Bovine Papillomavirus Type 1 Genome in Hamster Sarcoma Cells *in vivo* and *in vitro*: Variation in the Level of Transcription

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SUMMARY

The physical state and expression of the bovine papillomavirus type 1 (BPV-1) genome were analysed in two transplantable hamster sarcomas (HT1 and HT2) after a low number of transplantations, in two tumourigenic cell lines derived from the first transplant of HT2 sarcoma and another transplantable sarcoma (HT3) and in tumours obtained by grafting these cells. Blot hybridization experiments indicated the presence of multiple free copies of the whole viral genome in HT1 and HT2 tumour transplants (100 and 25 copies/cell respectively), irrespective of the number of *in vivo* or *in vitro* passages. In contrast, HT3 cells, and tumours induced by these cells, at early and late passages *in vitro* contained viral sequences probably integrated in the cell genome, in addition to the free viral DNA sequences. Polyadenylated viral transcripts were easily detected in HT1, HT2 and HT3 tumours obtained at early passages *in vivo* and *in vitro*, with electrophoretic mobilities corresponding to 1200 to 1400 bases (HT1, HT2 and HT3), 1750 bases (HT1) and 2500 bases (HT2). Homologous sequences of the transcripts were localized in the transforming *BamHI-HindIII* fragment of BPV-1 DNA, mainly in the *BamHI-EcoRI* fragment (0.31 to 0.602 map units). In contrast, almost no viral transcription was detected in HT2 and HT3 cells after 50 subcultures and in the tumours induced by these cells. This suggests that the tumourigenicity of the HT2 and HT3 cells is compatible with a very low level of expression of the transforming region of the BPV-1 genome.

The bovine papillomavirus types 1 and 2 (BPV-1 and BPV-2) induce under natural conditions fibropapillomas in cattle and connective tissue tumours in horses. Under experimental conditions, they cause non-virus-producing connective tissue tumours in mice, hamsters, pikas, rabbits, cattle and horses (Olson *et al.*, 1969; Puget *et al.*, 1975; Breitburd *et al.*, 1981). In addition, these viruses can transform bovine and murine cells *in vitro* (Boiron *et al.*, 1964). Cells transformed by BPV-1 or BPV-2 *in vivo* or *in vitro* contain only multiple free copies of the viral genome (Law *et al.*, 1981; Moar *et al.*, 1981; for review, see Lancaster & Olson, 1982). The existence of a transforming region in the BPV-1 genome has been demonstrated by transfection with a cloned subgenomic fragment (Lowy *et al.*, 1980), and the viral RNAs transcribed from this region have been characterized in BPV-1-induced bovine warts, hamster tumours, and transformed mouse and hamster cells (Amtmann & Sauer, 1982a, b; Freese *et al.*, 1982; Heilman *et al.*, 1982). In this paper, the physical state and the transcription of the BPV-1 genome were analysed in two transplantable hamster sarcomas (HT1 and HT2) after a low number of transplantations, in two tumourigenic cell lines derived from the first transplant of HT2 sarcoma and of other transplantable sarcoma (HT3), and in tumours obtained by grafting these cells. The transplantable HT1 sarcoma was derived from a tumour induced by the subcutaneous inoculation of purified BPV-1 into a newborn hamster (about 10^{11} complete particles in 0.1 ml glycerine–saline). Attempts to establish a cell line from this sarcoma have been unsuccessful. The transplantable hamster sarcomas HT2 and HT3 and the cell lines established from the first

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Fig. 1. Blot hybridization analysis of BPV-1 DNA sequences in untreated or endonuclease-treated tumour cell DNAs. (a) Untreated BPV-1 DNA mixed with 10 μg of hamster liver DNA (0.63 ng, 50 copies per diploid cell DNA content); (b) 0.2, (c) 1, (d) 10 and (e) 100 copies per diploid cell DNA content of BamHI-treated BPV-1 DNA mixed with 10 μg of hamster liver DNA; aliquots of 10 μg of (f) untreated hamster liver DNA; (g to j) HT1/5h DNA (g) untreated or cleaved by (h) SalI, (i) BamHI, (j) BamHI + HindII; (k to o) HT2/4h DNA (k) untreated or cleaved by (l) SalI, (m) BamHI, (o) BamHI + HindII; (p) HT2/52c DNA cleaved by BamHI; (p to x) HT3/6c, 1h DNA (p) untreated or cleaved by (q) SalI, (r) BamHI, (t) EcoRI, (w) SmaI and (x) BamHI + HindII; (s) HT3/60c DNA cleaved by BamHI. The fragments are designated by their sizes (kbp), and the sizes corresponding to additional bands, detected in HT3/6c, 1h and HT3/60c DNAs, are underlined.

Table 1. Physical state and transcription of BPV-1 genome in tumour cells

<table>
<thead>
<tr>
<th>Tumour cell</th>
<th>Number of passages</th>
<th>Designation</th>
<th>Copy number</th>
<th>Physical state</th>
<th>Viral transcript (kb)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour 1</td>
<td>5</td>
<td>HT1/5h</td>
<td>100</td>
<td>Free</td>
<td>1.2-1.4, 1.75</td>
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<tr>
<td></td>
<td>6</td>
<td>HT1/6h</td>
<td>100</td>
<td>Free</td>
<td>1.2-1.4, 1.75</td>
</tr>
<tr>
<td>Tumour 2</td>
<td>4</td>
<td>HT2/4h</td>
<td>25</td>
<td>Free</td>
<td>2.1-2.4, 2.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>HT2/52c</td>
<td>25</td>
<td>Free</td>
<td>ND‡</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>HT2/58c, 1h</td>
<td>25</td>
<td>Free</td>
<td>ND</td>
</tr>
<tr>
<td>Tumour 3</td>
<td>1</td>
<td>HT3/6c, 1h</td>
<td>100</td>
<td>Free and integrated</td>
<td>1.2-1.4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>HT3/60c</td>
<td>100</td>
<td>Free and integrated</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>HT3/62c, 1h</td>
<td>100</td>
<td>Free and integrated</td>
<td>Trace (1-2)</td>
</tr>
</tbody>
</table>

* The copy number of BPV-1 DNA per diploid cell DNA content and physical state of viral genome were determined by blot hybridization experiments.
† The level of transcription of BPV-1 DNA and the size of viral transcripts were analysed by blot hybridization experiments.
‡ ND, Not detected.

The transplant of these tumours have been described previously (Breitburd et al., 1981). Total high molecular weight DNAs were prepared, according to the method of Wold et al. (1978), from the 5th and 6th transplants of HT1 tumour (HT1/5h, HT1/6h) and the 4th transplant of HT2 tumour (HT2/4h), from HT2 and HT3 cell lines after 52 and 60 subcultures respectively (HT2/52c and HT3/60c), and from tumours obtained by grafting HT2 and HT1 cells after 58 subcultures (HT2/58c, 1h) and 6 or 62 subcultures (HT3/6c, 1h and HT3/62c, 1h respectively) (Table 1).

Blot hybridization experiments were performed using tumour cell DNAs either unrestricted or cleaved with endonucleases which recognize no (SalI), one (BamHI, EcoRI, HindIII, HpaI, SmaI) or several restriction sites (HindII) on the BPV-1 genome (Lancaster, 1979). DNA
preparations were subjected to electrophoresis for 15 h at 35 V in 1% agarose slab gels, depurinated, denatured and transferred to diazotized paper (Alwine et al., 1979). The viral DNA sequences were detected by hybridization with nick-translated $^{32}$P-labelled BPV-1 DNA ($1.2 \times 10^8$ ct/min/µg; $2 \times 10^4$ ct/min/cm$^2$) followed by autoradiography as previously described (Orth et al., 1980; Favre et al., 1982). In unrestricted HT$_1$/5h, HT$_2$/4h, HT$_2$/52c, HT$_3$/6c, 1h and HT$_3$/60c DNAs and in these DNAs treated with SalI endonuclease, the radioactivity was found associated with the bulk of the cellular DNA (Fig. 1g, h, k, l, p and q), as observed on ethidium bromide-stained gels. Free circular monomers were observed in trace amounts in HT$_3$ DNA, in contrast to the large amount of free monomers previously found in HT$_2$ and HT$_3$ tumour cells (Breitburd et al., 1981). No hybridization was detected with normal hamster liver DNA (Fig. 1f). After cleavage of the tumour DNAs with a one-cut enzyme for BPV-1 DNA (BamHI), a band migrating like viral DNA form III was observed in all cases (Fig. 1i, m, n, r and s). From the reconstruction experiments (Fig. 1b to e), the number of viral genome equivalents per diploid cell DNA content may be estimated at about 100 for the HT$_3$/5h, HT$_3$/6c, 1h and HT$_3$/60c tumour cells, and at about 25 for the HT$_2$/4h and HT$_2$/52c tumour cells. Cleavage of tumour DNAs with a mixture of BamHI and HindIII yielded the four expected fragments for BPV-1 DNA (Fig. 1j, o and x) (Lancaster, 1979). These results indicate that no major deletion or rearrangement had occurred in the viral DNA present in the tumour cells. Similar data were obtained with HT$_1$/6h, HT$_2$/58c, 1h and HT$_3$/62c, 1h tumour cells (Table 1). Different viral copy numbers were previously reported for HT$_2$ cells after 58 subcultures (100 to 500 copies) and for HT$_3$ cells after 50 and 55 subcultures (10 to 100 copies) (Breitburd et al., 1981), suggesting that viral genome content may vary during cell passage.

No additional band was observed in BamHI-treated HT$_1$/5h, HT$_2$/4h and HT$_2$/52c DNAs (Fig. 1i, m and n), and HT$_1$/6h and HT$_2$/58c, 1h DNAs (data not shown), under conditions where a 0-2 viral genome equivalent per diploid cell could have been detected (Fig. 1b). These results render unlikely the integration of viral DNA sequences in the cellular genome. The slow-migrating species, observed in unrestricted HT$_1$ and HT$_2$ DNAs, may represent free catenated or oligomeric BPV-1 DNA molecules, as previously discussed in the case of the viral DNA complexes found in cells of tumours induced by cottontail rabbit papillomavirus (CRPV) (Favre et al., 1982; Wettstein & Stevens, 1982) and by BPVs (Breitburd et al., 1981; Law et al., 1981).

In contrast, when HT$_3$/6c, 1h and HT$_3$/60c DNAs were cleaved with one-cut enzymes (Fig. 1r to w) or with BamHI + HindIII (Fig. 1x), several bands were observed in addition to form III BPV-1 DNA. The labelling intensity of the bands, corresponding to about one copy per diploid cell (Fig. 1c), and the sizes of the bands argue against the existence of rearranged viral DNA molecules. These results indicate, rather, that integration of viral DNA sequences may occur in at least one site of the cellular DNA, a feature not yet reported for BPV-transformed cells (Amtmann & Sauer, 1982a; Lancaster & Olson, 1982; Law et al., 1981; Moar et al., 1981). Similar results suggesting integration were obtained in the case of HT$_3$/62c, 1h tumour (data not shown) and in HT$_3$ cells after 50 and 55 subcultures (Breitburd et al., 1981). However, unintegrated viral DNA sequences were also present, since free, unit-length, circular viral DNA molecules were observed in HT$_3$/6c, 1h (Fig. 1p, q), HT$_3$/60c tumour cells (data not shown) and in HT$_3$ cells after 50 or 55 subcultures (Breitburd et al., 1981).

Virus-specific RNA species were extracted according to the method of Auffray & Rougeon (1980) from the early transplants (HT$_1$/5h, HT$_2$/4h), from HT$_2$ and HT$_3$ cells after 52 and 60 subcultures (HT$_2$/52c and HT$_3$/60c), and from tumours obtained by grafting cells after a low or a high number of in vitro subcultures (HT$_3$/6c, 1h, HT$_3$/58c, 1h and HT$_3$/62c, 1h) (Table 1). Any contaminating DNA was eliminated by incubating the RNA preparation with deoxyribonuclease I (50 µg/ml in 10 mM-Tris-HCl pH 7-5, 10 mM-MgCl$_2$, for 2 h at 37 °C). The poly(A$^+$) RNAs were separated from poly(A$^-$) RNAs by oligo(dT)-cellulose chromatography (Favaloro et al., 1980). In some experiments, poly(A$^+$) and poly(A$^-$) RNA fractions (20 µg of each) were treated with pancreatic RNase (50 µg/ml, 30 min at 37 °C). RNase-treated and untreated RNA samples (20 µg), as well as DNA markers, were denatured at 65 °C for 5 min in 6% formaldehyde, 20 mM-MOPS pH 7-0, 5 mM-sodium acetate, 1 mM-EDTA (MOPS buffer) containing 50% deionized formamide. They were submitted to electrophoresis on denaturing
Fig. 2. Blot hybridization analysis of BPV-1-specific RNAs found in transplanted hamster tumours. (a) Untreated poly(A⁺) RNAs (20 μg) extracted from: lane 1, HT₁/₅h; lane 4, HT₂/₄h; lane 6, HT₂/₅₈c, 1h; lane 7, HT₃/₆c, 1h; lane 9, HT₃/₆₂c, 1h. RNase-treated poly(A⁺) RNAs obtained from: lane 2, HT₁/₅h; lane 5, HT₂/₄h; lane 8, HT₃/₆c, 1h. Poly(A⁺) RNAs extracted from: lane 3, HT₁/₅h. Blots were hybridized with nick-translated ³²P-labelled, cloned pBR322/BPV-1 DNA (2.3 × 10⁹ ct/min/μg; 6 × 10⁶ ct/min/cm²). (b) BPV-1-specific transcripts were mapped on the viral genome using three nick-translated ³²P-labelled BPV-1 DNA fragments. A physical map of the BPV-1 DNA used in these studies is shown at the bottom of the figure. The digestion products of BPV-1 DNA (200 ng) treated with BamHII + HindIII (lane 1), BamHI + EcoRI (lane 5) and BamHI + EcoRI + HindIII (lane 9), as well as poly(A⁺) RNAs from HT₁/₅h (lanes 2, 6 and 10), HT₂/₄h (lanes 3, 7 and 11) and HT₃/₆c, 1h (lanes 4, 8 and 12) were hybridized with: lanes 1 to 4, ³²P-labelled fragment B (sp. act. 0.8 × 10⁸ ct/min/μg); lanes 5 to 8, fragment C (sp. act. 1 × 10⁸ ct/min/μg); lanes 9 to 12, fragment A (sp. act. 0-9 × 10⁸ ct/min/μg). Molecular weight standards were provided by hybridization of ³²P-labelled pBR322/BPV-1 DNA probe to BamHII + AvaI-treated cloned BPV-1 DNA fragments, run in the same denaturing gels; they are expressed as nucleotide numbers on the left or right of the figure.

1% agarose gel in MOPS buffer at 250 V for 4 h, and transferred onto nitrocellulose filters in 10 × SSC. After prehybridization, the blots were hybridized with nick-translated, ³²P-labelled, cloned pBR322/BPV-1 DNA as described by Thomas (1980). A broad band was observed in the poly(A⁺) RNA fractions from tumours HT₁/₅h, HT₂/₄h and HT₃/₆c, 1h with electrophoretic mobility corresponding to about 1200 to 1400 nucleotides (Fig. 2a, lanes 1, 4 and 7 respectively). In addition, less intensely labelled bands were detected with HT₁/₅h and HT₂/₄h RNAs, with migration corresponding to about 1750 and 2500 nucleotides respectively (Fig. 2a, lanes 1 and 4). No hybridization was detected with poly(A⁺) RNA fractions after treatment with pancreatic ribonuclease (Fig. 2a, lanes 2, 5 and 8), or with the poly(A⁻) RNA fraction (Fig. 2a, lane 3), indicating the existence of BPV-1 polyadenylated RNA in these tumours. In contrast, no BPV-1-specific transcript was detected in the HT₂/₅₈c, 1h tumour (Fig. 2a, lane 6) or in HT₂/₅₂c and HT₃/₆₂c tumour cells (data not shown), while only trace amounts of an RNA species of about 1200 nucleotides were detected in HT₃/₆₂c, 1h (Fig. 2a, lane 9).

In order to localize homologous sequences of the viral transcripts on the BPV-1 DNA, ³²P-labelled probes were prepared from fragments corresponding to the transforming region (Lowy et al., 1980) of BPV-1 DNA (fragment A and C, from 0-602 to 1-0 and from 0-31 to 0-602 map units respectively), and the non-transforming region (fragment B, from 0 to 0-31 map units) (Fig. 2b). Fragments B and C were obtained by digestion of BamHII-linearized BPV-1 DNA with HindIII or EcoRI respectively, followed by two centrifugations in a 5 to 21% sucrose gradient in the presence of ethidium bromide (Orth et al., 1980). For the preparation of fragment A, digestion products of BamHII-linearized BPV-1 DNA with EcoRI and HindIII were electrophoresed and fragment A was electro-eluted and further purified by sucrose gradient
centrifugation. As a control, each of the 32P-labelled fragments A, B and C were hybridized with electrophoretically separated DNA fragments obtained by digestion of BamHI-linearized BPV-1 DNA with HindIII, EcoRI or a mixture of HindIII and EcoRI (Fig. 2b, lanes 1, 5 and 9). After autoradiography, only the expected fragments were revealed. No hybridization was observed between the non-transforming fragment B and the poly(A+) RNAs extracted from HT1/5h, HT2/4h, and HT3/6c, 1h tumours (Fig. 2b, lanes 2 to 4). In contrast, all the RNA species, detected in these tumours by a total BPV-1 DNA probe, also hybridized with the C and A fragments, the transforming region of BPV-1 genome (Fig. 2b, lanes 6 to 12). The highest labelling intensity of the RNA species was observed with fragment C (0·31 to 0·602 map units) (Fig. 2b, lanes 6 to 8), indicating that the main part of these RNA species corresponds to this fragment.

The presence of BPV-1-specific RNA species have been previously reported in virus-induced bovine warts, hamster tumours, and transformed hamster and mouse cells (Amtmann & Sauer, 1982a, b; Freese et al., 1982; Heilman et al., 1982). The major viral transcripts found in non-virus-producing transformed cells or tumours, ranging between 1050 and 1800 nucleotides, were mapped within the 69% transforming fragment of the BPV-1 genome, i.e. mostly between 0·31 and 0·602 map units. These viral RNA species have been shown to be transcribed from the same DNA strand, and their 3'termini have been located between 0·30 and 0·40 map units (Amtmann & Sauer, 1982b; Heilman et al., 1982).

The study of three hamster sarcomas, induced after a low number of in vitro or in vivo passages, confirms the observation that only the transforming region of the viral genome is transcribed in BPV-1-induced tumour cells, cells, mostly from a region mapped between 0·31 and 0·602 map units. Almost no viral transcription was detected in the case of the two tumourigenic HT2 and HT3 cell lines, or in tumours induced by grafting these cells after more than 50 subcultures, although the copy number and the physical state of the viral genomes remained unchanged. This, and the lack of evidence for virus-coded antigens in HT2 and HT3, tumour cells (Breitburd et al., 1981), suggest that the maintenance of the transformed state and the tumourigenicity of these cells are compatible with a very low level of expression of the viral genome.

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REFERENCES


Short communication


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