Bovine papillomavirus type 1-transformed primary mouse fibroblasts show
no correlation between tumorigenicity and viral gene expression, but c-myc
gene expression is elevated in tumorigenic cell lines

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Bovine papillomavirus type 1 (BPV-1) causes fibropapillomas in cattle (Lancaster & Olson, 1982) and transforms a variety of cells in culture (Howley, 1983; Lambert et al., 1988). BPV-1-induced transformation in cultures of continuous cell lines has provided a convenient model to investigate papillomavirus-induced transformation and latent viral replication. Extensive genetic analyses of BPV-1 have identified two independent transforming genes, one corresponding to the E6 open reading frame (ORF) and the other to the E5 ORF of the early region (Bergman et al., 1988; Schiller et al., 1984, 1986; Yang et al., 1985). BPV-1-transformed derivatives of continuous cell lines express low levels of BPV-1-specific transcripts of sizes 1 to 4 kb which can cause tumours in nude mice (Baker, 1990; Heilman et al., 1982; Stenlund et al., 1985). However, no virus particles are produced as late gene expression is limited to terminally differentiated epithelium (Baker & Howley, 1987). BPV-1 transcription is self-regulated by the products of the E2 ORF (Lambert et al., 1987; Spalholz et al., 1985, 1987) and, in transformed cells and in warts, has been analysed in detail by cDNA cloning, electron microscopy and S1 nuclease protection assays (Baker & Howley, 1987; Sousa et al., 1990; Stenlund et al., 1985; Yang et al., 1985). Transformation studies of wild-type and mutant BPV-1 genomes in rat FR3T3 cells suggest that activation of viral transcription may be a prerequisite for transformation (Binétruy et al., 1990). Two viral elements appear to play a role in this activation: the activator function of the full-length E2 protein and cis-acting sequences in the late region (Binétruy et al., 1990). Factors such as the site of viral DNA integration and activation of cellular oncogenes can also influence viral transcription, and these probably play an important role in malignant transformation (Couturier et al., 1991; Doeberitz et al., 1991). It has been proposed that the primary role of the virus in malignant transformation may be the initiation of transformation, but the action of cellular genes may be required for the maintenance of the malignant phenotype (Smith & Campo, 1989).

The tumorigenic potential of BPV-1-transfected primary LSH hamster embryo cells was found to correlate with the levels of viral gene transcription and the E5 transforming protein (Zhang et al., 1987). Leptak et al. (1991) were also able to induce morphological and tumorigenic transformation in C127 cells and in a continuous mouse keratinocyte line with the BPV-1 E5 gene. On the other hand, a study on hamster cell lines derived from transplantable tumours showed that neither the presence of the BPV-1 genome nor the viral transcripts correlated directly with tumorigenicity (Jaureguiberry et al., 1983). The role of viral transcription in determining the tumorigenic phenotype in the transformed cells still requires further studies.

Although a considerable amount of information has accumulated about BPV-1-induced transformation and transcription of the viral genes in C127 and NIH3T3 cells, less is known about transformation in primary fibroblasts, especially about the relationship between BPV-1 gene expression and tumorigenicity of the
transformed cells. Our previous studies revealed that several of our transformed primary mouse fibroblast cell lines were tumorigenic in nude and syngeneic mice (Laatikainen et al., 1990). In one of the lines (and its clones), the BPV-1 DNA was integrated into the cell genome (Agrawal et al., 1992). In the present study, cell lines containing episomal or integrated BPV-1 sequences were analysed for BPV-1-specific transcripts and c-myc gene expression. The results were correlated with the tumorigenicity. A high level of c-myc gene expression was found in all lines exhibiting a tumorigenic phenotype. Total BPV-1-specific expression was high in cell lines containing episomal BPV-1 DNA as compared to lines containing integrated BPV-1 sequences. This was mostly due to a higher expression of the E6/E7 region. No correlation was found between BPV-1 transcription and tumorigenicity, although BPV-1 gene expression occurred in all lines.

The five parental cell lines (B6B21, B6B22, B6B31, B6B71 and B6B81) established by transformation with BPV-1 from primary fibroblast cultures of C57BL/6 mice (Mäntyläärvi et al., 1988) were used in the present experiments. Viral DNA is episomal in all the parental lines except B6B31 (and its clones); in this line viral DNA is integrated at a long interspersed repetitive sequence (Agrawal et al., 1992) with a deletion of the cis-acting enhancer sequences proposed to be required for autonomous viral replication and transformation (Lusky & Botchan, 1984, 1986). Clones obtained from line B6B31 by terminal dilution were named by adding a capital letter, e.g. B6B31-A, B6B31-B, etc.

Northern blot analysis (Maniatis et al., 1982) of 5 µg of RNA from each of the BPV-1-transformed cell lines was done with the BPV-1 subgenomic probes shown in Fig. 1 (g). Three major groups of transcripts expressed from the early region were observed with the BPV-E probe (Fig. 1a). Group A consisted of transcripts of sizes greater than 3-5 kb, group B transcripts of sizes between 1.7 and 3.5 kb and group C transcripts of sizes smaller than 1.7 kb (Fig. 1 and Fig. 2). To characterize these transcripts in more detail, the filters were hybridized sequentially with the probes FII and FIV (Fig. 1 b and c). Hybridization with FII and FIV, which detect transcripts containing sequences from the E6/E7 and E2/E5 ORFs, respectively, revealed that the E6/E7 transcripts were mainly confined to groups A and B (Fig. 1b, Fig. 2), whereas the E2/E5 transcripts were found in all three size groups, A, B and C (Fig. 1 c, Fig. 2). Very low levels of viral expression were detected with the FII probe in cell lines B6B22 and B6B71 (episomal viral DNA) and in clones B6B31-A and B6B31-C (integrated viral DNA; data not shown). When FII was used as a probe, no viral expression was seen in any of the cell lines (data not shown), supporting previous observations that E1 ORF expression is very low or absent in BPV-1-transformed cell lines (Burnett et al., 1987). Transcripts coding for the E5 protein should constitute the majority of RNA detected by FIV in group C (Baker, 1990).
BPV-1 early gene expression was compared in cell lines containing episomal (B6B22 and B6B71) or integrated (B6B31 and its clones) BPV-1 sequences by densitometric analysis (Fig. 2). The amount of RNA loaded in each lane was standardized by using the glyceraldheide-3-phosphate dehydrogenase (GAPDH) probe (Fort et al., 1985). Comparison of peak areas obtained with the BPV-E probe to those of the control probe GAPDH indicated that the total BPV-1 early expression was higher in cell lines containing episomal BPV-1 DNA than that in cell lines with integrated DNA (Table 1, probe BPV-E). When BPV-1 E6/E7 expression of these cell lines was estimated by densitometry using FI as a probe, the expression was similarly higher in cell lines containing episomal BPV-1 DNA and extremely low in the clones containing integrated viral DNA (Table 1, probe FI). In autoradiography, an exposure time of 9 days was required to visualize transcripts containing BPV-1 E6/E7 sequences in Fig. 1(b), whereas an exposure of 4 h was enough to show the transcripts containing the BPV-1 E2/E5 sequences in Fig. 1(c). Densitometric comparisons were therefore not feasible. The expression of BPV-1 sequences did not seem to be determined by the physical form of viral DNA in the cells (Table 1).

The two episomal cell lines B6B22 and B6B71 exhibit a very low and a high tumorigenicity in nude mice, respectively (Laatikainen J. et al., 1990). Lines derived from B6B71-induced nude mouse tumours produce tumours in B6 mice. Transcription of BPV-1 early genes was lower in B6B71 than in B6B22 cells, and no specific features of expression could be associated with the tumorigenic phenotype. The tumour-inducing capacity of clones containing integrated BPV-1 DNA is variable (Mäntyjärvi H. et al., 1988; Laatikainen J. et al., 1990).

Table 1. Comparison of BPV-1-specific expression in transformed primary mouse fibroblast cell lines

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Cell line</th>
<th>BPV-1 DNA</th>
<th>Probe</th>
<th>A + B + C</th>
<th>A + B</th>
<th>B</th>
<th>C</th>
<th>A + B + C</th>
<th>A + B</th>
<th>B + C</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>B6B22</td>
<td>E</td>
<td>BPV-E</td>
<td>6.2†</td>
<td>6.1</td>
<td>2.6</td>
<td>3.5</td>
<td>53.6</td>
<td>52.5</td>
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<td></td>
<td>B6B31</td>
<td>I</td>
<td></td>
<td>0.3</td>
<td>0.3</td>
<td>0.7</td>
<td>1.4</td>
<td>2.1</td>
<td>2.1</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>B6B31-A</td>
<td>I</td>
<td></td>
<td>2.1</td>
<td>0.7</td>
<td>0.7</td>
<td>1.4</td>
<td>8.2</td>
<td>8.2</td>
<td>16.3</td>
</tr>
<tr>
<td></td>
<td>B6B31-C</td>
<td>I</td>
<td></td>
<td>1.7</td>
<td>0.6</td>
<td>0.6</td>
<td>1.1</td>
<td>7.5</td>
<td>7.5</td>
<td>15.0</td>
</tr>
<tr>
<td>II</td>
<td>B6B31-J</td>
<td>I</td>
<td>BPV-E</td>
<td>0.8</td>
<td>0.8</td>
<td>1.2</td>
<td>1.2</td>
<td>45.2</td>
<td>45.2</td>
<td>90.4</td>
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<tr>
<td></td>
<td>B6B31-J NuTu A</td>
<td>I</td>
<td></td>
<td>0.05</td>
<td>0.05</td>
<td>0.1</td>
<td>0.1</td>
<td>1.3</td>
<td>1.3</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>B6B31-J NuTu A B6Tul</td>
<td>I</td>
<td></td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>8.2</td>
<td>8.2</td>
<td>16.4</td>
</tr>
<tr>
<td></td>
<td>B6B71</td>
<td>E</td>
<td>FI</td>
<td>3.9</td>
<td>1.7</td>
<td>1.5</td>
<td>2.2</td>
<td>30.6</td>
<td>29.9</td>
<td>60.5</td>
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<tr>
<td></td>
<td>B6B71 NuTu A</td>
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<td>0.5</td>
<td>0.5</td>
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<td>8.7</td>
<td>17.4</td>
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<td></td>
<td>B6B71 NuTu A B6Tul</td>
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<td></td>
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<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>8.7</td>
<td>8.7</td>
<td>17.4</td>
</tr>
</tbody>
</table>

* Data are only comparable within experiment I or II.
† E, Episomal; I, integrated.
‡ Relative values from densitometric analyses with viral probes, after standardization with the control (GAPDH) probe. A, B and C indicate the three size groups of transcripts (see text).
Table 2. Comparison of BPV-1 and c-myc gene expression in transformed primary mouse fibroblast cell lines

<table>
<thead>
<tr>
<th>Tumorigenicity</th>
<th>Cell line</th>
<th>BPV-1 DNA</th>
<th>BPV-E</th>
<th>c-myc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>B6B21</td>
<td>E†</td>
<td>0±1†</td>
<td>0±1†</td>
</tr>
<tr>
<td></td>
<td>B6B22</td>
<td>E</td>
<td>5±4</td>
<td>0±8</td>
</tr>
<tr>
<td></td>
<td>B6B31</td>
<td>I</td>
<td>0±3</td>
<td>0±3</td>
</tr>
<tr>
<td></td>
<td>B6B31-B</td>
<td>I</td>
<td>1±2</td>
<td>1±2</td>
</tr>
<tr>
<td></td>
<td>B6B31-E</td>
<td>I</td>
<td>1±1</td>
<td>1±1</td>
</tr>
<tr>
<td>Positive</td>
<td>B6B31</td>
<td>E</td>
<td>0±1</td>
<td>2±0</td>
</tr>
<tr>
<td></td>
<td>B6B71</td>
<td>E</td>
<td>4±2</td>
<td>1±5</td>
</tr>
<tr>
<td></td>
<td>B6B31-A</td>
<td>I</td>
<td>2±2</td>
<td>2±7</td>
</tr>
<tr>
<td></td>
<td>B6B31-C</td>
<td>I</td>
<td>2±0</td>
<td>3±3</td>
</tr>
<tr>
<td></td>
<td>B6B31-J</td>
<td>I</td>
<td>0±8</td>
<td>1±2</td>
</tr>
</tbody>
</table>

* Data are only comparable within experiments with BPV-E or c-myc.
† E. Episomal; I, integrated.
‡ Relative values from densitometric analyses with viral or c-myc probes obtained after standardization with the control (GAPDH) probe.

B6B31-A, B6B31-C and B6B31-J can all form tumours in nude mice although the parental line B6B31 is non-tumorigenic. The parental line B6B31 expressed low levels of BPV-1-specific transcripts from both the E6/E7 and E2/E5 ORF regions as compared to the tumorigenic clones (Table 1). On the other hand, in vivo passage of the tumorigenic clone B6B31-J resulted in cell lines (B6B31-J NuTu A and B6B31-J NuTu A B6Tu1) with very low levels of expression of the viral early genes (Table 1, Fig. 1 d, e and f). The same relationship was, in fact, observed between the parental cell line B6B71 with episomal viral DNA and its sublines obtained from a tumour in a nude mouse (B6B71 NuTu A) or from a tumour after a further passage in a B6 mouse (B6B71 NuTu A B6Tu1; Table 1, Fig. 1 d, e and f).

Expression of the c-myc gene in the transformed cell lines was analysed with linearized pSV-c-myc as a probe (Land et al., 1983; Fig. 3a). Total RNA loaded on each lane was standardized by hybridizing with the GAPDH probe (Fort et al., 1985). Levels of c-myc gene expression were clearly higher in clones with a tumorigenic phenotype as compared to the non-tumorigenic clones (Fig. 3b). A statistical comparison showed the difference to be at the level of 0·05 > P > 0·01 (U 0·5; Mann-Whitney U test). When the BPV-1 and c-myc gene expression was compared for tumorigenicity in these cell lines (Table 2), no correlation was found between tumorigenicity and viral gene expression but c-myc gene expression was high in all lines with a tumorigenic phenotype (Fig. 3a and b).

BPV-1-specific transcripts were easily detected in all the BPV-1-transformed primary mouse fibroblast lines. The transcription of viral genes was more efficient in transformed cell lines with episomal BPV-1 DNA than in lines where the viral DNA was integrated into the host genome. There are several factors that could influence the viral gene expression in these lines. Regulatory features provided by site of integration may significantly
influence the transcriptional regulation of integrated viral genes (Doeberitz et al., 1991). There can be interactions between cellular factors and viral regulatory sequences (Sousa et al., 1990) present in the long control region. Thus, integration of viral DNA can result in a change in the expression of the viral genes. It has been proposed that at least in the case of human papillomaviruses (HPV), viral gene expression and cell growth may differ among cancers of independent clonal origins as a result of viral integration (Doeberitz et al., 1991). Our results also show that clones derived from a cell line containing integrated BPV-1 sequences can exhibit different levels of viral gene expression and tumorigenicity.

In BPV-1, E5 is the primary viral gene involved in focus formation and transformation, whereas ORF E6 and the 3' end of ORF E7 are expressed primarily in inducing anchorage independence (Neary & DiMaio, 1989). Our results are in agreement with others in that, at least at the viral transcription level, sequences containing the BPV-1 E2/E5 ORF were expressed in all cell lines, although at variable levels (Table 1, FIV probe). One significant feature of the integrated BPV-1 DNA is the deletion of 534 bp (nucleotides 6478 to 7013) from the middle of L1 to the 3' end of the L1 ORF. This deletes the cis-acting replication enhancer sequence (6673 to 6848) reported to be required for efficient replication and transformation of BPV-1 DNA (Lusky & Botchan, 1984, 1986). In BPV-1-transformed FR3T3 rat cells, transformation was associated in the majority of the transfected cells with elevated levels of viral transcription which depended not only on the E2 trans-activator but also on sequences within the late region of the viral genome (Binétruy et al., 1990). Dependence of the transformation and the autonomous replication of BPV-1 DNA on the late viral sequences has been observed previously (Binétruy et al., 1982; DiMaio et al., 1982).

The tumorigenic potential of BPV-1-transformed primary hamster embryo cells was found to correlate directly with the level of viral gene expression and the synthesis of the BPV-1 E5 transforming protein by Zhang et al. (1987). The BPV-1 E5 gene also induced tumorigenicity in C127 cells and in a continuous mouse keratinocyte line (Leptak et al., 1991). However, the tumorigenic phenotype may not be regulated by E5, although other properties characteristic of transformation may still depend on a certain critical level of BPV-1 E5 expression (Binétruy et al., 1990). In our results, no direct correlation was found between the viral gene expression and tumorigenicity. Similarly, Heilman et al. (1982) did not find any correlation between tumorigenicity of transformed hamster cells and viral transcription. Our results also agree with Tada et al. (1989), who demonstrated that in transformed C127 cells there was no correlation between the in vitro parameters of transformation and viral transcription. The authors attributed the degree of expression of the transformed phenotype to changes in the expression of cellular genes.

Our cell lines originate from primary mouse fibroblasts transfected with linearized BPV-1 DNA. Among these cell lines, c-myc oncogene expression was higher in the lines with a tumorigenic phenotype, as compared to the variable expression of viral genes. HPV integration and activation of c-myc oncogene sequences in cervical cancers has been demonstrated (Couturier et al., 1991). In BPV-1-induced transformation, a secondary activation of a cooperating cellular oncogene has been proposed (Binétruy et al., 1990). A majority of C127 cell lines transformed by BPV-4 were negative for viral DNA (Smith & Campo, 1988) leading the authors to propose that viral gene products are necessary for the initiation of transformation but not for the maintenance of the malignant phenotype. Even though our cell lines have in vitro properties of fully transformed cells (Mäntyjärvi et al., 1988), our results indicate that transcription of viral genes may be essential for transformation, but tumorigenicity may depend more on cellular factors than on the activity of viral genes. However, the role of viral and cellular factors at the protein level requires further analyses.

Tumorigenicity of a cell line in an immunocompetent host is also affected by immunological mechanisms, and we have addressed that question in a parallel series of studies (Laatikainen et al., 1990). In our analyses, clones containing integrated viral DNA sequences showed low levels of E6/E7 gene expression suggesting selective down-regulation of these genes on integration. Furthermore, there was a decrease in the viral gene expression in clones obtained from tumours of parental clones containing either episomal or integrated BPV-1 sequences; E6/E7 expression, particularly, was negligible in tumours from B6 mice (Table 1, Fig. 1e). In the case of HPV, the E7 gene has been proposed to code for a transplantation antigen (Chen et al., 1991). If the E7 protein also acts as a transplantation antigen of BPV-1-transformed cells, the decrease in E6/E7 expression in the tumour clones of B6 mice might enable the cells to avoid the host immune response.

In conclusion, there was no correlation between the viral gene expression and the tumorigenicity in BPV-1-transformed mouse cell lines containing either episomal or integrated viral sequences. However, higher levels of c-myc oncogene expression were found in cell lines with a tumorigenic phenotype.

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