The Genomes of Bovine Papillomaviruses Types 3 and 4 are Colinear

By L. W. COGGINS,1 I. HETTICH,2 K. T. SMITH,1 A. A. SLATER,1 F. A. ROE,1 H. PFISTER3 AND M. S. CAMPO1.*

1Beatson Institute for Cancer Research, Wolfson Laboratory for Molecular Pathology, Garscube Estate, Bearsden, Glasgow G61 1BD, Scotland, U.K., 2Institut für Virologie, Zentrum für Hygiene, Universität Freiburg, Hermann Herder Strasse 11, 7800 Freiburg, F.R.G., and 3Institut für Klinische Virologie, der Universität Erlangen-Nürnberg, Loschgestrasse 7, 8520 Erlangen, F.R.G.

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SUMMARY

The 7.2 kb genomic DNA of bovine papillomavirus type 3 (BPV-3) was molecularly cloned using its unique EcoRI site, and the 7.3 kb genome of BPV-4 was cloned using its single BamHI site. The viral genomes were compared by liquid hybridization, Southern blot hybridization and heteroduplex mapping. Low stringency hybridization conditions revealed that the genomes are colinear but the sequences are extensively mismatched. The relative alignment of the restriction endonuclease maps of the two viral genomes has been determined. It was found that the genomes as linearized for cloning are out of phase by 1.7 kb, so that the single EcoRI site of BPV-3 appears to coincide with the BPV-4 EcoRI site at 0.22 map units. It is concluded that the genomes of BPV-3 and BPV-4, both of which cause true epithelial warts, share the same physical organization but exhibit sequence divergence.

The six types of bovine papillomaviruses (BPV-1 to 6) described so far can be divided into two groups. The first group comprises BPV-3, 4 and 6 which cause true epithelial warts involving proliferation of epidermal cells only (Jarrett et al., 1978; Pfister et al., 1979; Campo et al., 1980; W. F. H. Jarrett et al., unpublished results). Members of the other group, which contains BPV-1, 2 and 5, cause fibropapillomas in which both epidermal cells and fibrocytes proliferate (Lancaster & Olson, 1978; Jarrett et al., 1980; Campo et al., 1981). The DNA sequence of BPV-1 has been determined, and analysis of the open reading frames suggests that the genome is transcribed unidirectionally from one strand (Chen et al., 1982). We have shown previously that the genomes of BPV-1 and 2 are colinear, though mismatched, and thus these viruses probably share a similar genetic organization (Campo & Coggins, 1982). In this paper we compare the genomes of BPV-3 and 4. BPV-3 has been found so far only in Australian cattle where it causes cutaneous warts (Pfister et al., 1979) whereas BPV-4 was originally isolated from true frond papillomas of the upper alimentary canal (Jarrett et al., 1978; Campo et al., 1980).

The genome of BPV-3 was cloned both in λL47 and in the plasmid pAT153 (Twigg & Sherratt, 1980) using the unique EcoRI site, following the protocols of Gissmann et al. (1982) and Campo & Coggins (1982) respectively. The recombinant plasmids pBV3a and pBV3b contain the viral sequences in opposite orientations (Fig. 1). BPV-4 DNA was cloned in pAT153 using the unique BamHI site, and is referred to as pBV4 (Fig. 1; Campo & Coggins, 1982). The size of the cloned BPV-3 genome, determined electron microscopically, was 7.16 ± 0.16 kb (n = 25). The BPV-4 genome has been found to be 7.27 kb (Campo & Coggins, 1982). The size of both viral genomes estimated by electron microscopy is larger than the 6.6 to 6.8 kb determined by gel electrophoresis (Pfister et al., 1979; Campo et al., 1980), but this discrepancy does not affect the overall interpretation of results obtained by the two methods.

Liquid hybridization experiments were performed with either 32P-labelled BPV-3 or 32P-labelled BPV-4 DNA and a 12-fold excess of the heterologous DNA. Reassociation of probe
Fig. 1. Restriction endonuclease maps of SalI-digested pBV3a, 3b and 4. 'A' indicates region of the BPV-4 genome subcloned in pBV4A. The dashed lines show the alignment of homologous sequences in pBV3b and pBV4. E, EcoRI; B, BamHI; H, HindIII; HII, HindII.

Fig. 2. Reassociation of 32P-labelled BPV-3 DNA in the presence of 50 ng BPV-3 DNA (□), 600 ng BPV-4 DNA (●), or salmon sperm DNA (■). The BPV-3 and 4 DNAs were separated from their respective phage and plasmid vectors by restriction endonuclease digestion and were isolated from a low-melting point agarose gel. BPV-3 DNA was labelled by nick-translation (Rigby et al., 1977) and, after denaturation and neutralization, both viral DNAs were reassociated in 0.5 M-NaCl, 17 mM-EDTA pH 8.1 and 0.1% Sarkosyl at 68 °C. Aliquots were analysed by hydroxylapatite chromatography and the fraction of DNA remaining single-stranded ($f_s$) was plotted according to Wetmur & Davidson (1968).

DNA deviated from second-order kinetics (Fig. 2), and analysis according to Sharp et al. (1974) indicated 12% sequence homology between BPV-3 and 4.

In order to determine the relative orientation and position of putative related sequences in the two viral genomes, heteroduplex mapping was carried out. The recombinant plasmids pBV3a, pBV3b and pBV4 were digested with SalI, which cuts each once in the vector region. Under standard conditions of heteroduplex preparation containing 40% formamide (Davis et al., 1971), most hybrid molecules showed duplex DNA only in the vector regions (data not shown).
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This was always the case for pBV4 hybridized to pBV3a, but in the pBV3b–pBV4 preparations some heteroduplexes showing pairing between the viral regions were also observed. To encourage duplex formation in such molecules, the stringency of hybridization was reduced by decreasing the formamide concentration in the spreading solution to 35%. In such preparations, most heteroduplexes between pBV3b and pBV4 showed hybridization in both vector and viral regions (Fig. 3a), establishing that the genomes were cloned in the same relative orientation and contain related sequences. Two single-stranded loops were present, one at each position expected for the vector–virus junctions, flanking the duplexed viral segments. The loop near the long vector arm was \(2.12 \pm 0.17\) kb and that near the short vector arm was \(2.10 \pm 0.25\) kb \((n = 20)\). These data suggest that the two genomes as linearized for cloning are permuted with respect to their related sequences. As different vector restriction endonuclease sites were used for cloning, it is possible to tell which viral genome is responsible for each loop. Since the 0.38 kb EcoRI–BamHI plasmid segment did not form a separate unpaired strand at either vector–virus junction, it had to be part of each 2.1 kb loop and must be adjacent to the unpaired viral segments. Thus part of the BPV-4 DNA must be non-hybridized near the long vector arm, and part of the BPV-3 DNA near the short vector arm. Subtraction of the 0.38 kb vector component from the size of each loop indicates that the genomes as cloned are permuted relative to each other by about 1.7 kb. A short \((72 \pm 16\) bp) unpaired region was found at the base of some \((17/20)\) of the loops near the short vector arm. It could be due to a small insertion or substitution between the two genomes, or to a local denaturation of the sequences. Between the two single-stranded loops is a duplex region containing the remaining 5.5 kb of the viral genome. It is
Fig. 4. Southern blot hybridization between BPV-3 and BPV-4 DNAs. The BamHI, HindIII and HindI fragments of EcoRI-digested BPV-3 DNA were hybridized overnight to labelled pBV3 (a and c) or pBV4 DNA (b and d) in relaxed conditions, (6 × SSC, 50 °C; a and b) and in stringent conditions (2 × SSC, 70 °C; c and d). Lanes 1, BamHI; 2, HindIII; 3, HindII. EcoRI cleaves the HindIII B fragment of BPV-3 DNA asymmetrically into B' and B", the HindII C fragment into two equal fragments, and the BamHI A fragment into A and D (see diagram in Fig. 5).

double-stranded along most of its length in the majority of heteroduplexes, although individual molecules varied in the amount of hybridization exhibited. In partially denatured heteroduplexes, the major unpaired region was 9.0±2.2% to 21.4±3.3% (n = 20) along the paired viral segment, measuring from the junction with the long vector arm.

These observations do not reveal whether the viral sequences in the two single-stranded loops are also homologous. However, heteroduplexes were occasionally seen in which these sequences were also hybridized (Fig. 3b), but often showed short denatured regions near the junction with the short vector arm. This suggests that these sequences are related but the region farthest from the BPV-4 EcoRI site is most mismatched in the 1.7 kb segment. To confirm this homology, a subclone, pBV4A, was used which contains the BPV-4 1.6 kb BamHI–EcoRI segment in question cloned between the BamHI and EcoRI sites of pAT153 (Fig. 1). Compared to the same viral sequence in pBV4, it was in the opposite orientation in the vector and so was hybridized to pBV3a. In 35% formamide, each heteroduplex consisted of three components (Fig. 3c): a long duplex arm of the size expected for the SalI–EcoRI vector region and the adjacent hybridized 1.7 kb viral sequences; a single-stranded loop which contains the remaining pBV3a viral sequences and the EcoRI–BamHI vector region; and a short duplex BamHI–SalI vector segment. No deletion loop was observed in the long duplex arm at the position expected for the vector–viral junctions. The EcoRI sites which bounded these two viral segments must therefore be in almost homologous positions (within 100 bp) in the two genomes.

The results obtained by heteroduplex mapping were confirmed by blot hybridization (Southern, 1975). Restriction fragments of BPV-3 DNA (Fig. 1 and 4) were hybridized to 32P-labelled pBV3 and pBV4 in both relaxed and in stringent conditions, which allow the formation of hybrids with up to 35% and 12% mismatch respectively (Bonner et al., 1973). Homologous hybridization was similar in the two conditions (Fig. 4a and c) whereas there were marked qualitative and quantitative differences in the pattern of heterologous hybridization (Fig. 4b and d). All the BPV-3 restriction fragments that hybridized to pBV3 DNA hybridized to pBV4 DNA only under relaxed conditions. However, only BPV-3 BamHI fragment A, HindIII fragments A and C and HindII fragments A and C hybridized in stringent conditions (Fig. 4d),
indicating that these regions of BPV-3 DNA or portions of them, have a higher degree of homology with BPV-4 DNA than does the rest of the BPV-3 genome.

The colinearity of the two viral genomes was established by hybridizing four pBV4 subclones to restriction fragments of BPV-3 DNA under relaxed conditions. The results (Fig. 5) show that the two genomes as cloned in pAT153 are out of phase by approximately 1.5 kb, consistent with the 1.7 kb permutation found by electron microscopy. Colinearity is thus established when the EcoRI site at 0.22 map units in BPV-4 DNA is aligned with the single EcoRI site of BPV-3 DNA, although the data do not reveal whether these sites are truly homologous.

In relaxed conditions only, the subclone pBV4A hybridized to a limited extent also to BPV-3 BamHI-B, HindII-B and HindIII-A fragments (Fig. 5a) suggesting the presence of diverged repetitive sequences in either viral genome. Comparison of the nucleotide sequences of these viruses will eventually clarify this point.

It is concluded that the genomes of BPV-3 and BPV-4 have a similar overall physical organization but have undergone sequence divergence which probably underlies their different cytopathological effects. This parallels the situation previously found between the BPV-1 and BPV-2 genomes (Campo et al., 1981; Campo & Coggins, 1982), which are colinear but mismatched. Human papillomavirus type 1 and BPV-1 also appear to share the same sequence organization, although their genomes have diverged to a much greater extent (Croissant et al., 1982; Chen et al., 1982; Danos et al., 1982, 1983). Our data suggest that the BPV-3 and BPV-4 genomes are less homologous overall than are the BPV-1 and BPV-2 genomes. The least homologous sequences in BPV-1 and BPV-2 occur in the regions containing the L1 and L2 genes (Chen et al., 1982; Campo & Coggins, 1982), although sequences in this part of the genome are conserved between papillomaviruses from different host species (Law et al., 1979; Croissant et
In BPV-3 and 4 the 'left-hand' segments on the restriction maps contain the regions which show least homology between the two genomes. By analogy with BPV-1 and 2, these regions may code for the structural polypeptides. This hypothesis is supported by the observation that a sub-genomic clone, containing the 3' end of the transforming region of BPV-1, is homologous, albeit to a low extent, to the 'right-hand' segment of BPV-3 (H. Pfister, unpublished results). Otherwise, BPV-1 and BPV-2 do not show sequence homology with BPV-3 or BPV-4 (Pfister et al., 1979; Campo et al., 1980, 1981; Campo & Coggins, 1982), so the two groups of papillomaviruses appear to have diverged considerably. Thus it cannot yet be concluded that BPV-3 and 4 have a common organization with other papillomaviruses and further interpretation awaits determination of the position and transcriptional orientation of the viral genes.

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