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Recombinant DNA molecules comprising bovine papilloma virus type 1 DNA linked to plasmid DNA are maintained in a plasmidial state both in rodent fibroblasts and in bacterial cells

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Transformed cells obtained after transfecting FR3T3 rat fibroblasts with DNA of bovine papilloma virus type 1 (BPV1) maintained only free copies of the viral genome. Transfection with BPV1 DNA inserted in a bacterial plasmid (pBR322 or pML2) did not produce transformants at a detectable rate, unless the viral sequences had been first excised from the plasmid. In contrast, transfer of the same plasmids by polyethylene glycol-induced fusion of bacterial protoplasts with FR3T3 rat or C127 mouse cells led to significant transformation frequencies. A total of eight cell lines were studied, three rat and five mouse transformants, obtained with various BPV1-pML2 recombinants. In all cell lines, both BPV1 and plasmid sequences were maintained as non-integrated molecules, predominantly as oligomeric forms of the transforming DNA. In the three rat transformants and in two of the mouse lines, parts of the non-transforming viral region and some bacterial sequences were deleted. In the remaining three mouse lines, the monomeric repeat was a non-rearranged plasmid molecule which could be re-established as a plasmid in *Escherichia coli* after cleavage with "one-cut" restriction endonucleases and circularization of the molecule.
Key words: bovine papilloma virus type 1/eukaryotic plasmid/protoplast fusion/shuttle vector/transformation.

Introduction

Bovine papilloma virus type 1 (BPV1) (Lancaster and Olson, 1978) induces proliferative epidermal lesions in cattle and other mammals (see review by Howley *et al.*, 1980) and transforms cells in culture. The transformed and tumor cells maintain only non-integrated viral genomes (Amtmann *et al.*, 1980; Lancaster, 1981; Law *et al.*, 1981). The plasmidial BPV1 replicons thus provide a model system for the analysis of DNA replication and mitotic segregation in mammalian cells and a tool for the construction of genetic vectors.

The virus cannot be propagated in cell culture and studies at the molecular level require amplification of its DNA in bacteria. Transfection by the calcium phosphate co-precipitation technique of BPV1 DNA synthesized in *Escherichia coli* led to the oncogenic transformation of mouse and rat fibroblasts and only part of the genome (*Bam*HI-*Hind*III 69% fragment, see Figure 1) was shown to be required (Howley *et al.*, 1980; Law *et al.*, 1981). The *in vitro* ligation of this fragment with foreign DNA molecules, such as the rat preproinsulin gene or a bacterial HGPRT gene, allowed these genes to be maintained in the transformed cells in a stable plasmidial state (Sarver *et al.*, 1981; Howley *et al.*, 1981). In these experiments, BPV1 DNA had first to be excised from the bacterial plasmid (Lowy *et al.*, 1980; Law *et al.*, 1981). Transfection with the complete BPV1-pML2 recombinant DNA does not produce transformants at detectable frequencies (this report).

Gene transfer by DNA transfection has relatively low efficiency and selects for a limited and poorly defined population of "competent" cells (Wigler *et al.*, 1980). As an alternative, we assayed the transforming ability of BPV1-pML2 recombinants by polyethylene glycol-induced fusion of rodent fibroblasts with bacterial protoplasts (Schaffner, 1980). As previously reported (Rassoulzadegan *et al.*, 1982), this method leads, in our hands, to high efficiencies of transfer of SV40 and polyoma virus early genes (up to 100%), as checked by short-term expression and long-term transformation.

Results

Transformation of FR3T3 rat fibroblasts with BPV1 DNA

FR3T3 fibroblasts were transfected with 0.5 – 1 μg of form I BPV1 DNA and transformed derivatives were selected either by focus formation or by colony formation in agarose medium. Transformation frequencies were in the range of 10^{-5} – 10^{-4} transformants/treated cell (Table I).

Three independent cell lines were established from foci (FR3T3 1 – 3), and three others (FR3T3 4 – 6), from colonies grown in agarose medium. These lines exhibited a fully transformed phenotype. Their growth pattern and tumorigenic properties will be described elsewhere (manuscript in preparation).

The state of the viral genomes in these lines was determined by blot hybridization of total DNA after agarose gel electrophoresis and transfer to nitrocellulose sheets, using as probe a high specific radioactivity BPV1 DNA. The experimental conditions used would have allowed detection of <1 integrated BPV1 genome equivalent per haploid cell genome (Mougneau *et al.*, 1980). Results are shown in Figure 2. They

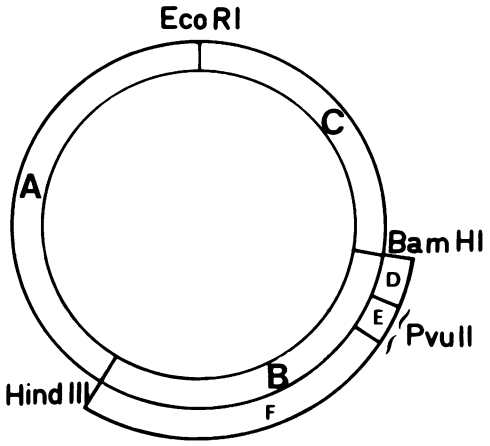

Fig. 1. Restriction enzyme map of BPV1 DNA. *Bam*HI, *Eco*RI, and *Hind*III sites according to Howley *et al.*, 1980. Fragment sizes: A: 3.2 kb, B: 2.5 kb, C: 2.4 kb, D: 0.3 kb, E: 0.25 kb, F: 1.9 kb.

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Table I. Transformation of FR3T3 rat cells with BPV1 DNA

Transfecting DNA	μg DNA /plate	Selection procedure[a]	Transformants /10^6 cells[b]
pBR322	1	A/F	0
BPV1 DNA[c]	0.5	A	25
	1	A	60
	0.5	F	200
	1	F	300

[a]A: colonies in agarose medium; F: foci on confluent monolayers.
[b]Total number of foci/colonies in five plates seeded with 2 x 10^5 cells and transfected with the indicated amounts of DNA.
[c]DNA extracted from viral particles.

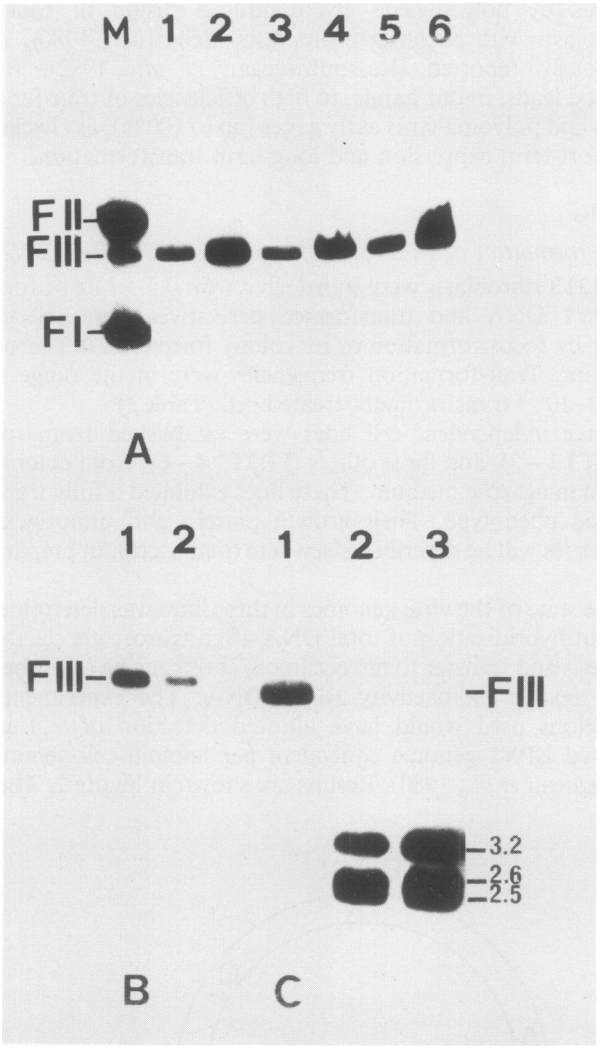

Fig. 2. Viral DNA sequences in FR3T3 rat cells transformed with viral DNA purified from virions and in cells derived from tumors. Blot-hybridization of ^{32}P-labeled BPV1 DNA to 20 μg of *Eco*RI-digested cellular DNA. **A**: Total cellular DNA from lines BPV-FR3T3-1 to -6 (**lanes 1** through **6**) digested with *Eco*RI and submitted to agarose gel electrophoresis for 15 h at 1.5 v/cm. **B**: Comparative analysis of total cell DNA (20 μg, **lane 1**) and low mol. wt. DNA (Hirt, 1967) from an equivalent amount of cells (**lane 2**) in the case of BPV-FR3T3-2. Electrophoretic migration of BPV1 marker DNA prepared from virions: (FI) superhelical, (FII) relaxed circular, (FIII) linear molecules. **C**: Blot-hybridization of BPV-FR3T3-2 DNA immediately after establishment of the line (**lanes 1** and **2**) and after ~60 cell generations (**lane 3**); (**1**): cleavage with *Eco*RI, (**2**) and (**3**): cleavage with a mixture of *Eco*RI, *Bam*HI, and *Hind*III.

indicate that, like the previously described BPV1 transformed and tumor cells (Amtmann *et al.*, 1980; Lancaster, 1981; Law *et al.*, 1981), FR3T3 transformants maintain only non-integrated copies of the viral DNA. In addition to forms I and II of the viral DNA, small amounts of the high mol. wt. forms previously described by Law *et al.* (1981) were present (data not shown). The number of form I and II molecules was estimated from the intensity of the bands on the autoradiograms (not shown), compared to those produced by calibrated amounts of marker DNA. Values varied from one cell line to another from ~10 to >100 copies/haploid rat genome equivalent and were quite reproducible for a given line between different DNA preparations. The autonomous DNA molecules were stably maintained upon prolonged growth in cell culture (Figure 2), as well as during growth of the cells as a tumor in the animal (manuscript in preparation).

Transformation of FR3T3 and mouse C127 fibroblasts with BPV1-pBR322 recombinant DNA

Knowing that BPV1 DNA can be stably maintained as a plasmid in FR3T3 rat fibroblasts, we enquired whether transformation and subsequent maintenance could be achieved using recombinant DNAs that include either the complete viral genome or its transforming region (*Bam*HI-*Hind*III) linked to a bacterial plasmid DNA (Table II).

Transformation was first attempted by the calcium phosphate co-precipitation method. No transformed colony could be detected, indicating an efficiency at least two orders of magnitude lower than with viral DNA (Table III). This result was expected in the case of recombinant pB1, where the pBR322 sequences are inserted at the *Eco*RI site of BPV1 (Figure 1), but not for recombinant pB2, that carries an intact transforming region (Lowy *et al.*, 1980). In fact, transformation frequencies comparable to that of native BPV1 DNA were observed when pB2 was cleaved with *Bam*HI endonuclease prior to cell transfection. These negative results could not be explained by the presence in pBR322 of the same "poison" sequences that inhibit replication of SV40 DNA in monkey cells (Lusky and Botchan, 1981): recombinants pMB2, pMH4, and pM69, derived from a plasmid pML2, lacking the "poison" sequences (Lusky and Botchan, 1981, M. Botchan, personal communication), were also negative.

Transformation was then attempted using the protoplast fusion technique. As shown in Table IV, transformed derivatives were obtained both from rat FR3T3 and from mouse C127 fibroblasts with the same recombinant plasmids that had been registered as essentially non-transforming by transfection. As expected (see above), plasmid pB1 was still unable to transform.

BPV1 and linked bacterial sequences are maintained in the transformed cells obtained by protoplast fusion as autonomous multimeric molecules

The state of the viral and bacterial sequences was first studied in a set of four independently established cell lines, three from FR3T3 rat fibroblasts and one from C127 mouse cells (Table V). Neither pML2, nor BPV1 integrated DNA sequences could be detected: hybridization patterns were in each case identical in the total cell DNA extract and in the Hirt supernatant fraction (data not shown) and the same hybridization pattern was observed using different restriction endonucleases that have no recognition site on the transforming plasmid ("no-cut" enzymes) (see Figure 3). In all cases, cleavage of total cellular DNA by "one-cut" restriction endonucleases (see below) generated a unique linear molecule,

Table II. Recombinant DNA molecules constructed from BPV1 and bacterial sequences

Recombinant plasmid	Size (kb)	Bacterial vector	BPV1 DNA insert	Number of cleavage sites					
				*Bam*HI	*Eco*RI	*Hind*III	*Pvu*II	*Sal*I	*Xba*I
pB1	12.4	pBR322	Genomic viral DNA linearized at *Eco*RI site	2	2	2	3[a]	1	0
pB2	12.4	pBR322	Genomic viral DNA linearized at *Bam*HI site	2	2	2	3[a]	1	0
pMB2	11	pML2	Genomic viral DNA linearized at *Bam*HI site	2	2	2	2[a]	1	0
pMH4	11	pML2	Genomic viral DNA linearized at *Hind*III site	2	2	2	2[a]	1	0
pM69	8.5	pML2	*Bam*HI-*Hind*III 69% fragment	1	2	1	0	1	0

[a] *Pvu*II has two cleavage site close to each other in BPV1 DNA (Figure 1)

Table III. Frequency of transformation after transfection of FR3T3 cells with BPV1 DNA and BPV1-pBR322 recombinants

Transfecting DNA	μg DNA /plate	Selection procedure[a]	Transformants /10^6 cells[b]
pBR322	1	A/F	<1
pB1	1	F	<1
	1	A	<1
pB2	1	F	<1
	1	A	<1
pMB2	1	F	<1
	1	A	<1
pM69	1	F	<1
	1	A	<1
BPV1 DNA	0.5	F	180
from pB2 DNA[c]	1	F	250

[a] and [b] see Table I.
[c] pB2 DNA (see Table II) was cleaved with *Bam*HI endonuclease and the mixture of DNA products was used for transfection.

Table IV. Transformation efficiency of BPV1-pBR322 recombinants after transfer by protoplast fusion

Plasmid	Transformants per 10^6 cells[a]	
	FR3T3	C127
pBR322	<1	<1
pB1	<1	<1
pB2	30	40
pMB2	20	35
pM69	10	10
pMH4	25	40

[a] See Table I.

without the additional junction fragments that must be generated by cleaving an integrated structure. As in the cell lines transformed with native BPV1 DNA, the number of autonomous copies varied between ~10 and 100 genome equivalents/cell.

In these four transformants, the only or the main autonomous form (in DNA of cells RH1, RH3, and RH4, a faint band could be detected at the monomer position (form I), that could not be seen in S69 cells) was of a size larger than the original plasmid. This is exemplified in Figure 3 after

Table V. Cell lines transformed with BPV1-pML2 recombinants: non-rearranged and deleted plasmids

Species	Cell line	Transforming DNA		Autonomous DNA
		Plasmid	Size (kb)	Size (kb) of monomer
Deletions				
Rat	RH1	pMH4	11	7.5
Rat	RH3	pMH4	11	8.5
Rat	RH4	pMH4	11	7.5
Mouse	SH105	pMH4	11	10
Mouse	SB6	pMB2	11	6.5
Intact plasmids				
Mouse	SB5	pMB2	11	11
Mouse	S694	pM69	8	8
Mouse	S69	pM69	8	8

treatment of the total DNA with endonucleases that do not cleave the plasmid DNA (*Xba*I cleavage of RH3 DNA and *Xba*I and *Pvu*II cleavage of S69 DNA). In spite of that fact, autonomous molecules in cell line S69 cleaved by "one-cut" and "multi-cut" endonucleases yielded patterns identical to those of the original plasmid (Figure 3). These results suggest that oligomeric forms of the transforming DNA are maintained in these cells. They appear to be heterogeneous in size, but their precise distribution could not be accurately deduced from these experiments, due to the difficulty of accurate size determination in this mol. wt. range. Their minimal size was estimated to be that of a trimer of pM69 DNA. This was confirmed when the products of partial digestions with "one-cut" enzymes were studied by agarose gel electrophoresis and sucrose gradient zonal ultracentrifugation (Figure 4). Molecules of dimeric, and trimeric, length could be detected in the cleavage reaction products (Figure 4B).

Non-rearranged and deleted oligomeric circular forms

As indicated above, cleavage of the oligomeric molecules maintained in S69 cells with various enzymes (Figure 3) produced the same hybridization patterns as that of the transforming plasmid DNA (pM69), indicating that the repeated monomer is a non-rearranged copy of the transforming plasmid.

In contrast, cleavage of the autonomous molecules present in RH1, RH3, and RH4 cells with *Pvu*II endonuclease, which has no recognition site in pML2 and two sites very close to

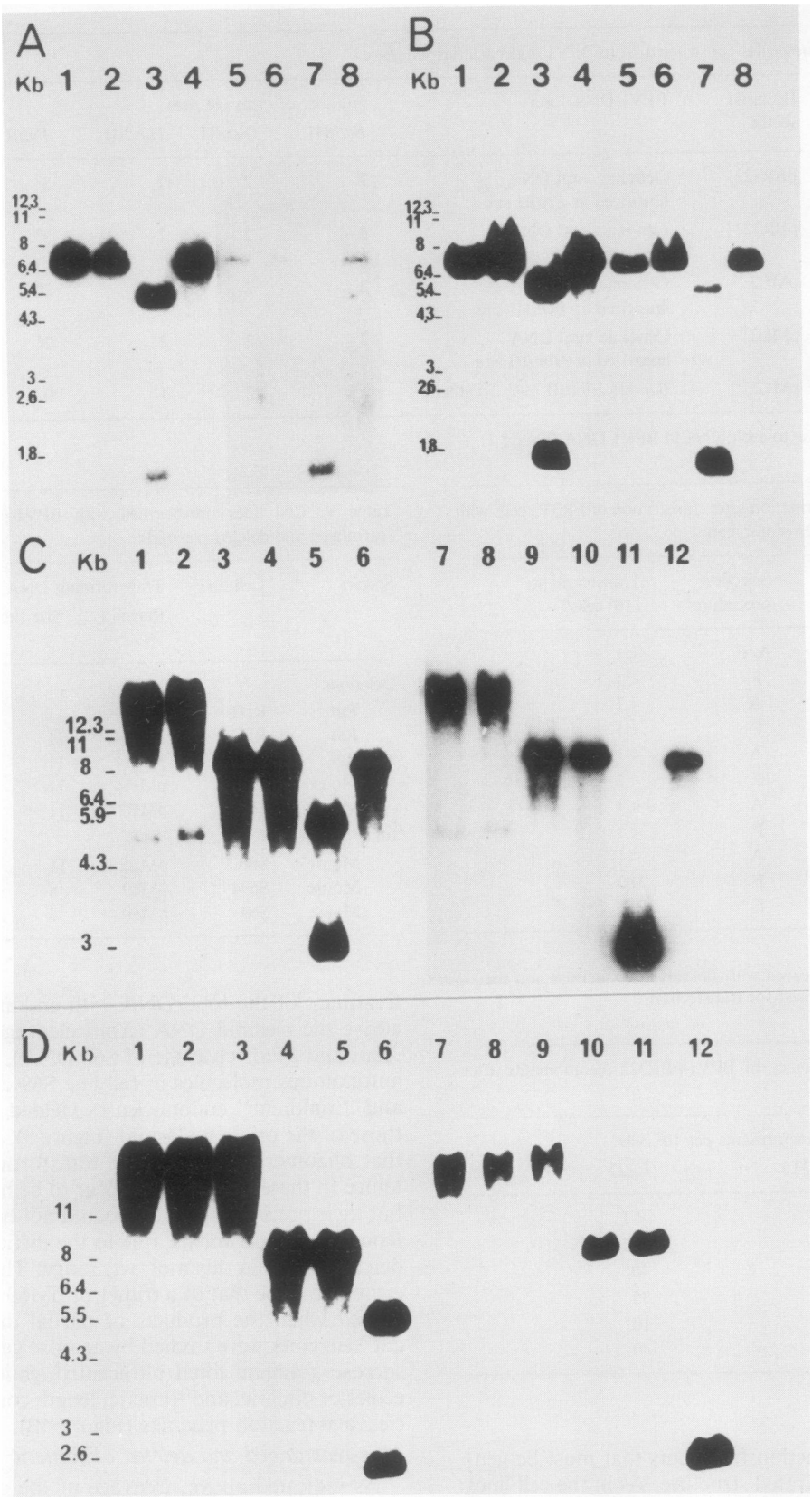

Fig. 3. Free viral DNA in FR3T3 rat and C127 mouse transformed fibroblast cells obtained by protoplast fusion. **Panels A** and **B**: total DNA from RH1 (**A**) and RH4 cells (**B**) was digested with *Bam*HI (**1** and **5**), *Hind*III (**2** and **6**), *Bam*HI plus *Hind*III (**3** and **7**), *Pvu*II (**4** and **8**). After electrophoresis and transfer, the nitrocellulose sheet was first incubated (**1–4**) with ^{32}P-labeled pB2 plasmid DNA (BPV1 and pBR322 sequences). After autoradiography, it was treated with NaOH (see Materials and methods) and a second hybridization was performed with a ^{32}P-labeled pBR322 DNA probe (**5–8**). **Panel C**: same experiment with DNA from cell line RH3, after cleavage with *Xba*I (**1** and **7**), *Sal*I (**2** and **8**), *Bam*HI (**3** and **9**), *Hind*III (**4** and **10**), *Bam*HI and *Hind*III (**5** and **11**) and *Pvu*II (**6** and **12**). **Panel D**: same experiment with DNA from cell line S69, after cleavage with *Xba*I (**1** and **7**), *Pvu*II (**2** and **8**), *Xba*I and *Pvu*II (**3** and **9**), *Bam*HI (**4** and **10**), *Hind*III (**5** and **11**) and *Bam*HI and *Hind*III (**6** and **12**). In **C** and **D**: **lanes 1–6**: hybridization with a pB2 probe, **lanes 7–12**: hybridization with a pBR322 probe.

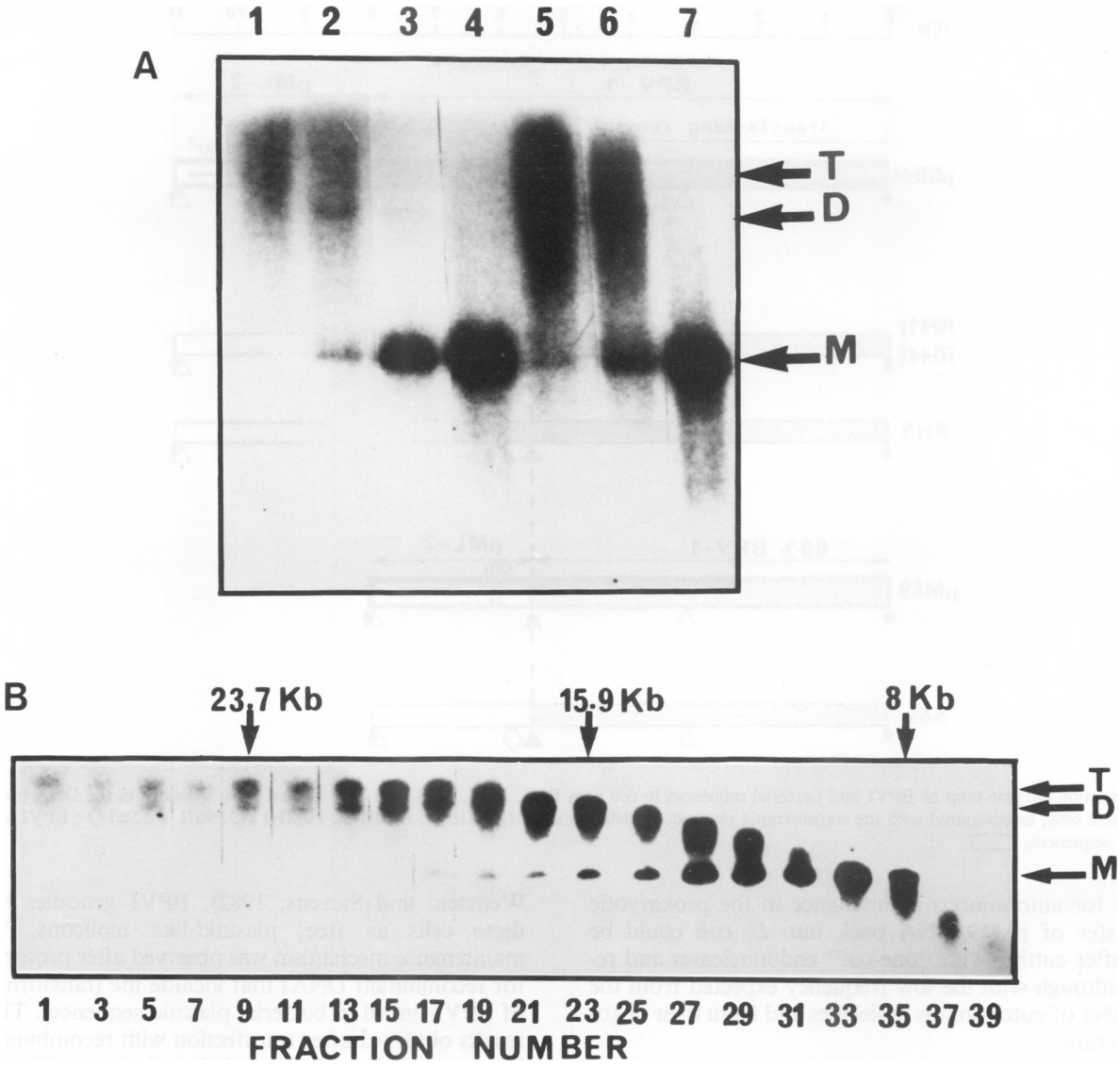

Fig. 4. Partial digestion with "one-cut" restriction endonucleases of the autonomous DNA in cell line S69. **Panel A**: 20 μg of total cellular DNA prepared from S69 cells was treated with 20 U of either *Bam*HI or *Hind*III for the indicated time at 37°C in a total volume of 1 ml. Under these conditions, complete cleavage required 1 h. Samples were analyzed by agarose gel electrophoresis. A parallel sample was digested to completion with the "no-cut" *Xba*I enzyme. **1**: *Xba*I; **2**: *Bam*HI, 5 min; **3**: *Bam*HI, 10 min; **4**: *Bam*HI, 3 h; **5**: *Hind*III, 5 min; **6**: *Hind*III, 10 min; **7**: *Hind*III 3 h. **Panel B**: total DNA was extracted from S69 cells, digested with *Bam*HI, *Pvu*II (no cleavage site in pM69 DNA), and pancreatic RNase and centrifuged through a 5–20% sucrose gradient in 20 mM Tris-HCl buffer pH 7.9, 0.2 M NaCl, 20 mM EDTA for 16.5 h at 18°C in a Beckmann SW 27 rotor (33 ml), 0.5-ml fractions were collected. A 50-μl aliquot of every second fraction was analyzed by agarose gel electrophoresis, transfer to nitrocellulose and hybridization with a ^{32}P-labeled pB2 DNA probe. Sedimentation markers (arrows on horizontal axis): "8 kb": *Bam*HI-cleaved pM69 DNA; "15.9 kb": large *Ava*I fragment from lambda phage DNA; "23.7 kb": large *Hind*III fragment from lambda phage DNA. Electrophoresis mol. wt. markers (arrows on vertical axis): "M", "D", and "T" correspond to the electrophoretic migration of linear monomeric, dimeric, and trimeric pM69 DNA, calculated from the migration of the same markers.

each other in BPV1 sequences, produced a unique fragment shorter than the transforming DNA (Figure 3). Its size was 7–7.5 kb for RH1 and RH4 and 8.5–9 kb for RH3, as compared with 11 kb for plasmid pMH4. The same result was observed after cleavage of the DNA of these cells with either *Bam*HI or *Hind*III, indicating that one of the two *Bam*HI sites and one of the two *Hind*III sites of the transforming plasmid had been lost (Figure 5). The DNA molecules maintained in these cells appear therefore to be oligomers of deleted recombinant molecules. This was confirmed by further restriction mapping (Figures 3 and 5): in the three lines, a region of the recombinant genome, which overlaps part of the non-transforming region of BPV1 (*Pvu*II to *Hind*III) and part of the pML2 sequences, had been lost.

In view of these results, an additional set of four cell lines, all derived from mouse C127 cells, were analyzed to check whether the maintenance of an undeleted DNA was characteristic of C127 transformants. It appears from the results summarized in Table V that this is not the case. In two cell lines (SB5, S694) the same results were obtained as in S69 (complete oligomers), but the other two lines (SH105, SB6) exhibited oligomeric forms of a deletion mutant comparable to those maintained in the three FR3T3 transformed lines.

Back-transfer into E. coli of plasmid molecules prepared from transformed mouse cells

We next asked whether the bacterial replicons, in which no deletion could be detected by blot-hybridization, were indeed

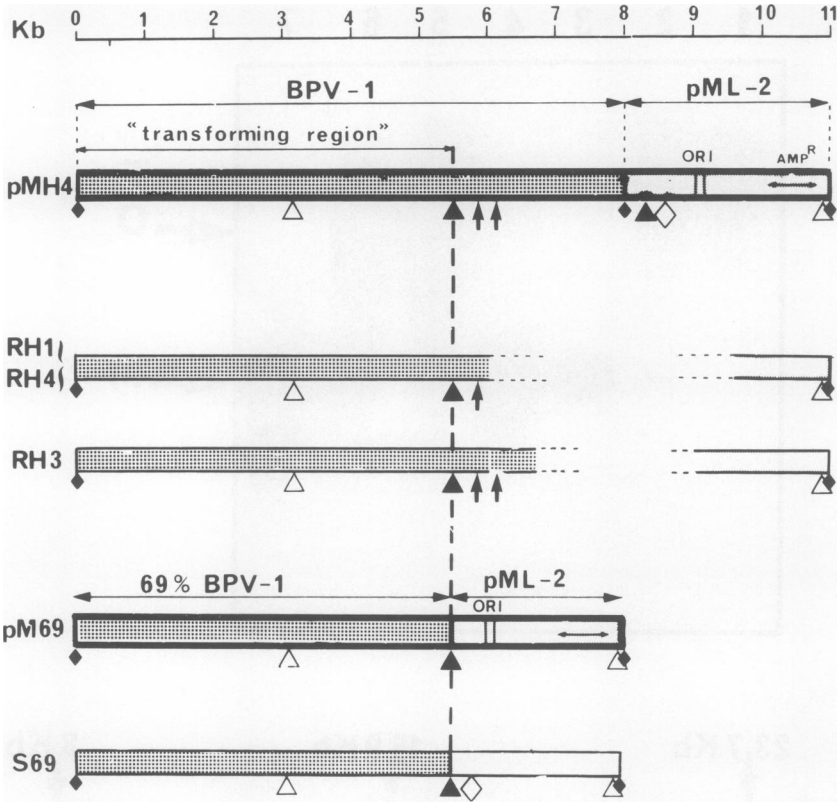

Fig. 5. Restriction enzyme map of BPV1 and bacterial sequences in cell lines RH1, RH3, RH4, and S69. Dotted lines: deletions in the DNA prepared from the transformed cells, as compared with the transforming plasmid. Restriction sites: *Hind*III ♦ ; *Eco*RI △; *Bam*HI ▲; *Pvu*II ↑; *Sal*I ◊ ; BPV1 sequences: ▨; pML2 sequences: ▭

functional for autonomous maintenance in the prokaryotic cell. Transfer of pM69 DNA back into *E. coli* could be achieved after cutting with "one-cut" endonucleases and religation, although with the low frequency expected from the small number of autonomous molecules and from their oligomeric structure.

Total DNA from S69 cells was cleaved with *Bam*HI and fractionated by velocity sedimentation as shown in Figure 4. Fractions corresponding to the linear monomer (30–36) were pooled and dialyzed against 60 mM Tris-HCl buffer pH 7.4, 1 mM EDTA. The resulting solution was adjusted to 0.6 mM ATP, 6 mM $MgCl_2$, 10 mM dithiothreitol and incubated overnight in the presence of 6 units of T4 phage DNA ligase (Boehringer). The mixture was used for transfection of competent *E. coli* cells (strain 1106) that were plated onto ampicillin plates. Four resistant colonies were obtained out of 5×10^{10} bacteria plated. The structure of the plasmids carried in these clones was determined by restriction cleavage and agarose gel electrophoresis on cleared lysates. Two clones analyzed exhibited the same fragment pattern as the original pM69 plasmid after cleavage with either *Bam*HI, or *Hind*III, or with both enzymes. Blot-hybridization against BPV1 and pBR322 probes confirmed the presence of the expected bacterial and viral fragments (Figure 6).

Discussion

BPV1 DNA appears to be highly transforming in rat fibroblasts. Transformed derivatives can be readily selected either by focus formation or by growth in suspension. As previously demonstrated for various BPV1-transformed and tumor cells, as well as for other papillomaviruses (Moar *et al.*, 1981; Wettstein and Stevens, 1982), BPV1 genomes replicate in these cells as free, plasmid-like replicons. The same maintenance mechanism was observed after protoplast fusion for recombinant DNAs that include the transforming region of BPV1 linked to bacterial plasmid sequences. The negative results observed after transfection with recombinant plasmid DNA are likely to be due to the relative inefficiency of this transfer procedure. The protoplast fusion method, that leads to frequencies of transfer at least one order of magnitude higher, produces BPV1-pML2-transformed lines from either mouse or rat fibroblasts with frequencies well above the background levels of detection.

A feature common to all the BPV1-pML2 transformants studied was the oligomeric structure of the autonomous recombinant genomes. The restriction data indicated that a majority of these high mol. wt. forms are head-to-tail oligomers. We cannot exclude that some of the oligomers are catenated molecules, as previously suggested by Law *et al.* (1981) for cell lines transformed by BPV1 DNA. The accumulation of either circular or catenated oligomers, or both, was in fact observed in many instances when autonomous circular DNA replicates in a transformed cell (Hudson and Vinograd, 1969; Cuzin *et al.*, 1970; Jaenisch and Levine, 1971).

Except for their oligomeric structures, some of the chimaeric replicons maintained in fibroblast cells did not exhibit detectable rearrangement (see Table V), and thus may prove useful as genetic vectors. Of particular interest in this perspective is the fact that these plasmids can be transferred both ways between the fibroblast cell and the bacterium after cutting and re-ligation.

Extensive deletions were observed in five cases out of eight.

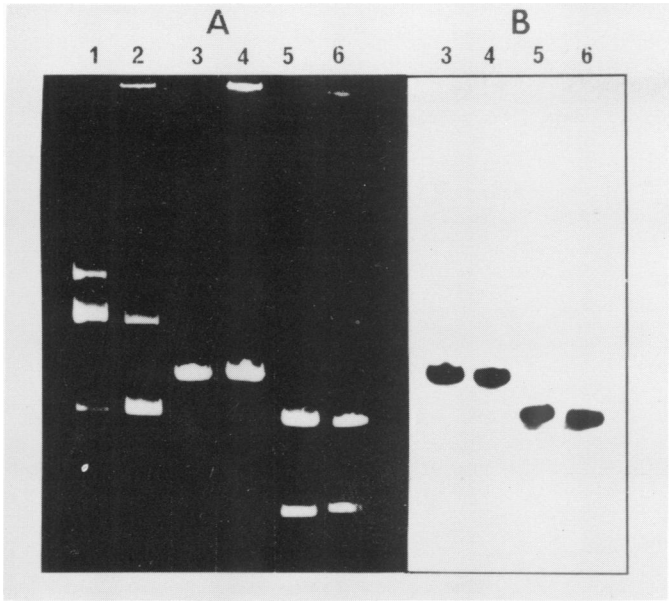

Fig. 6. Comparative analysis of plasmid pM69 and of the plasmid recovered after back transfer into *E. coli*. **Panel A**: ethidium bromide stained agarose gel after electrophoresis of the original plasmid pM69 (**lanes 1, 3,** and **5**) and of the plasmid carried in bacteria transfected with S69 cell DNA (**lanes 2, 4,** and **6**). **Lanes 1, 2:** no restriction cleavage; **3, 4:** *Bam*HI; **5, 6:** *Bam*HI + *Hind*III. **Panel B:** hybridization with ^{32}P-BPV1 DNA after transfer of **lanes 3 – 6** of the gel shown in **Panel A.**

Since any part of the transforming plasmid that was deleted in a given line was maintained in other transformants, we can exclude selection against discrete nucleotide sequences that would hinder the correct replication or segregation of the recombinants. On the other hand, the occurrence of deletions did not appear to be preferentially associated with a given plasmid and they occurred both in the rat and in the mouse cells (Table V). They, in fact, affected the genes for which there was no selective pressure during establishment and growth of the transformants: these DNA sequences (bacterial sequences and viral genes in the small *Bam*HI-*Hind*III fragment) are involved neither in the replication and mitotic segregation of the plasmidial molecule, nor in the expression of the transformed phenotype.

A possible hypothesis is that either the presence of the bacterial sequences, or the transfer by fusion, triggers recombination events in the recipient cell and produce deletions in part of the incoming genomes. If one assumes an equal probability of establishment for any structure that carries the basic elements of the BPV1 replicon (*ori* region, regulatory genes) and the selected transforming gene(s), the maintenance of unselected sequences would depend on the proximity of sequences maintained by a selective pressure. This would be consistent with the observation that, in the limited sample of lines tested, deletions were observed more often in the larger plasmid molecules than in the smaller one (Table V). If this is the case, the probability of establishing a non-rearranged genome should be increased by adding to the larger plasmids additional selectable markers, interspersed with the bacterial DNA and other inserts. Recombinants are now being tested that include one or more of the known dominant resistance markers (bacterial xanthine:guanine phosphoribosyltransferase (Mulligan and Berg, 1981), aminoglycoside phosphotransferase (Colbère-Garapin *et al.*, 1981) and dihydrofolate reductase genes (O'Hare *et al.*, 1981)).

Materials and methods

Cell culture

FR3T3 Fischer rat fibroblasts (Seif and Cuzin, 1977), C127 mouse fibroblasts (Lowy *et al.*, 1978), and their transformed derivatives were grown in Dulbecco modified Eagle's medium supplemented with 10% newborn calf serum (GIBCO).

Molecular cloning of BPV1 DNA

BPV1 DNA purified from virions was recombined *in vitro* with pBR322 and pML2 DNA (Lusky and Botchan, 1981) using standard techniques (Morrow, 1979). Biohazards associated with the experiments described in this publication have been examined previously by the French National Committee and the experiments were carried out according to the rules established by this Committee.

Gene transfer techniques

(i) DNA transfection: the calcium phosphate co-precipitation technique (Graham and Van Der Eb, 1973) was used with 5 – 10 μg of DNA/6-cm Petri plate (2 x 10^5 cells). (ii) Fusion with bacterial protoplasts: the procedure was derived (Rassoulzadegan *et al.*, 1982) from that described by Schaffner (1980).

Selection of transformants

Transformed cell lines were selected as previously described, either by focus formation or by colony formation in agarose medium (Seif and Cuzin, 1977).

Blot hybridization

DNA preparation from cells in culture, restriction enzyme cleavage, electrophoresis, transfer to nitrocellulose, and hybridization with high specific radioactivity ^{32}P-labeled DNA probes were performed as previously described (Mougneau *et al.*, 1980; Meneguzzi *et al.*, 1981). For successive hybridization with different probes, the nitrocellulose sheet, after autoradiography, was treated for 15 min at 42°C with 0.1 N NaOH, extensively washed with 2 x SSC buffer (pH 7.0), and re-hybridized.

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