Cooperation between bovine papillomavirus type 4 and ras in the morphological transformation of primary bovine fibroblasts

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Primary bovine fibroblasts derived from foetal palate can be transformed by bovine papillomavirus type 4 DNA only in the presence of an activated ras gene, indicating that the virus does not encode all the information required for morphological transformation of non-established cells. A subgenomic fragment containing the complete E8 and E7 open reading frames (ORFs) induces transformation in cooperation with activated ras but transformation is abolished when the E7 ORF is deleted at the 3' end, showing that this ORF encodes a necessary transforming function. Transformation is more aggressive when the E8 and E7 ORFs are placed under the transcriptional control of the long terminal repeat of the mouse Moloney leukaemia virus, suggesting that the degree of transformation is dependent on the level of expression of these genes.

Bovine papillomavirus type 4 (BPV-4) induces papillomas in the upper alimentary canal of cattle (Jarrett et al., 1978a; Campo et al., 1980) which can progress to cancer in animals feeding on bracken fern (Jarrett et al., 1978b). Viral DNA is absent both in naturally occurring and experimentally induced bovine carcinomas (Campo et al., 1985; Campo & Jarrett, 1987) and in in vitro transformed mouse C127 cells (Smith & Campo, 1988, 1989), leading us to propose that BPV-4 transforms cells by a 'hit and run' mechanism.

Despite several similarities between the in vivo and the in vitro system, in nature BPV-4 infection is restricted to the mucous epithelium of the alimentary canal of cattle (Jarrett, 1985) and, therefore, in vitro transformation of established mouse fibroblasts would appear a rather remote system from the natural one. We have therefore investigated the capability of BPV-4 to transform primary bovine cells from the foetal palate. Although fibroblastic, these cells have the distinct advantages of being non-established, belonging to the natural host of BPV-4 and deriving from one of the main sites of BPV-4 infection (Jarrett, 1985). In addition, primary human cervical fibroblasts have been useful in the analysis of the transforming properties of human papillomavirus type 16 (HPV-16) (Matlashewski et al., 1988).

Fibroblasts were explanted from the palate of a bovine foetus (Pal cells) as previously described (Jarrett et al., 1990), routinely grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum and passaged once a week, care being taken not to allow them to reach confluence. The cells were transfected at passage nine with linearized plasmids (Fig. 1) by calcium phosphate-DNA precipitation (Campo & Spandidos, 1983). The precipitate was left on the cells for between 16 and 20 h, after which the cells were washed twice with serum-free medium, refed, replated at a dilution of 1:6 24 h later and grown for at least 21 days, after which time plates were scored for dense foci.

The BPV-4 open reading frame (ORF) assignment shown in Fig. 1 is different from that previously published (Patel et al., 1987), owing to DNA sequence corrections (Stamps & Campo, 1988). The BPV-4 genome lacks an identifiable E6 ORF, a feature shared by BPV-3, another member of the subgroup B bovine papillomaviruses (our unpublished results).

Introduction of BPV-4 DNA into Pal cells consistently had no effect; the cells looked no different from controls and, after prolonged culture, senesced and died (Table 1). This is in contrast to the ability of the fibropapillomaviruses BPV-1 and BPV-2 to transform primary bovine fibroblasts rapidly to full malignancy (Jarrett et al., 1990; W. F. H. Jarrett, unpublished results). The lack of transformation by BPV-4 DNA cannot be attributed to the interruption of the E1 ORF in pBv4 BI, with the consequent loss of some critical transforming function, as the same result was obtained with BPV-4 DNA linearized in the L1 ORF (Table 1).

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It has been shown by Matlashewski et al. (1987) that human papillomavirus type 16 (HPV-16) can transform primary rat kidney epithelial cells only in the presence of an activated ras gene, confirming the necessity for cooperation between two or more oncogenes for transformation of primary cells (Land et al., 1983). Moreover, the ras gene is activated in BPV-4-associated carcinomas of cattle (Campo et al., 1990) suggesting synergism between viral and cellular oncogenes.

When BPV-4 DNA and the activated human ras gene of pT24 (Santos et al., 1982) were cotransfected into Pal cells, areas of giant cells appeared approximately 3 weeks after transfection (Fig. 2b and c). These cells resemble the giant C127 cells described by Burnett et al. (1989), in which maximal replication of BPV-1 DNA takes place. We are currently investigating whether the extraordinary enlargement of Pal cells may similarly be due to BPV-4 DNA replication. Approximately 5 weeks after transfection, the areas of giant cells developed into foci of tightly packed cells morphologically different from the controls (Fig. 2d; Table 1). The same result was obtained with ras together with the activated myc gene of pSVmyc (Land et al., 1983) but not with ras or myc alone, or BPV-4 DNA with myc (Table 1). These foci were picked and expanded but the cells soon reverted to normal morphology, were contact-inhibited and were non-tumorigenic when injected subcutaneously into nude mice at 1 x 10⁷ cells/mouse.

Since non-transformed cells have, in certain cases, been shown to inhibit focal growth of transformed cells (Dotto et al., 1988), the above experiments were repeated in the presence of pSV2neo (Southern & Berg, 1982) and cells were selected for G418 resistance using 500 µg G418/ml of medium. Selection was maintained for 21 days, with two changes of medium per week, and

Table 1. Transformation of Pal cells by BPV-4

<table>
<thead>
<tr>
<th>DNA</th>
<th>G418' colonies/5 x 10⁵ cells</th>
<th>Morphology of G418' colonies</th>
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</tr>
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</table>

* —, Not done.
† NA, Not applicable.
Fig. 2. Transformed Pal 7 cells. (a) Control cells; (b) BPV-4 DNA plus ras, giant cells; (c) a giant cell; (d) BPV-4 DNA plus ras, tight focus; (e) pSVE8+E7+ plus ras; (f) pZipE8+E7+ plus ras. All cells were selected for G418 resistance. Cells were fixed in methanol and stained with Giemsa. Bar markers represent 40 μm.

colonies were scored. When only ras was used, a few small G418 colonies were obtained (Table 1) which died on continuous culturing, a result essentially identical to that obtained with pSVneo alone (Table 1). When BPV-4 alone was used, approximately 20 G418 colonies were obtained (Table 1); all of them showed a flat phenotype, could not be expanded, senesced and died. BPV-4 DNA with ras gave rise to similar numbers of G418 colonies with a tight phenotype similar to that observed in a focus assay (Table 1). These colonies could be expanded only as a pool, suggesting dependence on cell density and cell-cell cooperation, and were not capable of tumour induction in nude mice. Thus, BPV-4 DNA confers a limited growth advantage to primary cells but, even in the absence of surrounding normal cells, cooperation between BPV-4 and ras functions is not sufficient to induce their morphological transformation. Similar results have been obtained by Matlashewski et al. (1988) with HPV-16 DNA and human primary fibroblasts.

We have shown that a subgenomic fragment of BPV-4 containing the long control region (LCR) and ORFs E8 and E7 efficiently transforms C127 cells (Smith & Campo, 1988). A similar fragment was cloned in pSV2neo and in pZipneoSV(XI) (Cepko et al., 1984), generating pSVE8+E7+ and pZipE8+E7+ respectively (Fig. 1). In pSVE8+E7+ the BPV-4 genes are under the transcriptional control of the BPV-4 LCR, which contains both positive and negative regulatory elements (M. Jackson & M. S. Campo, unpublished results), although the enhancer in the simian virus 40 (SV40) early promoter region may be expected to influence their expression. In pZipE8+E7+ the BPV-4 genes have been placed under the powerful transcriptional control of the Moloney leukaemia virus (MoLV) 5' long terminal repeat (LTR).

In both focus and G418-resistance assays, pSVE8+E7+ behaved like the full BPV-4 genome (Table 2). When cotransfected with ras it gave rise to a few tight G418 colonies, morphologically similar to those obtained with BPV-4 DNA plus ras (Fig. 2e; Table 2). Of these, two were expanded and proved to be non-oncogenic in nude mice. The overall qualitative and quantitative similarity of the results obtained with BPV-4 DNA and with pSVE8+E7+ indicates that the effects observed using the
full-length viral genome can be attributed to the functions of the E8 and E7 ORFs and this confirms previous findings (Smith & Campo, 1988) that the other early ORFs and the late region are not necessary for cell transformation. When pZipE8+E7+ alone was used, no foci but large numbers of G418+ colonies were obtained (Table 2). However, when cotransfected with ras, it gave rise to morphologically transformed foci and to large numbers of G418+ colonies, 60% of which showed a distinct transformed piled-up phenotype (Fig. 2f; Table 2).

The greater activity of pZipE8+E7+ compared to pSVE8+E7+, suggests that a higher level of expression of the E8 and E7 ORFs from the retroviral LTR is sufficient to drive the cells to a non-contact inhibited phenotype in cooperation with ras. However, although their phenotype would suggest a higher degree of transformation, these cells are not tumorigenic in nude mice. These results are very similar to those originally reported by Matlashewski et al. (1987) with HPV-16 and ras in rat epithelial cells.

The EcoRI fragment (nucleotides 905 to 1138) was removed from pSVE8+E7+ and pZipE8+E7+, generating pSVE8+E7− and pZipE8+E7−, respectively, in which the 3′ third of the E7 ORF is deleted (Fig. 1). The deleted plasmids showed no transforming activity (Table 2); a few small G418+ colonies were observed with pSVE8+E7− but they were all flat and died rapidly. No G418+ colonies were observed with pZipE8+E7−; the reasons for this are unknown. These results show that an intact E7 ORF is absolutely necessary for cell transformation, confirming earlier observations that digestion of BPV-4 DNA with EcoRI completely abolishes transformation of both NIH3T3 and C127 cells (Smith & Campo, 1988). The deletion removed the second of the two Cys-X-X-Cys motifs of the E7 protein (Fig. 3), which has been shown to bind Zn2+ (Barbosa et al., 1989) and whose mutation in HPV-16 leads to the abolition of E7-driven transformation (Storey et al., 1990; Watanabe et al., 1990). We infer from these results that, as in HPV-16, BPV-4 E7 is responsible for the morphological transformation of primary cells. The E7 protein of HPV-16 binds the cellular protein p105Rb (Dyson et al., 1987). However, although Ser 31 and Ser 32 which are normally phosphorylated in the cellular protein p105Rb (Dyson et al., 1987) are expected to bind p105Rb, their mutation in HPV-16 leads to increased E7 expression and higher levels of cell transformation, bypassing the need for strong heterologous promoters; BPV-4 E2 does trans-activate the homologous LCR (M. Jackson & M. S. Campo, unpublished results) when expressed under the transcriptional control of the SV40 early promoter in pSVE2, or

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### Table 2. Transformation of Pal cells by BPV-4 subclones

<table>
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<tr>
<th>DNA</th>
<th>Foci/5 × 10⁵ cells</th>
<th>G418+ colonies/5 × 10⁵ cells</th>
<th>Morphology of G418+ colonies</th>
</tr>
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<tr>
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</tbody>
</table>

* −, Not done.
† NA, Not applicable.
under the control of the MoLV LTR in pZipE2 (Fig. 1). Cotransfection of Pal cells with pSVE8+E7+, activated ras and pZipE2 approximately doubled the number of G418, colonies, 35% of which were morphologically transformed (Table 2), suggesting that expression of E2 does influence the function of the transforming ORFs, as is the case for HPV-16. No effect was detected with pSVE2 (data not shown), in agreement with the finding that pZipE2 is four times as active as pSVE2 in transactivation assays (M. Jackson, unpublished results).

The DNA of four cell lines transformed by the E8+E7+ plasmids and ras was analysed by Southern blot hybridization for the presence of BPV-4 sequences. The neo' gene sequences were present in all cell lines but no viral DNA could be detected, even when one genome equivalent of the E8 and E7 ORFs could be easily detected in a reconstruction experiment (data not shown). This confirms that in primary, as in established cells, BPV-4 functions are necessary for initiating the transformation process but not for its maintenance.

Cumulatively our results indicate that BPV-4 cannot transform primary fibroblasts in vitro by itself but needs the cooperation of an activated oncogene; the main transforming function is encoded by the E7 ORF, although a role for E8 cannot be excluded. The results also suggest that increased expression of E7 leads to a greater degree of transformation. However, even in the presence of activated ras, the transformed cells are not tumorigenic in nude mice, suggesting that additional events may still be needed for full malignant conversion.

This is in accordance with the requirement for the action of several cofactors during the long delay between primary infection and neoplasia (Jarrett et al., 1978b; Campo & Jarrett, 1987).

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References


