Stability of a Bacterial Gene in a Bovine Papillomavirus-based Shuttle Vector Maintained Extrachromosomally in Mammalian Cells

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(Accepted 15 September 1986)

SUMMARY

In order to analyse the stability of cloned genes in a viral vector we have constructed a shuttle vector based on bovine papillomavirus and the Escherichia coli gene lacZ. Propagation of this vector in mouse C127 cells and analysis of vector sequences in bacteria produced no detectable mutations in the lacZ gene in over 6137 clones analysed. This is 100-fold less than the mutation frequency observed when the same and similar target genes are replicated in monkey COS cells using a simian virus 40-based shuttle vector.

Extrachromosomal vectors based on viral replication origins are becoming increasingly important for the expression of cloned genes and the analysis of complex host processes such as the molecular basis of mutation in mammalian cells. Of particular importance are shuttle vectors which can replicate in both mammalian cells and in bacteria; hence bacterial, molecular and genetic systems can be used for the construction of vectors and the rapid analysis of events that arise in mammalian cells.

This approach has been used to study the important question of the stability of extrachromosomal DNA sequences in vectors propagated in monkey (Ashman & Davidson, 1984), rodent and human cells (Calos et al., 1983) using simian virus 40 (SV40)-, polyoma- or BK papovavirus-based shuttle vectors. In each case the stability of the vector as measured by the spontaneous mutation frequency in the target gene lacI (Calos et al., 1983), supF (Sarkor et al., 1984), galK (Razzaque et al., 1983), gpt (Ashman & Davidson, 1984) or lacZ (unpublished observations) has been observed to be between 0.5 and 3.7%. This value is significantly greater than the spontaneous mutation frequency observed in chromosomal genes in cultured mammalian cells, e.g. the hypoxanthine phosphoribosyl transferase and adenosine phosphoribosyl transferase (aprt) loci (Chasin, 1974). Recent studies have shown that the high frequency of mutations observed in papovavirus-based shuttle vectors may be due to a combination of factors such as DNA damage acquired during the transfection procedure and the subsequent fixation of pre-mutagenic lesions through replication of the vectors in the host cell nucleus (Miller et al., 1984).

In an attempt to derive a shuttle vector system with a high degree of stability we have adapted pCGBPV9 (Matthias et al., 1983), a shuttle vector based on the full length bovine papillomavirus type 1 (BPV) genome and ColE1 plasmid sequences. By inserting into this vector a fragment of the lacZ gene from M13 mp2 (Messing et al., 1977) as an indicator gene we have constructed the shuttle vector pGM8. BPV offers advantages over other papovaviruses in that papillomaviruses once established in the nucleus of a cell may be maintained at a steady copy number, usually in the range of 10 to 200 per cell, and that cell lines harbouring exclusively monomeric extrachromosomal plasmids can be obtained. We show here that the vector pGM8 in an...
Fig. 1. Map of BPV shuttle vector pGM8. Restriction endonuclease sites for EcoRI (Eco) and HindIII (Hd) are indicated as are the BPV sequences (I), lacZ sequences (II) and bacterial plasmid sequences (III). The arrows indicate the direction of transcription of the apt gene, lacZ gene and the BPV major early transcripts. Promoters are designated P.

Fig. 2. Twenty micrograms of DNA from individual cloned pGM8-transfected cell lines were digested with EcoRI and analysed on a 0.7% gel. The gel samples were transferred and hybridized with radiolabelled pGM8. (a) 1 ng of pGM8, (b) C127 T9, (c) T6, (d) T7, (e) T8, (f) T1, (g) T3 and (h) S6, a C127 line transfected with pSV2neo. The major pGM8 bands are indicated at 6.0 kb, 4.5 kb and 0.9 kb.

established C127 transfected cell line, C127:T1, has a very high degree of stability. No mutations within the lacZ target gene contained in pGM8 have been observed in over 6000 bacterial clones analysed. This degree of stability is nearly two orders of magnitude higher than that observed for the same target gene in a simian virus 40 (SV40)-based vector after replication in simian COS-7 cells (unpublished observations).

The BPV-based shuttle vector pGM8 (Fig. 1) was constructed from M13 mp2 and the plasmid pCGBPv9 BS, which consists of the aminoglycoside phosphotransferase (apt) gene of the transposon Tn5 under the control of both the bacterial P1 and herpes simplex virus thymidine...
kinase (TK) promoters, and the full length BPV-1 genome. This plasmid confers kanamycin resistance in *Escherichia coli* and resistance to G418 (1.2 mg/ml) in mouse C127 cells. The plasmid pCGBP9 B5 contains a single *BamHI* site located between the ColE1 replication origin and the promoter for the *apt* gene, into which was cloned a *Sau3AI* fragment carrying the *lacZ* sequence from M13 mp2. This vector, pGM8, was selected on the basis of kanamycin resistance and its ability to complement the *lacZ* M15 deletion mutation in a *recA* bacterial strain, FX1. Blue bacterial colonies are observed when FX1 harbouring pGM8 is plated on agar containing the chromogenic β-galactosidase indicator 5-bromo-4-chloro-3-indoyl-D-galactopyranoside (X-Gal). To study the rate of mutation of the *lacZ* gene in pGM8 after propagation in mammalian cells the vector was transfected into non-transformed C127 cells.

Sub-confluent monolayers of C127 cells (2 × 10^5 cells per 9 cm dish) were transfected with 10 μg of pGM8 by the calcium phosphate co-precipitation procedure (Graham & van der Eb, 1973) without added carrier DNA, and the transfected cells were selected and maintained in G418. Twelve days after selection, G418-resistant colonies were scored indicating a transfection frequency of 1.1 × 10^-3 transformants per microgram of DNA. Ten independent clones were picked for analysis. Of the ten, nine exhibited a transformed phenotype (refractile and not contact-inhibited), and one was normal (flat, epithelial-like and contact-inhibited). C127 cells transfected with pSV2neo (Southern & Berg, 1982) produced clones at a frequency of 3 × 10^-3, and none had a transformed phenotype.

To determine whether pGM8 was maintained in an extrachromosomal state within any of the clones isolated, DNA was prepared from nine of the transfected clones after 23 days in continuous culture (one clone failed to grow after being picked). As observed by Matthias *et al.* (1983), with the parental vector pCGBP9 most cell lines examined contained slow migrating, high molecular weight DNA sequences that hybridized to pGM8 and only three lines (T1, T7 and T9) contained significant quantities of monomeric episomal pGM8. Interestingly, these three clones grew at a faster rate than the other cell lines and of the nine cell lines examined they contained the largest number of pGM8 plasmid sequences. The cell line with the untransformed phenotype (T6) contained very little material that hybridized to pGM8 and no episomal material was detected. In order to determine whether the high molecular weight pGM8 DNA within the transformed clones was integrated into chromosomal sequences, or whether pGM8 was present as multimeric episomal copies, DNA extracted from clones T7 and T9 was further analysed by sodium chloride density gradient centrifugation. This indicated that most of the pGM8-hybridizing material existed as multimeric episomes, and was not integrated into the genome (data not shown). The monomeric episomes represent only a small proportion of the total pGM8-hybridizing material. Restriction analysis of T1, T7 and T9 showed that the cell lines T7 and T9 contained pGM8 sequences that were grossly rearranged when compared to the plasmid pGM8. An *EcoRI* digest of pGM8 produced three bands of 6.0, 4.5 and 0.9 kb. Fig. 2(f) shows that digestion of T1 DNA with *EcoRI* produced the same arrangement of bands, suggesting that at this level of sensitivity, the DNA had not been rearranged. *EcoRI* digestion of DNA from cell lines T7 and T9 produced the three *EcoRI* bands expected, but also produced other predominant bands suggesting that rearrangements had taken place in the pGM8 sequence contained in these cell lines.

After 7 weeks in continuous culture and G418 selection, analysis of extracted material (Hirt, 1967) from C127 T1 showed unrearranged episomal pGM8 at a copy number of 10 to 20 per cell, whereas T7 and T9 contained exclusively deletion derivatives of pGM8. Consequently, the T1 cell line was selected to generate material for the analysis of deletion and mutation events within the *lacZ* target gene in pGM8.

From Southern (1975) analysis, the pGM8 sequences contained within the T1 cell line appeared not to be rearranged after long periods in continuous culture (Fig. 3). To obtain a more accurate measure of the frequency with which rearrangements, small deletions or point mutations occurred in the *lacZ* gene cloned in pGM8, pGM8 was extracted from C127 T1, transfected (Hanahan, 1983) into bacteria (FX1) and plated on agar containing kanamycin (25 μg/ml), X-Gal and isopropyl β-D-thiogalactopyranoside (IPTG). Plasmids with a functional *lacZ* gene gave rise to kanamycin-resistant clones that were blue, while those plasmids with non-
Fig. 3. Undigested Hirt extract from the equivalent of 5 × 10^6 T1 cells was electrophoresed through a 0-4% agarose gel, transferred and hybridized with nick-translated pGM8. (a) Hirt extract, undigested pGM8 plasmid markers are shown in lane; (b) 10 ng, (c) 1 ng, (d) 100 pg, (e) 10 pg.

Fig. 4. Plasmid 'mini-preparations' from bacteria transformed with Hirt-extracted nucleic acids from C127 T1 cells were electrophoresed through a 0-4% gel and stained with ethidium bromide. Lane m shows the position of native pGM8 plasmid DNA.

### Table 1. Analysis of lacZ clones*

<table>
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<tr>
<th>Sample number</th>
<th>Population doublings</th>
<th>Kanamycin-resistant clones</th>
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<th>Number white</th>
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<td>6137</td>
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</table>

* Hirt extracts from the cell line T1 were transfected into E. coli FX1 cells which were then plated on agar containing X-Gal, IPTG and 25 μg/ml kanamycin. Sample numbers indicate different Hirt extracts prepared at various population doublings after transfection.

silent mutations in the lacZ gene gave rise to white clones. In total, 6137 bacterial colonies produced by transfection of FX1 with DNA extracted from C127 T1 over a period of 120 population doublings were analysed (Table 1).

Without exception, all the bacterial colonies produced were blue, indicating the absence of deletions or non-silent mutations within the lacZ region of pGM8 propagated in the T1 cell line. This compares with a mutation frequency of approximately 1% which we observed after passage of an SV40-based vector containing the identical M13 mp2 lacZ target gene in simian COS-7 cells (data not shown).
To measure whether gross rearrangements outside the \( lacZ \) gene were occurring within pGM8 sequences contained in the T1 cell line which did not disrupt the complementation function of the \( lacZ \) gene, we analysed a number of blue colonies produced by the transfection of pGM8 extracted from T1. At an early passage (40 days post-transfection), agarose gel analysis of DNA from 16 of the 226 blue, kanamycin-resistant clones showed that two out of the 16 samples migrated aberrantly. One plasmid contained a deletion, the other a small insertion. Mapping of these mutants indicated that both the deletion and the insertion had occurred within BPV sequence (data not shown). However, subsequent gel analysis of 209 pGM8 clones isolated from C127 T1 after approximately 120 cell population doublings showed that all plasmids were, within the limits of resolution of the gel, the same size as the input pGM8 (Fig. 3).

This suggests that the two variants of pGM8 observed in Hirt-extracted material rescued from the C127 T1 line at an early passage, although capable of replication in the mammalian cell system, may have been at a proliferative disadvantage and were consequently lost through dilution in subsequent cycles of host cell division.

Shuttle vectors have been used in several laboratories to analyse the stability of foreign genes in mammalian cells (for review, see Lehmann, 1985). In all cases to date either papovaviruses or retrovirus sequences have been used as the replication origin. In these studies, the rate of mutation within the bacterial target gene of SV40 host vectors ranged from 1.0 to 3.7\( \times \) 10\(^{-3}\) for \( lacI \) (Calos et al., 1983), or 1\% for galactokinase (Razzaque et al., 1983). In general these mutations fall into two classes, deletions and point mutations. Point mutations generally arise as a consequence of G:C to A:T transitions or G:C to T:A transversions in non-replicating systems whereas deletions are the major class in replicating systems (Miller et al., 1984). It has been reported that the mutation frequency in C127 cells (as used in this study) is the same as that observed in COS-7 (1 to 2\%\(^{-1}\)) cells using the \( lacI \) gene as the target for mutagenesis in polyoma-based shuttle vectors (Lebkowski et al., 1984). The frequency we have observed, less than 1.0 \( \times \) 10\(^{-4}\), is approximately two orders of magnitude below this value and is approaching that reported for the chromosomal \( aprt \) gene in hamster cells (Chasin, 1974; Meuth & Arrand, 1983). This suggests that the observed frequency of spontaneous mutations in mammalian shuttle vector systems may depend upon the eukaryotic replication origin and position of the target gene in the vector and, to a lesser degree, on cell type. During the preparation of this manuscript, Ashman & Davidson (1985) reported that the frequency of mutation within the \( gpt \) gene in a BPV \( gpt \) vector was between 3 \( \times \) 10\(^{-3}\) and 16 \( \times \) 10\(^{-3}\), a frequency higher than we observe with pGM8. This may reflect either the differences in the bacterial plasmid sequences, or the nature of the target gene and method of selection for transfected C127 colonies.

Similarly Lazo (1985) reported an unpublished observation of a mutation frequency of 2.0\% in a BPV vector transfected into B cells.

The high degree of stability and low frequency of mutation that we observed in the pGM8 vector once a stable cell line has been selected suggests that this system will be appropriate for the propagation of foreign DNA and the study of induced mutagenesis in a manner that may reflect chromosomal mutagenesis. The ability to re-introduce the T1 cell line into mice and extract pGM8 from tumours may also allow the use of shuttle vectors to study stability and mutagenesis in whole animals.

REFERENCES


Short communication


(Received 1 August 1986)