to the two different phenotypes with respect to transformation. Preliminary data from RNA analysis showed differences in the pattern of BPV-1 transcripts. These results suggested that signals for either initiation or processing of transcripts might have been located within this part of the BPV-1 genome (see the legend to Fig. 8), and the precise structural analysis of the transcripts from this part of the viral genome may give a clearer picture.

TABLE 3. Replication and transformation of BPV-1 mutant *dl*306 in C127 cells

Plasmid	Mutation	No. of foci per DNA (μg): 0.1 Expt 1	0.1 Expt 2	1 Expt 1	1 Expt 2	Plasmid maintenance	Free copies per cell
pMLBPV-5	WT	7	6	150	135	+	100–150
*dl*306	E1	206	220	>2,000	>2,000	+/−	Variable

Several of the *cop*$^-$ and *rep*$^-$ mutants described here, however, implied a more distinct interaction between the gene products of the replication system and those of the viral transforming system.

The efficiency of the *cop*$^-$ mutants (E7) to induce foci was decreased 10- to 20-fold compared with that of the WT genome (Table 1). Furthermore, when foci induced by these mutants were expanded into cell lines, we observed a change in the morphology of these cells; e.g., the initially spindle-shaped cells obtained an increasingly flat appearance. A simple interpretation could be that the function defined by mutations in E7 was directly involved in transformation in that it had transforming activity. Alternatively, the low copy number of the mutant DNAs in C127 cells could have influenced the transformed state of the cells.

The ability of mutant *dl*306 (E1) to transform C127 cells was increased 20- to 30-fold over that of WT DNA (Table 3). However, this positive activation of transformation seemed to be recessive, as shown in the complementation analysis when *dl*306 DNA was cotransfected with *dl*576 DNA (Table 2).

DISCUSSION

We have shown in this report that the *trans*-acting factors required for BPV-1 replication and stable plasmid maintenance with a high copy number are encoded by at least two distinct viral genes belonging to different complementation groups, *rep* and *cop*. Mutants (*rep*$^-$) affecting the E1 ORF abolish extrachromosomal replication and lead to integration of the mutant genomes into chromosomal DNA. In contrast, mutants (*cop*$^-$) affecting the E7 ORF maintain the ability of the viral genome to replicate extrachromosomally, but the copy number of these mutant genomes is reduced 100-fold compared with the copy number of WT DNA. The presence of both functions together, as shown by complementation analysis in cotransfection experiments, can rescue the WT phenotype. *rep*$^-$ and *cop*$^-$ mutant genomes replicate extrachromosomally and stably with high copy numbers within the same cell.

The results obtained with the *rep*$^-$ mutants described here indicate that the E1 function is absolutely necessary for

autonomous replication, prevention of integration, or both. Mutant *dl*306, however, appears only partially defective in this function. The mutation in *dl*306 is the result of an in-frame deletion, thus leading to a deleted form of the WT gene product. Therefore, it could be possible that, in this situation, the *rep* gene product is partially intact, thus allowing to some extent for extrachromosomal replication. The nature of this deletion might also account for the increased transformation frequency of mutant *dl*306 (Table 2). This phenotype of mutant *dl*306 raises the interesting possibility that the gene product defined by this mutation is somehow involved in modulating the expression of the viral transforming functions. However, we do not believe the *rep* gene to be simply a negative regulator since other *rep*$^-$ genomes do not display this enhanced transformation. Information is needed about the structure of this gene on the molecular level. It could be possible that this protein has a complex set of domains which display both positive and negative aspects of gene regulation.

Taken together, the mutational analysis of the E1 region with respect to BPV-1 replication leads us to conclude that integration of the viral genome occurs when the function of this particular gene product is destroyed; e.g., when E1 functions properly, the viral genome does not integrate but replicates autonomously, and the two processes seem to be exclusive, at least in the C127 cell system. This notion is supported by the behavior of the *cop*$^-$ mutants. With these mutant genomes, transformation efficiencies are reduced relative to the WT genome, and the copy number is decreased at least 100-fold. Nevertheless, integration of viral genomes in these transformed cells is still not detected; thus, it is tempting to speculate that the E1 gene product is one of the factors required in *trans* to specifically preclude integration of the viral genome into chromosomal DNA. Although in most cases papilloma virus infections lead to persistence of the viral DNA in the extrachromosomal state, in several systems integration of the viral DNA has been observed. Breitburd et al. (5) have reported the integration of BPV-1 DNA in one hamster tumor cell line. Shope papilloma virus DNA has been found to be associated with HMW chromosomal DNA in rabbit carcinomas and carcinoma cell lines (29). Furthermore, it has been reported that human papilloma virus 18 DNA, in several biopsies and cell lines derived from cervical cancers, appears to be integrated into chromosomal DNA (4). In light of our results obtained with the E1 mutants described here, we suggest that integration of the viral DNA is neither a consequence of nor a prerequisite for morphological transformation but depends on the proper function of E1. In addition, it is likely that certain host cell factors are interacting with the BPV-1 replication proteins and that alterations in these cell factors may lead to loss of viral plasmid DNA. Thus, in the progression and selective amplification of tumor cells transformed by papilloma virus in vivo, either loss of permissivity or mutations in the *rep* function could necessitate integration of the viral DNA.

Our results obtained with the mutant i471, which is characterized by a 4-bp insertion in the E6 ORF, do not allow us at present to assign a function to a putative E6 gene. The behavior of this mutant in C127 cells is indistinguishable from WT DNA with respect to both parameters, extrachromosomal replication and morphological transformation. However, further mutations in this region should be analyzed, since our mutation maps to the extreme C-terminal part of the putative E6 gene. Thus, it could be possible that the C-terminal 8 to 10 amino acids of the putative protein are not crucial for its function and, therefore, that the mutation remained undetected in our assays. Recent evidence suggests that the E6 gene, when linked to heterologous promoters, can induce transformation in C127 cells (Y.-C. Yang and P. Howley, personal communication; J. Schiller and D. Lowy, personal communication). The analysis of cDNA clones from transcripts in this region revealed, in addition to the presence of unspliced transcripts, one class of spliced transcripts, leading to a fusion of the E6 and E7 ORFs and thus creating an E6-7 gene (Berg et al., manuscript in preparation; Y.-C. Yang and P. Howley, personal communication; A. Stenlund and U. Pettersson, personal communication). Since the mutation at BPV-1 position 471, therefore, also maps within the intron of this E6-7 gene, this could also explain the WT phenotype of mutant i471 in contrast to the mutant phenotype of *dl*576 and *dl*54.

Mutations within the E7 ORF have several effects on the behavior of the viral genome. Two mutants have been analyzed, *dl*576 and *dl*54. The extrachromosomal maintenance of these mutant genomes (*cop*$^-$) with very low copy numbers does not seem to reflect a simple defect in segregation upon cell division. If this were the case, one would expect to find within the population individual cells with different copy numbers; most importantly, true revertants with zero copies would be expected. However, all of the single-cell subclones analyzed from the initial lines exhibit the same phenotype (Fig. 5). We analyzed over 60 subclones, and each contained the mutant genome, apparently stable with one to two copies of plasmid DNA per cell. This phenotype could be the result of the establishment of the mutant DNA within the cells at an initial, inherent low and stable copy number. Alternatively, the transfection could introduce the viral genome into the cell at a high copy number; however, this situation might be unstable due to the mutant *cop* function. As a result, this would then lead to an actual loss of viral plasmids until a final number of copies is reached. In this respect, we have tested the ability of PMS1-*neo* to replicate in the presence of the mutant *dl*576 genome. Preliminary evidence suggests that PMS1 can be maintained in the extrachromosomal state together with *dl*576 (M. Lusky and M. Botchan, unpublished data); however, the reversion frequency to neomycin sensitivity appears to be high (J. Reynolds and M. Botchan, unpublished data). We do not know at present how the *cop* function mediates the stable maintenance of the BPV-1 genome at a high copy number. The effect could be direct, for example, by some interaction of this gene product with other viral functions such as the *rep* gene product. Alternatively, the effect could be an indirect one and be mediated by an interaction of the *cop* function with specific host cell factors or by induction of such factors.

The foci obtained upon transfection with *cop*$^-$ mutant DNAs cannot be distinguished morphologically from those obtained with WT DNA. However, upon expansion of these foci into cell lines, the individual cells gradually lose their spindle-shaped appearance, typical for BPV-1-transformed cells, and become flat. A simple interpretation of these results is that the function defined by mutations in E7 is important not only for copy number control but also plays a role in the transformation process; e.g., this gene product could also have transforming activity. An alternative possibility is that for the permanent expression of the oncogenic functions, one copy or a few copies of the viral genome in the extrachromosomal state are not sufficient to render the cells fully transformed. In contrast, when BPV-1 is integrated, it has been shown by others (21) and observed by us (M. Lusky, L. Berg, and M. Botchan, unpublished data) that

one copy or a few copies of the viral genome can indeed be sufficient to allow for the stably transformed phenotype. Thus, it is very tempting to speculate that in the extrachromosomal state the expression of the viral transforming function is somehow limited and that this situation with the WT genome (BPV-1) is overcome by maintaining a high number of viral genomes in each cell. The unstable nature of the phenotype with respect to morphological transformation as mediated by mutants *dl*576 and *dl*54 could then reflect a gene dosage effect. This notion is directly supported with results which show that the cDNA of the E6-7 transcript alone, when expressed from a retroviral promoter, is unable to induce transformation of C127 cells. However, it can complement the mutation in *dl*576. These points will be discussed in detail in an independent report (Berg et al., in preparation).

The mutants *dl*576 and *dl*54 lead to very-low-copy-number maintenance. If, indeed, there is a threshold requirement for the viral transforming functions, it should be possible to find other more leaky alleles of the *cop* gene which can maintain the transformed state of the cells.

The results obtained with the complementation analysis allow us to unambiguously define the E7 and E1 coding regions as two distinct genes or at least as parts thereof. Both functions act in *trans* and are necessary for replication and stable plasmid maintenance of the viral genome. We cannot conclude from our data that both functions are also sufficient for these processes. This issue can only be solved when each function can be tested uncoupled from the entire BPV-1 genome. However, this requires a detailed knowledge of the structure of these genes at the molecular level and points to the limitations of the approach used here.

As more information about the structure of the *cop* and *rep* genes becomes available, it should be possible to express them individually, and through the proper use of genetic techniques, more knowledge about their functions should be obtained. Moreover, interesting questions such as the following can be approached. Are both factors required continuously during the cell cycle, and do either of these gene products interact with the viral DNA, thereby exerting their function in replication or segregation?

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