The EMBO Journal Vol.2 No.9 pp.1487 – 1492, 1983

A bovine papilloma virus vector with a dominant resistance marker replicates extrachromosomally in mouse and *E. coli* cells

Patrick D. Matthias*, Hans U. Bernard, Angela Scott, Ged Brady, Tamotsu Hashimoto-Gotoh and Günther Schütz

Institute of Cell and Tumor Biology, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, FRG

Communicated by G. Schütz
Received on 5 May 1983; revised on 14 June 1983

We describe the construction of a bovine papilloma virus-based vector (pCGBPV9) which contains a dominant selectable marker and replicates autonomously in both mouse and *Escherichia coli* cells. This vector contains the complete bovine papilloma virus genome, a ColE1 replication origin and a dominant selectable marker conferring resistance to kanamycin in bacteria and G418 in eukaryotic cells. A high number of G418^R colonies are obtained after transfer of pCGBPV9 into mouse C127 cells. These G418^R colonies contain vector DNA which replicates autonomously at ~10 – 30 copies per cell. The molecules are in most cases unrearranged and can be rescued into *E. coli* cells by bacterial transformation.

Key words: bovine papilloma virus/shuttle vector/dominant selectable marker/cell transfection

Introduction

Bovine papilloma virus (BPV-1) DNA replicates exclusively as an extrachromosomal molecule in virally induced tumors (Amtmann *et al.*, 1980; Lancaster, 1981) as well as in transformed mouse fibroblasts in culture (Law *et al.*, 1981; Lancaster, 1981). The complete viral genome or a 69% *Hind*III-*Bam*HI fragment thereof have been used as vectors to introduce cloned prokaryotic or eukaryotic genes into mammalian cells in culture (Sarver *et al.*, 1981, 1982; Zinn *et al.*, 1982; DiMaio *et al.*, 1982; Pavlakis and Hamer, 1983; Wang *et al.*, 1983). These recombinant molecules replicate as multicopy plasmids in stably transformed cells. This suggests that a broad potential exists for BPV-1 DNA-derived vectors. The functional analysis of cloned eukaryotic genes would be greatly facilitated if the normal and *in vitro* mutated copies of these genes could be kept in a controlled genetic environment (extrachromosomal maintenance) once stably reintroduced into eukaryotic cells. In addition, it may be possible to rescue BPV recombinants as minichromosomes and thus study associated proteins and the chromatin structure. Thus, the function of *cis*-acting DNA sequences and *trans*-acting factors could be studied in a defined system, as the configuration of all transferred genes in the recipient cells remains the same.

An important limitation of the BPV vector system is that the efficiency of focus formation in mouse C127 fibroblasts is decreased by two orders of magnitude when BPV-1 DNA is linked to sequences of the bacterial plasmid pBR322 (Lowy *et al.*, 1980; Law *et al.*, 1981; Sarver *et al.*, 1982; DiMaio *et al.*, 1982; Binétruy *et al.*, 1982). To circumvent this inhibition of transformation, the BPV-1 DNA has to be freed from pBR322 DNA by enzymatic cleavage prior to transfection into eukaryotic cells. This renders the rescue of BPV recombinants into bacteria impossible. Recently, however, BPV vectors have been described that can be shuttled between eukaryotic and prokaryotic cells. They contain either the complete BPV genome and a derivative of pBR322 (pML2d, Sarver *et al.*, 1982), or the 69% BPV fragment linked to pBR322 sequences and some 'positively acting sequences' such as the human β-globin gene (DiMaio *et al.*, 1982) or the rat growth hormone gene (Kushner *et al.*, 1982) which apparently facilitate the extrachromosomal maintenance of the recombinants.

The second important limitation of all BPV-derived vectors described heretofore is that the identification of the transformants relies on the morphological transformation (focus formation) of contact inhibited mouse cells. This strongly limits the range of recipient cells for BPV recombinants. In this communication we describe the construction and preliminary analysis of a selectable, dually replicating BPV-derived vector. This vector can be shuttled between eukaryotic and prokaryotic cells and provides an alternative to the focus assay. This selectable BPV vector consists of the complete BPV-1 genome, a mini ColE1 replication origin, and, as a dominant selectable marker, the kanamycin gene of Tn5 engineered in such a way that it confers resistance to kanamycin in bacteria and to G418 in mammalian cells. Furthermore, the presence of the *cos* site of bacteriophage λ might allow the efficient cloning of very large DNA inserts. We have introduced this vector into cultured mouse C127 cells and show here that G418-resistant colonies can be otained at high frequencies. In these colonies the BPV vector is replicated autonomously and can be rescued back into *Escherichia coli* cells as an unrearranged molecule.

Results

Construction of BPV recombinants

As a dominant selectable marker for the BPV plasmids, we chose the aminoglycoside phosphotransferase gene of transposon Tn5 which confers resistance to the antibiotic G418 (Jimenez and Davies, 1980; Colbère-Garapin *et al.*, 1981; Southern and Berg, 1982). Starting from the plasmid pAG60 (Colbère-Garapin *et al.*, 1981) we made a cosmid derivative called pHSG272 (Figure 1A) whose detailed construction and characteristics will be published elsewhere (Brady *et al.*, in preparation). Briefly, pHSG272, a ColE1-derived vector, contains the kanamycin resistance gene of Tn5 under the control of two promoters: the eukaryotic HSVtk promoter (as in pAG60) and the prokaryotic P1 promoter of pBR322 (Stüber and Bujard, 1981). The antibiotic resistance marker of pHSG272 can thus be selected for in both prokaryotic and eukaryotic cells where expression confers resistance against 12.5 μg/ml kanamycin and >1.2 mg/ml G418, respectively (Brady *et al.*, in preparation). Furthermore, this plasmid contains the *cos* site of bacteriophage λ which allows the use of the *in vitro* packaging reaction to clone large DNA fragments. To extend this dual selection system towards a eukaryotic extrachromosomally replicating vector, full length BPV-1 DNA was cloned in either orientation as a *Hind*III fragment in the single *Hind*III site of pHSG272. The two

*To whom reprint requests should be sent.

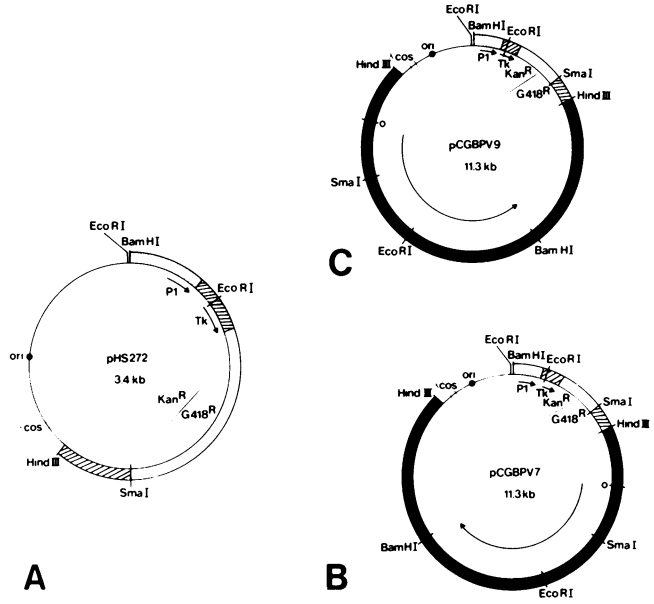

Fig. 1. Structure of the BPV-G418^R recombinants described in the text. (**A**) Cosmid pHSG272 (Brady *et al.*, in preparation). (**B,C**) Cosmids pCGBPV7 and pCGBPV9, respectively. Thin line: DNA segment containing the ColE1 replication origin (●) and the λ *cos* region (□). Stippled bar: pBR322 DNA segment containing the P1 promoter (short arrow: Stuber and Bujard, 1981). Hatched bar: HSV *tk* DNA segment containing a eukaryotic promoter (short arrow) and polyadenylation signals (Colbère-Garapin *et al.*, 1981). Open bar: kanamycin resistance gene of transposon Tn5. Solid bar: BPV-1 DNA; the long arrows indicate the approximate location of the BPV-1 early transcripts (Chen *et al.*, 1982).

resulting constructs were called pCGBPV7 and pCGBPV9 (Figure 1B and C). Similar recombinants have also been made that contain the same BPV fragment and kanamycin/G418 resistance unit but have, instead of a ColE1 replication origin, a pML2 (pBR322) replication origin and, in addition, the ampicillin resistance gene (pMGBPV plasmids, structure not shown).

Transfer of pCGBPV7 and pCGBPV9 into mouse C127 cells

We used mouse C127 cells as recipients to study whether transformation and stable maintenance, as determined by G418 resistance or focus formation, could be achieved using these BPV-containing plasmids. Plasmid-$CaPO_4$ precipitates were prepared with or without carrier DNA, in order to examine the influence of calf thymus carrier DNA on the transformation efficiency and on the state of the DNA in the transformed cells. Cells were transfected with 1 μg plasmid DNA per 10 cm dish (10^6 cells) and after 14 days of incubation in G418-containing medium, G418-resistant colonies were scored. Foci were counted after 21 days of growth in medium lacking this antibiotic. The number of G418^R colonies obtained was compared with that obtained after transfection of the cells with pAG60 or pHSG272, which lack any BPV1 DNA. Results from a typical experiment (one out of five similar experiments) are shown in Table I.

In all experiments the number of G418-resistant colonies was much higher than the number of foci. The addition of calf thymus carrier DNA reproducibly led to a 5- to 20-fold increase in the number of G418-resistant colonies with the plasmids pAG60, pHSG272 and pCGBPV7 (Table I and data not shown). In contrast, the number of G418-resistant colonies obtained with pCGBPV9 was increased only ~2.5-fold (Table I) and in two experiments no effect of the calf thymus

Table I. Transformation of mouse C127 cells to G418 resistance with various plasmids

Plasmid DNA	Calf thymus carrier DNA	G418^R colonies	Foci
pAG60	–	111	0
	+	510	0
pHSG272	–	181	0
	+	1248	0
pCGBPV7	–	368	0
	+	3441	176
pCGBPV9	–	1721	257
	+	4529	573
None	+	0	0

Mouse C127 cells (10^6/dish) were transfected with 1 μg of plasmid DNA with or without the addition of calf thymus carrier DNA (20 μg/dish); the $CaPO_4$ DNA co-precipitate was removed after 16 h and the cells were replated 48 h after DNA addition at a density of 10^6/10 cm dish and challenged with G418 containing or normal medium. G418-resistant colonies were scored after 14 days and foci after 21 days. The numbers are expressed per pmol of plasmid DNA.

carrier DNA was observed. We have also tested, in a similar manner, several other BPV-G418^R plasmids which contain the Amp^R gene, the origin of replication of pML2 and no λ *cos* site (pMGBPV plasmids). None of these recombinants showed this relative carrier independence and the results obtained were comparable with those obtained with pAG60, pHSG272 or pCGBPV7 (data not shown).

The G418-resistant colonies derived from BPV vectors were of two morphological types. Some colonies displayed a transformed phenotype, whereas others had a normal appearance. The ratio of colonies with the normal or transformed phenotype varied from experiment to experiment. On average, with pCGBPV9, 50–90% of the colonies displayed the transformed phenotype. With pCGBPV7 this percentage was always significantly lower. When analyzed by Southern blotting, both types of G418-resistant colonies resulting from pCGBPV9 transformation were shown to contain the vector DNA in an extrachromosomal state (see below).

State of pCGBPV7 and pCGBPV9 DNA in G418-resistant C127 transformants

To test for extrachromosomal maintenance of these plasmids in transformed eukaryotic cells, pCGBPV7- and pCGBPV9-derived G418-resistant clones were grown up to 2 x 10^7 cells, total DNA was prepared (Colbère-Garapin *et al.*, 1981), and analyzed by the Southern blotting technique. The data obtained from seven pCGBPV7 and 12 pCGBPV9 clones from one transformation experiment are shown in Figure 2. Undigested DNA from each clone was run on a 0.6% agarose gel, blotted onto nitrocellulose filters and hybridized to ^{32}P-labelled pCGBPV9 DNA (Figure 2A, lanes 5–16, 2B, lanes 2–8). pCGBPV9 DNA isolated from bacterial cells and mixed with calf thymus DNA was used as a size marker (Figure 2A, lane 1, 2B, lane 1). All 12 pCGBPV9 clones (Figure 2A, lanes 5–16) contain an extrachromosomal DNA species migrating at the same position as form I pCGBPV9 DNA (Figure 2A, lane 1). The addition of calf thymus carrier DNA during cell transformation had no apparent influence on the state of pCGBPV9 DNA in the

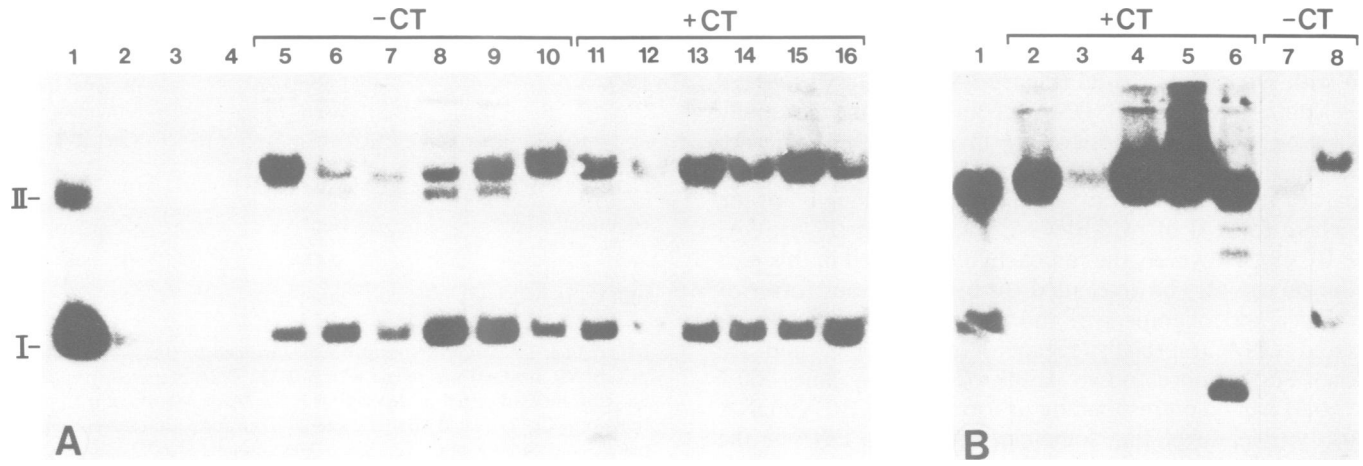

Fig. 2. Southern blotting analysis of total DNA from various G418^R cell lines containing pCGBPV9 or pCGBPV7 DNA. Total cell DNA was electrophoresed on a 0.6% agarose gel, blotted onto nitrocellulose and hybridized to nick-translated pCGBPV9 DNA. **Lanes 1** contain 200 pg pCGBPV9 from bacterial origin mixed with 1 μg calf thymus DNA. In **A**, cells were transfected with pCGBPV9 DNA in the absence (**lanes 5 – 10**) or in the presence (**lanes 11 – 16**) of calf thymus carrier DNA (20 μg/transfection). **Lanes 2 – 4** are pAG60 transformants. DNA of **lanes 5 – 7** and **11 – 13** was derived from colonies with the transformed phenotype, whereas DNA of **lanes 8 – 10** and **14 – 16** was derived from colonies with the untransformed phenotype. In **B**, cells were transfected with pCGBPV7 in the absence (**lanes 7,8**) or in the presence (**lanes 2 – 6**) of calf thymus carrier DNA (20 μg/transfection). I and II indicate the position of form I and form II of pCGBPV9, respectively.

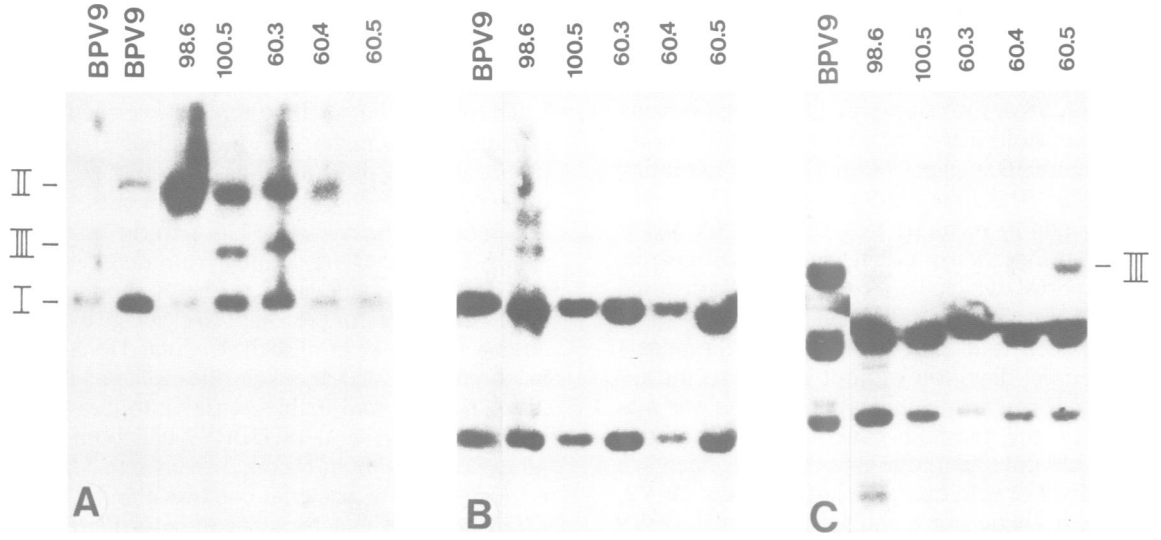

Fig. 3. Southern blot analysis of undigested (**A**), *Hind*III digested (**B**) and *Eco*RI digested (**C**) total DNA from various pCGBPV9 G418^R transformants. DNA (5 μg) was run on a 0.6% agarose gel, blotted onto nitrocellulose and hybridized to nick-translated pCGBPV9 DNA. The left-most BPV9 lane (**A**) contains 18 pg pCGBPV9 DNA, whereas the other BPV9 lanes (**A,B,C**) contain 180 pg of pCGBPV9 DNA, mixed with calf thymus carrier DNA. I, II and III indicate the position of the supercoiled, open circular and linear form of pCGBPV9, respectively. Sample 60.5 in **A** was underloaded (as judged by the ethidium bromide staining of the gel; not shown).

various transformants (compare lanes 5 – 10 of Figure 2A showing transformants made without carrier DNA with lanes 11 – 16 showing transformants made with carrier DNA). One of these clones contains an additional faster migrating DNA species which is likely to be a deleted plasmid (Figure 2A, lane 11, bottom). In contrast to that, only one out of seven pCGBPV7 clones contains a DNA species of the size expected for full length plasmid (Figure 2B, lane 8), while another one contains a faster migrating DNA species which is also probably a deleted plasmid (Figure 2B, lane 6). The remaining five pCGBPV7 clones analyzed here do not show any DNA molecules migrating similarly to supercoiled monomer pCGBPV9 DNA (Figure 2B, lanes 2 – 5 and 7). As expected, none of the three pAG60 G418-resistant colonies shows any extrachromosomal material (Figure 2A, lanes 2 – 4; note that the faint fast migrating band visible in lane 2 of Figure 2A is due to spillover from lane 1).

All of the 19 clones presented here also exhibit a complex array of slowly migrating DNA species, part of which migrate similarly to form II pCGBPV9 DNA (Figure 2A, lane 1, 2B, lane 1). Some of these bands might be form I and form II dimers of the plasmid used, whereas some co-migrate with the bulk of high mol. wt. cellular DNA and could thus represent integrated DNA (see Discussion).

The pMGBPV plasmids behaved similarly to pCGBPV7 when transfected into C127 cells. Only a minor proportion of the G418-resistant colonies contain unrearranged extrachromosomal monomer plasmid material (results not shown).

From another transformation experiment exhibiting identical characteristics, the DNAs of five pCGBPV9-derived

G418-resistant clones were analyzed in more detail by Southern blotting experiments with or without prior restriction with *Hind*III or *Eco*RI (Figure 3A, B, C). All five cell lines contain pCGBPV9 DNA as an extrachromosomally replicating plasmid, co-migrating in an agarose gel with supercoiled pCGBPV9 DNA (Figure 3A). As seen in Figure 2, part of the hybridizing material migrates in a region of the gel where form II of pCGBPV9 DNA migrates (Figure 3A, lane BPV9). However, the radioactivity observed in this part of the gel can also be accounted for by the various forms of the dimer and oligomers of the plasmid as well as by integrated DNA (especially Figure 3A, lane 98.6, and see below). Furthermore, in two samples (Figure 3A, lanes 100.5 and 60.3) a band corresponding to form III pCGBPV9 DNA is also visible, suggesting some cleavage of the DNA in the samples.

Digestion of pCGBPV9 DNA with *Hind*III gives two fragments of 7.95 kbp and 3.4 kbp in length, while *Eco*RI digestion produces three fragments which are 6.4, 4.5 and 0.45 kbp long (Figure 1C). Figure 3B and C show the results of the *Hind*III and *Eco*RI digests performed with the five cell lines DNAs described above. As a size marker we used pCGBPV9 plasmid DNA mixed with carrier DNA and digested with the appropriate restriction enzyme (Figure 3B and C, lane BPV9). All five cell lines show the expected *Hind*III or *Eco*RI fragments co-migrating with the control DNA (the third *Eco*RI fragment, 0.45 kbp long, ran out of the gel). One of the cell lines, 98.6, also shows additional hybridizing fragments of various molecular size reflecting several recombination events (Figure 3B, C, lane 98.6). The slow migrating band found in cell line 60.5 upon *Eco*RI digestion (Figure 3C, lane 60.5) corresponds to form III pCGBPV9 DNA and is most likely a partial digestion product, since it can also be observed in the control which was only partially digested (Figure 3C, lane BPV9). The hybridization seen at the top of the gel with undigested total DNA (Figure 3A) disappears upon *Hind*III or *Eco*RI digestion without leading to the appearance of 'junction' fragments, indicating that the vast majority if not all of the plasmid material is truly extrachromosomal and has not undergone gross rearrangements in the mammalian cells. For reference, the left-most slot BPV9, Figure 3A, shows a single copy equivalent of pCGBPV9 DNA (18 pg). DNA from cell line 98.6, however, shows upon *Eco*RI or *Hind*III digestion the two expected fragments and a variety of other hybridizing fragments of various sizes. These fragments may be the junction fragments arising from integration of pCGBPV9 at different locations in the cellular DNA or may reflect rearrangements within plasmid DNA maintained as oligomeric structures.

We did not precisely quantitate the amount of extrachromosomal material present in our transformed cell lines. However, by comparing the signals obtained with these cell lines with that obtained with known amounts of pCGBPV9 DNA, we estimate that G418-resistant transformants carry ~10–30 plasmid copies per cell. This number is in good agreement with other reports (Law *et al.*, 1981; Kushner *et al.*, 1982; DiMaio *et al.*, 1982).

Rescue of pCGBPV9 DNA from G418-resistant C127 cells

To obtain further evidence for the extrachromosomal maintenance of pCGBPV9 in G418-resistant transformed C127 cells a rescue in *E. coli* was attempted. Highly competent $CaCl_2$-treated HB101 cells (Dagert and Ehrlich, 1979) were incubated with undigested total DNA from 10 different G418-resistant transformants. Kanamycin (Kan)-resistant bacterial colonies were obtained from all DNA samples. Approximately 1 μg of total DNA yielded 10–20 Kan-resistant colonies. In each case the plasmid DNA from three HB101 colonies was prepared (Holmes and Quigley, 1981), examined by restriction analysis with up to five enzymes and was found to contain, in all cases, exclusively unrearranged pCGBPV9 DNA (data not shown).

Table II. Rescue of pCGBPV9 in bacterial cells

DNA	Restriction digest	Bacterial transformants (No.)	
		Kan	Tet
None	None	0	0
60.3 (Kan^R, Tet^S)	None	35	64
+	*Sau*3A	0	0
pBR322 (Kan^S, Tet^R)	*Mbo*I	2	72

60.3 cellular DNA (G418^R transformant obtained by transformation with pCGBPV9, see text) was mixed with pBR322 DNA prepared from *dam*$^+$ bacteria, mock-digested or digested with *Sau*3A or *Mbo*I and used to transform $CaCl_2$-treated HB101 bacteria. Transformation mixes were then plated onto either Kan or Tet plates. The amounts of 60.3 and pBR322 DNA had been adjusted so that they would give comparable numbers of transformants when not restricted.

To rule out the possibility that the rescued DNA was a contaminant stemming from the original DNA applied to the mouse cells in the transfection procedure and carried over during the growth of the G418^R colonies, we examined the sensitivity of the rescuable DNA to the enzymes *Sau*3A and *Mbo*I. Plasmid DNA coming from *dam*$^+$ *E. coli* cells (such as HB101), is methylated at the A residue in the sequence GATC and therefore resistant to cleavage by *Mbo*I but not by *Sau*3A (Peden *et al.*, 1980). Cellular DNA, or DNA which has been replicated in eukaryotic cells, has no methylation at the A residues and is thus sensitive to cleavage by *Mbo*I, as well as by *Sau*3A. If pCGBPV9 had been replicated in the mouse cells it should become sensitive to *Mbo*I digestion and no Kan-resistant bacterial colonies should be obtained after *Mbo*I cleavage and bacterial transformation. 15 μg of 60.3 cellular DNA (see Figure 3A, B and C) containing ~400 pg pCGBPV9 DNA (as estimated from Southern blots and bacterial rescue experiments) were mixed with 500 pg of pBR322 DNA prepared from *dam*$^+$ bacterial strain (HB101). This mixture was divided into several aliquots which were mock-digested or digested with *Sau3A or Mbo*I. After phenol extraction, ether extraction and ethanol precipitation the different samples were used to transform HB101 bacteria which were subsequently plated onto either Kan or tetracycline (Tet) plates. *Sau*3A digestion eliminated both the Kan and Tet transforming activities (pCGBPV9, pBR322 respectively), whereas *Mbo*I drastically reduced the Kan transforming activity (pCGBPV9) (Table II). This result confirms that the pCGBPV9 DNA which can be rescued in *E. coli* was passaged in mouse cells and was not a contaminant carried over during the growth of the G418 clones.

Discussion

Here we describe a BPV-1-derived vector, pCGBPV9, which carries a dominant selectable marker (Kan/G418) and

replicates autonomously in both mouse C127 and *E. coli* cells. Transfection of pCGBPV9 DNA into C127 cells and selection for G418 resistance yields high numbers of stable G418^R transformants (Table I). These transformants contain free pCGBPV9 DNA that is unrearranged as judged by restriction analysis and which can be rescued into *E. coli* cells by bacterial transformation. Occasionally, in addition to the input vector molecule, one can also detect rearranged or integrated molecules. One example of this is provided by cell line 98.6 in which the amount of free monomer pCGBPV9 DNA is small, although the absolute amount of plasmid hybridizing DNA is comparable with that contained in other G418^R transformants. Unrearranged pCGBPV9 DNA can be rescued from this cell line into *E. coli* (results not shown) proving that indeed some plasmid DNA is maintained extrachromosomally in this cell line.

Our belief that most of the plasmid hybridizing material found in the G418^R mouse cells is extrachromosomal, is based on several observations. In most cases a significant portion of the hybridizing material co-migrates with form I pCGBPV9 monomer DNA (Figure 2A). With undigested DNA, the remainder of the radioactivity is found in a region of the gel where high mol. wt. cellular DNA migrates. We know that various types of circular pCGBPV9 molecules (open circular monomer, supercoiled and open circular dimer) also migrate in this area of the gel and are not well resolved under these electrophoresis conditions. Following restriction digestion of the DNAs, the slowly migrating hybridizing material disappears completely, without leading to the appearance of unexpected fragments (Figure 3B, C). An exception to this behavior, however, is cell line 98.6 which upon digestion shows the expected fragments, but also a variety of weakly hybridizing fragments (Figure 3B, C). The intensity of these fragments corresponds approximately to a single copy signal and therefore probably represents junction fragments of an integrated structure, or perhaps reflects rearranged plasmids maintained extrachromosomally at very low copy number.

Surprisingly, pCGBPV7, which carries the BPV fragment in the opposite orientation relative to the plasmid moiety, behaves differently from pCGBPV9 when transfected into mouse cells. Only rarely can extrachromosomally maintained unrearranged or rearranged form I monomers be found. In the other transformants, plasmid hybridizing material can be found exclusively in the high mol. wt. region of the gel. We have no explanation for this different behavior of pCGBPV7 and pCGBPV9, but it is worth noting that in pCGBPV9 the BPV 'early' transcripts (Chen *et al.*, 1982) and the Kan/G418 transcripts are read in opposite orientation, whereas in pCGBPV7 they are both in the same orientation.

The other series of G418^R recombinants we made (pMGBPV plasmids) showed only rarely unrearranged plasmid DNA maintained extrachromosomally in transfected mouse cells. Therefore, it seems that the plasmid part contained in the pCGBPV vectors is more suitable for extrachromosomal maintenance than the one contained in the pMGBPV vectors. These latter plasmids differ from the pCGBPV constructs in at least three points. The origin of plasmid replication in pMGBPV is supplied by pML2 (pMB1/pBR322 derivative; Lusky and Botchan, 1981) whereas in pCGBPV it is supplied by pHSG262, a ColE1 derivative (Brady *et al.*, in preparation; Hashimoto-Gotoh and Inselburg, 1979). The pMGBPV plasmids contain an additional antibiotic resistance gene (the β-lactamase gene from pML2) and do not contain the *cos* site region of bacteriophage λ. Further experiments would be needed to delineate which of these differences explains the poor extrachromosomal maintenance of the pMGBPV plasmids.

While with the plasmids pAG60, pHSG272, pCGBPV7 and pMGBPV, the addition of calf thymus carrier DNA reproducibly stimulated the transfection rates by a factor of 5–20 for G418^R colonies, the rate with pCGBPV9 was already nearly maximal without added carrier DNA. Although a detailed understanding of this observation needs further study, these data may indicate that some of the effect of carrier DNA may be to facilitate integration of the selectable marker into chromosomal DNA, and therefore extrachromosomal maintenance, which was reproducibly observed only with pCGBPV9, bypasses this pathway.

The number of foci obtained in mouse C127 cells with our G418^R BPV plasmids was consistently lower than the number of G418^R colonies, generally by a factor of at least 10-fold (Table I). We believe that this discrepancy is not simply due to trivial reasons (e.g., the fact that G418^R colonies are easier to score than foci), but reflects post-transfectional events depending on the selection procedure. Preliminary data indicate that many foci did not exhibit resistance to G418 and had deleted part of the vector DNA (details of these observations will be published in a separate communication). Similar findings have also been made by Binétruy *et al.* (1982), who found, in many cases, deletions in the vector moiety or in the BPV non-transforming region.

The fact that pCGBPV9 is a cosmid allows use of the *in vitro* packaging reaction (Hohn and Murray, 1977) to efficiently clone large DNA fragments. At the present time we do not yet know the stability of pCGBPV9 with cloned inserts in mouse cells. We are currently analyzing this parameter using pCGBPV9 containing either very large inserts (~35 kbp mouse DNA) or the HSV1 *tk* gene. Furthermore, we are also using this vector to ask whether hormonally regulated expression of a tryptophan oxygenase-chloramphenicol acetyltransferase fusion gene (Danesch *et al.*, in preparation) can be obtained in an episomal state in transfected cells. This would allow analysis of the chromatin structure of this fusion gene in the induced and uninduced state, free from cellular interferences.

Materials and methods

Construction of BPV recombinants

The cosmid pHSG272 (Brady *et al.*, in preparation) was opened at its unique *Hind*III site, treated with calf intestine phosphatase (Weaver and Weissmann, 1979) and ligated to the gel-purified 7.95-kb BPV-1 DNA fragment (obtained by *Hind*III cleavage of the plasmid pBR-BPV1 which contains a complete copy of BPV-1 DNA in the *Hind*III site of pBR322). The two plasmids thus obtained, representing both orientations of the BPV insert, were called pCGBPV7 and pCGBPV9 (Figure 1B,C). pHSG272 confers kanamycin resistance to bacteria (HB101, DH-1: 12.5 μg Kan/ml) and G418 resistance to mammalian cells (up to 8 mg/ml in Ltk$^-$ cells, T. Jenuwein, unpublished data). The pMGBPV plasmids (structure not shown) were obtained similarly by cloning the entire BPV-1 DNA fragment into the *Hind*III site of a pML2 vector carrying the same Kan/G418 resistance unit as pHSG272.

Cell culture and DNA transfection

Mouse C127 cells (provided by P.M. Howley, *via* P. Gruss) were maintained at low density in Dulbecco modified Eagle's (DME) medium supplemented with 10% fetal calf serum (FCS), 10 mM Hepes (pH 7.4) and 100 μg/ml penicillin/streptomycin. For DNA transfections the plasmid DNA (1 or 2 μg) was mixed with $CaCl_2$ and Hepes Buffered Saline with or without calf thymus carrier DNA (20 μg) and the precipitate was allowed to form at room

temperature for at least 30 min (Wigler *et al.*, 1978). It was then added to the cells in 10 cm dishes containing 10 ml of fresh medium. After 16–20 h at 37°C, the $CaPO_4$ precipitate was removed, the cells were washed with phosphate buffered saline (PBS) and further incubated with fresh medium. G418 selection (1.2 mg/ml G418 in medium) was started 2 days after the addition of DNA and G418-containing medium was changed every 6 days. G418-resistant colonies were scored and picked after 14 days of selection. When grown in mass culture, the G418-resistant colonies were kept under selective pressure at all times. For foci selection, DME medium containing 5% FCS was used and the cells were refed ever 3–4 days until visible foci appeared (21–25 days).

DNA analysis

Total cellular DNA was extracted as described by Colbère-Garapin *et al.* (1981) or in some cases by a simplification of this procedure, followed by isopropanol precipitation (H.U.Bernard, unpublished data). DNAs were then resuspended and stored in Tris 10 mM (pH 8.0), EDTA 1 mM. The agarose gels were run in Tris-borate buffer and blotted to nitrocellulose filters. The conditions for hybridization and washing of the blots were as in Jolly *et al.* (1982). To ensure a comparable migration of the pCGBPV9 DNA used as size marker with that prepared from transformed cells, it was mixed with 2.5 μg calf thymus or mouse carrier DNA prior to loading on the gel.

Bacterial rescue

Highly competent *E. coli* HB101 cells were prepared by $CaCl_2$ treatment as described by Dagert and Ehrlich (1979). Transformation efficiency obtained was $\sim 5 \times 10^5$ colonies/μg pCGBPV9. From reconstruction experiments with a mixture of pCGBPV9 and C127 cellular DNA, it was concluded that the presence of high mol. wt. chromosomal DNA in large excess did not significantly decrease the overall transformation efficiency of pCGBPV9 under our experimental conditions.

Acknowledgements

We wish to thank H. Lehrach and A.M. Frischauf for hospitality to one of us at the initial stage of this work, P.M. Howley for gifts of material and unpublished information, P.J.L. Daniels (Schering Corporation) for generous gifts of G418, U. Danesch, R. Miksicek, R. Renkawitz and G. Scherer for critical comments on the manuscript and Ms.U.Joa for typing the manuscript. This work was supported by DFG grant Schü 51/4-1 and the Fonds der Chemischen Industrie.

References

Amtmann,E., Müller,H. and Sauer,G. (1980) *J. Virol.*, **35**, 962-964.
Binétruy,B., Meneguzzi,G., Breathnach,R. and Cuzin,F. (1982) *EMBO J.*, **1**, 621-628.
Chen,E.Y., Howley,P.M., Levinson,A.D. and Seeburg,P.H. (1982) *Nature*, **299**, 529-534.
Colbère-Garapin,F., Horodniceanu,F., Kourilsky,P. and Garapin,A.-C. (1982) *J. Mol. Biol.*, **150**, 1-14.
Dagert,M. and Ehrlich,S.D. (1979) *Gene*, **6**, 23-28.
DiMaio,D., Treisman,R. and Maniatis,T. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4030-4034.
Hashimoto-Gotoh,T. and Inselburg,J. (1979) *J. Bacteriol.*, **139**, 608-618.
Hohn,B. and Murray,K. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 3259-3263.
Holmes,D. and Quigley,M. (1981) *Anal. Biochem.*, **114**, 193-197.
Jimenez,A. and Davies,J. (1980) *Nature*, **287**, 869-871.
Jolly,D.J., Esty,A.C., Bernard,H.U. and Friedmann,T. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5038-5041.
Kushner,P.J., Levinson,B.B. and Goodman,H.M. (1982) *J. Mol. Appl. Genet.*, **1**, 527-538.
Lancaster,W.D. (1981) *Virology*, **108**, 527-538.
Law,M.F., Lowry,D.R., Dvoretzky,I. and Howley,P.M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2727-2731.
Lowry,D.R., Dvoretzky,I., Shober,R., Law,M.F., Engel,L. and Howley, P.M. (1980) *Nature*, **287**, 72-74.
Lusky,M. and Botchan,M. (1981) *Nature*, **293**, 79-81.
Pavlakis,G.N. and Hamer,D.H. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 397-401.
Peden,K.W.C., Pipas,J.M., Pearson-White,S. and Nathans,D. (1980) *Science (Wash.)*, **209**, 1392-1396.
Sarver,N., Gruss,P., Law,M.F., Khoury,G. and Howley,P.M. (1981) *Mol. Cell Biol.*, **1**, 486-496.
Sarver,N., Byrne,J.C. and Howley,P.M. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7147-7151.
Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **1**, 327-341.
Stüber,D. and Bujard,H. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 167-171.
Wang,Y., Stratowa,C., Schaefer-Rider,M., Doehmer,J. and Hofschneider, P.H. (1983) *Mol. Cell. Biol.*, **3**, in press.
Weaver,R.F. and Weissmann,C. (1979) *Nucleic Acids Res.*, **7**, 1175-1193.
Wigler,M., Pellicer,A., Silverstein,S. and Axel,R. (1978), *Cell*, **14**, 725-731.
Zinn,K., Mellon,P., Ptashne,M. and Maniatis,T. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4897-4901.