Characterization of bovine papillomavirus E1 region deletion mutants associated with neoplastic transformation in a murine cell line

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Spontaneous focus formation in the contact-inhibited C127 cell line, cl.2, harbouring multiple copies of a bovine papillomavirus type 1 deletion mutant, was associated with the evolution of further viral genomic deletions in addition to an amplification of the viral genome copy number. Three simple frameshift deletions of 308, 605 and 1291 bp, associated with separate transformation events, were mapped within the E1 open reading frame, implying a common mechanism of spontaneous transformation in this cell line. Furthermore, each transformed cell line also retained multiple copies of the intact E1 gene, suggesting that these novel deletion mutants might function by a dominant-negative mechanism to disrupt the normal control of viral DNA replication or viral transcription. These mutants had the potential to encode truncated E1 polypeptides with a common N-terminal region encoded by the 5′ end of E1, i.e. overlapping the previously described E1 modulator gene. A possible role for these mutants in diverting a lethal type of virus–cell interaction is discussed.

Bovine papillomavirus type 1 (BPV-1) readily transforms murine C127 and NIH 3T3 cell lines in vitro, yielding dense foci of transformed cells (Dvoretzky et al., 1980). Cells isolated from such foci are stably transformed, and retain multiple copies of the viral genome which replicate as plasmids at a constant copy number (Law et al., 1981; Turek et al., 1982). Under appropriate cell culture conditions, BPV-1 DNA amplification can be induced in a subpopulation of growth-arrested C127 cells (Burnett et al., 1989a). These cells are characterized by a large expansion in nuclear volume, frequently in association with c.p.e., and by abundant expression of the viral E2 antigen (Burnett et al., 1990). Although in this system virus production has not been detected, it nevertheless appears that certain events of the vegetative replication pathway are reproduced. Consistent with this proposal, we have found that abundant E2 antigen expression occurred also at the site of induction of vegetative viral DNA synthesis in virus-producing bovine wart tissue (Burnett et al., 1990).

The flat cl.2 line, isolated from a BPV-1 virion-infected C127 cell culture (Burnett et al., 1988), has played a central role in our studies on the regulation of BPV-1 early gene expression and viral DNA replication under specific cell growth conditions. These cl.2 cells harbour multiple copies of a mutant BPV-1 plasmid with a 277 bp deletion downstream of the E5 transforming gene, the most distal early open reading frame (ORF). As all of the recognized early region ORFs were retained in this mutant genome, it seemed likely that the minimal transformed characteristics of cl.2 cells were due to inefficient expression of a viral transforming gene(s) (Burnett et al., 1988). Maintenance of cl.2 cells in confluent culture led to the appearance of large non-proliferating cells with highly amplified viral DNA, similar to the effect observed in wild-type (wt) BPV-1-transformed cells after serum deprivation, in addition to the formation of foci of transformed cells with an amplified copy number. Preliminary analysis of cells derived from transformed cl.2 foci indicated that they contained novel viral mutants with deletions within the E1 ORF (Burnett et al., 1989a). We describe here the precise mapping analysis of three such deletion mutants, and discuss the mechanism of deletion formation in addition to their possible role in cellular transformation.

Three independent transformed foci were picked and established as cell lines, and their viral DNA content was analysed by Southern blotting using a BPV-I virion DNA probe labelled with [32P]dCTP by random priming. As shown in Fig. 1, each transformed line (lanes 1 to 3: lines b3, d4 and e2 respectively) contained a heterogeneous content of viral DNA compared with the parent non-transformed cell line (lanes C). After treatment with restriction endonucleases which do not cleave BPV-1 DNA (SalI and SstI), viral plasmids smaller than the resident mutant viral genome were detected in two of...
Fig. 1. Heterogeneous viral genome content of spontaneously transformed cells. Total cellular DNA was isolated from parent cl. 2 cells or spontaneously transformed cell lines by phenol extraction followed by ethanol precipitation. Ten micrograms of DNA was digested overnight with 20 units of restriction enzyme. The samples were electrophoresed through an 0.7% (w/v) agarose gel and blotted onto nitrocellulose (Southern, 1975). The nitrocellulose filter was hybridized overnight with a 32P-labelled BPV-1 virion DNA probe, washed under stringent conditions, and exposed to X-ray film (Burnett et al., 1989a). Lanes C, control parent cl.2 cell DNA; lanes 1 to 3, cellular DNA from transformants b 3, d 4 and e 2, respectively. Double arrowheads indicate novel viral circular plasmids (SalI and SstI) or linearized forms (EcoRI and BamHI).

three transformed cell lines (lanes 2 and 3). The third transformed line (lane 1) contained a lower level of monomeric viral plasmid DNA which corresponded in size to the resident viral genome, in addition to a predominant slowly migrating complex of viral DNA, which, on the basis of restriction endonuclease analysis (below), appeared to represent complex viral concatemers. Similar complex forms of episomal BPV-1 DNA have also been observed in wt virus-transformed cell lines (Law et al., 1981). Following cleavage with the endonuclease BamHI, which cuts the resident BPV-1 genome at a single site (base 4450), a simplified pattern of bands was obtained for each transformed line. These lines each contained a novel viral genomic species which migrated further than the resident linearized viral form, indicating the presence of deletion variants (the broad band seen in lane 1 was resolved into two narrowly spaced bands after a short exposure of the autoradiograph). Essentially the same pattern of bands as obtained with BamHI was also observed for lines b 3 and e 2 by cleavage with a second endonuclease which cleaves BPV-1 only once (EcoRI). In contrast, EcoRI digestion of d 4 cellular DNA resulted in the expected linearized resident viral genome band in addition to multiple slower migrating bands. These additional bands represented circular forms of the novel viral genomic species in this cell line, indicating that the EcoRI site (base 2113) had been deleted. The absence of the supercoiled putative monomeric form of the d 4 mutant species after EcoRI treatment may have been due to a non-specific nicking activity in the enzyme preparation.

By comparison of the band intensities in autoradiographs, we estimated that there was a limited increase (approximately two- to threefold) in the total viral genome copy number in each transformed line compared to the parent cell line. The copy number increases in lines b 3 and e 2 were largely due to the presence of amplified copies of the novel genomic species, and no or little amplification of the resident cl.2 viral genome was observed. Line d 4, however, also exhibited a two- to threefold amplification of the cl.2 parent genomic species. It is not possible to conclude from these observations that specific or preferential amplification of the novel viral genomic species had occurred within a single cell to initiate the transformation event in each case. A selection for cells containing multiple copies of two distinct viral genomic species may have occurred instead by unequal partitioning of plasmids during successive cell divisions until some kind of homeostasis was established.
Restriction enzyme mapping analysis was carried out by using enzymes recognizing several sites in the viral genome to characterize these new viral genomic species. Fig. 2 shows the results of Southern blot analysis of these transformed lines following cleavage with the endonucleases BgII (cuts BPV-1 at bases 612, 2812, 6525 and 7826), AvaI (bases 945, 3408, 4684 and 4839), and a combination of HincII (bases 1, 1008 and 7142) plus BamHII (base 4450). For each transformed line a unique novel restriction fragment was identified (arrows), consistent with the formation, in each case, of a simple deletion mutant derived from the cl. 2 viral variant. The sizes of these novel fragments were estimated by comparison of their mobility relative to the BPV-specific restriction fragments of known molecular size. The combined data were consistent with deletions of approximately 300 bp (line b3), 1250 bp (line d4) and 600 bp (line e2) within common restriction fragments (BgII fragment B, bases 612 to 2812; AvaI fragment B, bases 945 to 3408; HincII–BamHII fragment A, bases 1008 to 4450). We concluded that all of the deletions were contained within the region of the viral genome between the HincII site at base 1008 and the BgII site at base 2812.

By Southern blot analysis of an additional transformed cl. 2 line (e1), a fourth mutant viral genomic species was identified (data not shown). This cell line had an estimated fivefold amplification of the viral genome copy number relative to the parental cl. 2 cells. It contained multiple copies of the normal sized cl. 2 BPV species in addition to a novel species, in this case larger in size than the parent genome, indicating that an insertion or duplication event had occurred. The ratio of the two genomic species in line e1 was approximately 2:1 in favour of the parental cl. 2 genome. From detailed restriction endonuclease analysis it was concluded that the e1 mutant contained a 1350 ± 100 bp direct duplication of sequences forming the 3'-terminal region of E1 and approximately two-thirds of the E2 ORF. Thus, a novel BPV-1 restriction fragment of approximately 1350 bp was observed in Southern blots after digestion of e1 cellular DNA with the restriction endonucleases BstEI (cuts BPV-1 at nucleotide (nt) 2405), or FspI (nt 3023) or KpnI (nt 3455). This analysis therefore indicated that a collinear segment of the BPV-1 genome encompassing the BstEI and KpnI sites (nt 2405 to 3448) was duplicated in the e1 mutant genome. In contrast, neither the EcoRI site (nt 2113) nor the BstXI site (nt 3881) was duplicated.

Complete characterization of the novel E1 deletion mutants was accomplished by limited sequence analysis of cloned restriction fragments to define the deletion endpoints for each mutant. To generate purified viral DNA for cloning purposes, the polymerase chain reaction (PCR) was employed (Saiki et al., 1988). By using 20 nucleotide PCR primers with 5' termini at bases 892 (plus-strand primer) and 4456 (minus-strand primer), the entire E1–E2–E5 region spanning the deletions was amplified. As expected, for each transformed cell line two PCR products were identified by agarose gel electrophoresis in combination with ethidium bromide staining (data not shown). One PCR product corresponded in size to the collinear cl. 2 mutant fragment, and was common to each PCR reaction, whereas the second PCR product in each case corresponded to the expected size of the deleted viral fragment. The putative collinear PCR product obtained from each cell line was not analysed further, since its electrophoretic mobility was identical to that of the control PCR product obtained by performing an amplification reaction on molecularly cloned parent cl. 2 viral DNA. After further restriction analysis to refine the mapping of each deletion, specific fragments were generated by internal digestion of the PCR products with combinations of restriction endonucleases (SmaI–EcoRI, mutant e2; SmaI–SpeI, mutant d4; EcoRI–Stul, mutant b3), and were cloned between compatible sites within the polylinker of pSP64 (SmaI–EcoRI, mutants e2 and b3; SmaI–XbaI, mutant d4). Bacterial clones containing recombinant plasmids with the desired mutant genome fragments were identified by restriction enzyme analysis of plasmid DNA, and were subjected to limited DNA sequence analysis by using the chain-termination method in combination with appropriate sequencing primers. Attempts were also made to amplify the larger e1 mutant genome fragment by PCR. However, owing to the inherent bias in the PCR method leading to preferential amplification of shorter DNA fragments, we have thus far not succeeded in isolating and cloning genomic viral fragments containing the large duplicated region in this mutant.

Sequence analysis confirmed that each of the deletion mutants (d4, e2 and b3) had formed by a simple deletion mutation. The deletion endpoints were bases 1085 to 2377 (mutant d4), 1118 to 1724 (mutant e2) and 2271 to 2580 (mutant b3). Their localizations are shown in Fig. 3(a, b). All three deletions affected the E1 ORF, resulting in a frameshift mutation in each case. The duplicated sequences in mutant e1, described above, thus mapped to a similar region of the viral genome as the deletion mutants, although we have not yet determined whether the E1 ORF in mutant e1 was disrupted by the duplication event. Deletions e2 and b3 affected non-overlapping portions of E1, whereas the d4 deletion encompassed deletion e2, and overlapped the 5' end of deletion b3. The E8 ORF was completely deleted in mutants e2 and d4, but was retained in mutant b3. The 3' endpoint of deletion b3 was located just 2 bp upstream from the start of the E2 ORF. Previously characterized
cis-regulatory sequences affected include a plasmid maintenance sequence (PMS) element (PMS2, bases 1522 to 1582; Lusky & Botchan, 1984) and an adjacent negative control of replication (ncor) element (ncorII, bases 1494 to 1517; Roberts & Weintraub, 1986), which were deleted in mutants e2 and d4. Mutant b3 retained these elements, but lacked the P2443 transcriptional promoter region. Homologous sequences were observed immediately 5' to the deletion endpoints of mutants d4 (AGCAG) and b3 (TAGACTG) (Fig. 4), consistent with a mechanism of illegitimate recombination involving significant sequence homology. It is interesting to note the homology between the conserved pentameric endpoint sequences of mutant d4 (AGCAG) and the pentameric sequences abutting the deletion endpoints of mutant b3 (A[C]TG). No striking sequence homology was identified immediately adjacent to the upstream and downstream deletion endpoints of mutant e2. However the sequence TCTGAA was found 28 bases downstream of each deletion endpoint of this mutant, indicating that this deletion also had formed by pairing of partly homologous sequences. Furthermore, pentameric motifs related to those observed at the deletion endpoints of mutants d4 and b3 were also observed close to the endpoints of deletion e2 (Fig. 4, arrows). This may indicate a specific role for this pentameric sequence in the generation of the deletions, such as a direct involvement in a virus-specific replication or recombination process.

Closer inspection of the sequences surrounding the deletion endpoints led to the identification of the following sequence elements repeated within the E1 region: AGTATTG(n)13-15AGCAGCGGTTTC (bases 1085-1108)

![Fig. 3. Localization of the deletions within the BPV-1 genome. (a) Genetic map of BPV-1. The genetic organization of the circular dsDNA genome of 7945 bp is characterized by the presence of multiple translational ORFs (E, early; L, late) in the same transcriptional orientation. The positions of major mRNA start sites are shown by the arrows, and the positions of binding sites for the viral E2 transcriptional regulatory proteins by the small triangles. The early and late polyadenylation signals are represented as A0 and A1, respectively. The original cl. 2 'downstream' deletion, common to each novel mutant genome, is indicated by the stippled segment, and the deletions mapped in this paper by the unfilled segments. The locations of methionine codons within the E1 ORF are shown by vertical bars. (b) Identification of repeated sequence motifs adjacent to deletion endpoints. Arrows indicate locations of the partial DNA sequences shown in the lower half of the figure. The positions of conserved pairs of sequence motifs [AGTATTG(n)13-15AGCAGCGGTTTC] within the 5' and 3' regions of E1 are shown by asterisks and are also underlined in the partial DNA sequences. E2-binding sites are shown by the boxed sequences. Regions of homology to the topoisomerase II (TOP II) consensus sequence (Sander & Hsieh, 1985) were observed at the 3' deletion endpoint of mutant e2 (12/15 matches to consensus sequence), and overlapping the AGTATTG motif nearby the 3' deletion endpoint of mutant d4 (14/15 matches to consensus sequence).]
It is interesting to note that each mutant was disrupted in, or had lost, at least one pair of these repeats. Thus, it is conceivable that these elements are involved in the cis-repression of viral early gene expression. The d4 deletion was the result of recombination between an upstream AGCAG half-motif and the downstream AGCAGCGGTTC motif, leading to conservation of the AGCAGCGGTTC element. This was the only mutant in which recombination occurred within one of the conserved elements of these sequence repeats. The 5′ deletion endpoint of the e2 mutant occurred between the conserved elements of the upstream pair. The role of these sequences is unknown. However the AGCAGCGGTTC element displays similarity to the binding site for the cellular transcription factor AP4 (CAGCTG2GG: Mermod et al., 1988). The downstream pair of motifs is located close to the P2443 mRNA initiation sites, and might therefore be involved in the regulation of transcription from this promoter. The upstream copy of the AGCAGCGGTTC element overlaps an E2-binding site (boxed in Fig. 3b) previously identified at bases 1125 to 1136 (Li et al., 1989). Another E2-binding site is found adjacent to the downstream pair of motifs in the P2443 promoter region. Neither the entire sequence repeat nor the AGCAGCGGTTC motif was identified elsewhere in the BPV-1 genome when a computer search was conducted. The AGTATTG motif was found at a third location in the late region (bases 4916 to 4922). A potential topoisomerase II recognition sequence (Sander & Hsieh, 1985; Cockerill & Garrard, 1986) was identified overlapping the AGTATTG motif in the P2443 promoter region (see Fig. 3b). This observation may be of interest in view of the known role of this enzyme in cellular replication and transcription processes, as well as its proposed role in illegitimate recombination events (Sperry et al., 1989). A second topoisomerase II-like sequence was also observed at the 3′ deletion endpoint of mutant e2 (Fig. 3b), further supporting the possibility that this enzyme may have been involved in the formation of certain of these deletion mutants.

The E1 ORF encodes a factor implicated in viral DNA replication and viral transcriptional regulation. It has been found repeatedly by genetic manipulation of cloned genomic BPV-1 DNA that frameshift or deletion mutations within E1 destroy the ability of BPV-1 to replicate as a plasmid (Lusky & Botchan, 1985; Rabson et al., 1986; Sarver et al., 1984). Furthermore, it has been reported that similar E1 mutations lead to an increased transforming efficiency of the viral genome in a C127 focus assay (Lambert et al., 1988; Schiller et al., 1989). Cells transformed by such E1-negative mutants also displayed enhanced anchorage-independent growth compared to cells transformed by wt viral DNA. Consistent with an increased expression of a viral transforming gene(s), the integrated BPV-1 genomes were reported to be more actively transcribed than BPV-1 plasmid DNA in wt virus-transformed cells. It was concluded that the E1 ORF encodes a repressor of BPV-1 early gene transcription, and this conclusion was supported by experiments demonstrating that the high transforming phenotype of E1-negative mutants was suppressed by cotransfection with an expression vector designed to express a full-length E1 polypeptide (Schiller et al., 1989).

The transformation-associated BPV-1 deletion mutants characterized in the present paper probably function by a dominant-negative mechanism to result in increased levels of transcription of a viral transforming gene(s). In keeping with this proposal, we have detected elevated levels of viral early region mRNA in each of these transformed cell lines, and the relative amount of transcription from the P99 promoter (believed to control expression of the transforming E6 gene) was increased by several times over both the parent cell line and a wt BPV-1-transformed cell line (Burnett et al., 1990; A. C. Ström & S. Burnett, unpublished results). In theory, transcriptional derepression could be achieved by the removal of cis-suppressor sequences, as discussed above, by establishment of an increased viral genome copy number, or by the expression of trans-dominant mutant viral proteins capable of disrupting the function of the normal gene product in the repression of viral transcription. In view of existing data indicating that an E1 gene product can indeed suppress BPV-1-induced cell transformation, the latter explanation seems more likely at present. For example, such mutant E1 proteins might inactivate or inhibit a transcriptional repressor activity of E1, leading to enhanced expression of a viral transforming gene(s) by a quantitative increase in, or by a qualitative change in the pattern of, synthesis of viral early region mRNA. A second, not mutually exclusive, possible mechanism involves expression of a mutant E1 protein which inactivates or inhibits a viral DNA amplification function of the wt protein, to rescue cells which would otherwise die through over-replication of viral DNA. In this case, the cells might become transformed due to enhanced expression of a viral transforming gene(s) by establishment of a stable BPV genome copy number higher than that of the parent cells. Consistent with this model, we observed by Southern blot analysis that each transformed cell line had a limited increase in the BPV genome copy number (see above). Amplification of viral DNA in spontaneous transformed cl.2 foci was also detected by in situ hybridization analysis (Burnett et al., 1989a). This model would imply a fundamental connection between the appearance of spontaneous trans-

Such evolutionary variants may thus yield information on functionally important regions of the normal gene. The distribution of methionine codons within the E1 ORF is shown in Fig. 3(a). The coding capacity of E1 is 605 amino acids beginning from the first AUG codon. Recent biochemical evidence has been reported which indicates that a 68K to 72K E1 putative replication initiation factor is encoded by the entire E1 ORF (Santucci et al., 1990; Sun et al., 1990). Each deletion mutant genome had the potential to express truncated E1 proteins with a common N terminus encoded by the 5' end of the E1 ORF. The mutant with the largest deletion (d^4) could, in theory, express an N-terminal fragment of a full-length E1 protein consisting of 79 residues of E1 fused to 10 residues from a short ORF beyond the d^4 3' deletion endpoint, and a presumably irrelevant 15 residue peptide if translation were initiated from the last AUG codon in E1. Similarly, by initiation of translation from the first AUG codon in E1, mutants e^2 and b^2 could encode truncated E1 proteins of 91 (90 residues from E1 plus one residue from a distal ORF) and 475 (474 residues from E1 plus one residue from a distal ORF) amino acid residues, respectively.

A prediction of the above hypothesis, therefore, would be that the N-terminal region of E1 encodes a protein domain capable of entering into dimer formation with wt E1 protein, to give inactive heterodimers, or of competing with wt E1 for binding to its site of action as a replication factor or transcriptional repressor. Recent experimental data indicate a specific interaction between a full-length E1 replication factor and the E2 transcriptional activator protein (Mohr et al., 1990). This finding can potentially explain several previous observations related to the control of viral replication and transcription, such as the apparent coincidence of origins of BPV-1 DNA replication with specific E2-binding sites (Yang & Botchan, 1990) or promoter regions (Burnett et al., 1989b), and the role of E1 in viral transcriptional repression. The identification of dominant-negative E1 mutants capable of interfering with viral transcription or replication may provide an important tool in identifying domains of E1 involved in regulating either of these processes. The mutant E1 genomes characterized herein thus potentially indicate domains of an E1 polypeptide capable of interacting with the E2 transactivator protein.

Further investigation of these E1 deletion mutants may also clarify the mechanism of action of the BPV-1 modulator factor (M) which, interestingly, was also mapped to the 5'-terminal region of the E1 ORF (Berg et al., 1986; Lusky & Botchan, 1986; Thorner et al., 1988). Evidence has been presented from one laboratory that E1 M-minus BPV-1 mutants exhibit a lethal phenotype in C127 cells (Lusky & Botchan, 1986). This observation would be consistent with a role for the M factor in inhibiting the growth arrest-specific induction of viral early gene expression and viral DNA amplification observed in BPV-1-transformed cells in vitro (Burnett et al., 1990). An intriguing possibility is that our transformation-associated mutant BPV genomes overcome this block to cell proliferation observed upon induction of this vegetative-like viral replication phase, by expression of truncated E1 factors which function in a manner analogous to the M factor in the establishment of transformation after a primary virus infection. Based on our present observations, this model leads to the following predictions: (i) the normal M gene is silenced in the subpopulation of division-arrested cells in which viral DNA amplification is observed and (ii) a large E1 replication factor is expressed in these cells, which shares a common N-terminal domain with the M factor.

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References


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