Identification of L2 Open Reading Frame Gene Products of Bovine Papillomavirus Type 1 Using Monoclonal Antibodies

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SUMMARY

Four hybridoma cell lines producing monoclonal antibodies (MAbs) to bovine papillomavirus type 1 (BPV-1) L2 open reading frame (ORF) gene products have been established from mice immunized with a BPV-1 L2-β-galactosidase fusion protein. Hybridomas were selected and cloned (from over 700 hybridomas) on the basis of specific reactivity of supernatant fluids with BPV-1 L2 epitopes on disrupted BPV-1 particles and L2-β-galactosidase fusion proteins by ELISA and Western blotting, and with acetone-fixed frozen sections of BPV-1-induced fibropapillomas by immunofluorescence. These MAbs were not reactive with intact BPV-1 particles or BPV-1 L1-β-galactosidase fusion proteins by ELISA or with β-galactosidase by ELISA and Western blotting. The four MAbs detected viral structural proteins of Mr 76K, 68K and possibly 55K in purified BPV-1 preparations by Western blotting. Two of the four MAbs were cross-reactive with BPV-2-induced fibropapillomas. These findings suggest that (i) the BPV-1 L2 ORF encodes the minor capsid protein(s), (ii) the gene products of the BPV-1 L2 ORF have Mr values of 76K, 68K and possibly 55K, (iii) minor capsid epitopes are internal to the BPV-1 particle, and (iv) MAbs reactive with genetically engineered truncated BPV-1 L2 ORF gene products can distinguish between BPV-1 and BPV-2 productive infections.

INTRODUCTION

Papillomaviruses (PV) have a non-enveloped icosahedral capsid with a diameter of 55 nm which contains a single molecule of double-stranded circular DNA of approx. 8000 bp (Melnick et al., 1974). Among the various types of PV, bovine papillomavirus type 1 (BPV-1) has been studied most extensively because of the availability of viral particles in large quantities from bovine fibropapillomas and the capacity of BPV-1 virions to induce stable transformation of rodent cells in vitro (Dvoretzky et al., 1980; Pfister, 1984). The BPV-1 genome has been sequenced and the genetic organization of both early (E) and late (L) open reading frames (ORFs) has been predicted by DNA sequence analysis and confirmed by cDNA analysis of BPV-1 transcripts from both virus-transformed cells in culture and productively infected fibropapillomas (Chen et al., 1982; Baker & Howley, 1987). The L ORFs, designated L1 and L2, span most of the genome region not required for cell transformation. The L1 ORF encodes a 55K major capsid protein [immunologically related to the 96K and 46K structural viral proteins (Cowsert et al., 1987; Nakai et al., 1987)] which constitutes 80% of the structural protein content of the virion (Favre et al., 1975). The BPV-1 minor capsid protein(s) has been putatively identified as having an Mr, of approx. 74K by gel electrophoresis (Meinke & Meinke, 1981). However, the gene products of the BPV-1 L2 ORF have not been previously identified by antibodies specific for the minor capsid protein.
In this study, we used BPV-1 L2-β-galactosidase fusion proteins to generate monoclonal antibodies (MAbs) to identify the $M_r$ of BPV-1 L2-encoded gene products by Western blot analysis and to determine their topography in the BPV-1 capsid by ELISA. In addition, we examined the reactivity and specificity of these MAbs for BPV-1 and BPV-2 L2 epitopes in productively infected fibropapillomas by immunofluorescence (IF).

**METHODS**

**Virus and virus-infected tissues.** BPV-1 was purified from experimentally induced bovine fibropapillomas as previously described by Lancaster & Olson (1978). Virus was stored at −80 °C. Intact and disrupted BPV-1 particles were used for ELISA as described by Cowsert et al. (1987). Particles were disrupted using SDS and heated at 90 °C for 5 min to prevent re-encapsidation.

Acetone-fixed, frozen sections of bovine fibropapillomas experimentally induced by BPV-1 and BPV-2 were tested for intranuclear viral capsid antigens by IF. HPV-1-induced plantar warts shown to be positive for virus particles by electron microscopy and typed previously for HPV-1 by in situ hybridization were also available for IF studies.

**BPV-1 plasmid constructions.** Two plasmid constructions containing the BPV-1 L1 and L2 ORFs were previously described by Pilaciniski et al. (1984, 1986). The plasmid designated pFU41 was the expression vector that synthesized the BPV-1 L2–β-galactosidase fusion protein used as immunogen for production of the MAbs used in this study. In this plasmid, the L2 ORF begins 3 bp downstream from the start codon and is terminally fused with the *Escherichia coli* β-galactosidase gene. A construction of the L1 ORF is contained within a plasmid designated pC2V1. This plasmid contains the L1 ORF beginning 76 bp downstream from the start codon at bp 5686. The L1 construction in pC2V1 is terminally fused to the *E. coli* β-galactosidase gene.

Plasmids were transformed into *E. coli* K12 strain 1829 and both L1 and L2 regions of BPV-1 were expressed as either L1-β-galactosidase or L2-β-galactosidase fusion protein under the control of the lac promoter as described previously by Pilaciniski et al. (1984). The L1- and L2-encoded fusion proteins were identified by Western blotting (data not shown) with rabbit anti-SDS-disrupted BPV-1 (Dako) and anti-L2-β-galactosidase (courtesy of Molecular Genetics) polyclonal antibodies.

**Partial purification of fusion proteins.** Partial purification of fusion proteins was carried out using a modification of the method of Seth & Vande Woude (1985). Bacteria carrying the BPV-1 L1 and L2 expression vectors were used to prepare insoluble pellets of L1 and L2 fusion proteins as described by Lacal et al. (1984). These pellets were then resuspended in 8 M-urea and adjusted to 100 μg of protein in 0.1 ml of phosphate-buffered saline (PBS) as determined by the Bio-Rad Protein Assay.

**Immunization.** Female, 6-week-old BALB/c mice (Jackson Laboratories) were immunized subcutaneously with 100 μg (0.1 ml in volume) of partially purified L2–β-galactosidase fusion protein emulsified in equal volumes of complete Freund’s adjuvant on day 0. On days 14, 28 and 42, the mice were inoculated intraperitoneally with 100 μg fusion protein in equal volumes of incomplete Freund’s adjuvant. On day 50, several animals were sacrificed by cervical dislocation and the spleens were removed aseptically. Spleen cell suspensions were prepared for fusion as previously reported (Cowsert et al., 1987).

**Hybridoma production.** Spleen cells of animals immunized with the L2–β-galactosidase fusion protein were fused with P3/NS1/AG4-1 myeloma cells and hybrids were selected according to the method of Herzenberg & Milstein (1978). Hybrids producing antibodies reactive with both the L2–β-galactosidase fusion protein and disrupted BPV-1 virion by ELISA were scored as positive clones for this study. Clones that reacted with purified β-galactosidase by ELISA were scored as negative and excluded from the study. Positive clones were subcloned twice by limiting dilution. The four clones (3A10, 3E8, 3F8 and 6A8) selected for this study demonstrated positive reactivity of supernatant fluids with BPV-1-induced fibropapillomas by IF.

**ELISA.** ELISA was performed on 96-well plates (Immunolon II, Dynatech) coated with either 20 μg of L2–β-galactosidase, L1–β-galactosidase and β-galactosidase, or 50 μg of intact and SDS-disrupted BPV-1. The fusion protein, β-galactosidase, and disrupted BPV-1 were absorbed to 96-well plates and air-dried overnight at room temperature. Intact BPV-1 virions were coated on the plates at 37 °C for 1 h to prevent partial disruption of the capsid. All subsequent incubations were at 37 °C for 1 h. ELISA was then done as previously described by Cowsert et al. (1987). Absorbance was determined by a MR700 Dynatech Micro-ELISA reader at 410 nm wavelength.

MAb immunoglobulin isotypes were determined by ELISA using a MonoAB-ID EIA kit (Zymed).

**Immunofluorescence.** Indirect IF was performed on acetone-fixed frozen sections of BPV-1 and BPV-2-induced fibropapillomas and HPV-1-induced plantar warts as previously described (Jenson et al., 1980). Hybridoma supernatant, ascitic fluid or polyclonal serum diluted in PBS were used as primary antibody. Tissue sections were incubated with MAb or polyclonal antibody either for 1 h at room temperature or overnight in a humidified chamber at 4 °C. Fluorescein isothiocyanate-labelled affinity-purified goat anti-mouse or anti-rabbit IgG antibody was used as secondary antibody. Tissue sections were mounted in Gelvatol and viewed with a standard Olympus fluorescence microscope with the appropriate excitation and barrier filters.
Identification of BPV-1 L2 ORF gene products

**SDS–PAGE and Western blots.** Purified BPV-1 (7.5 µg), 10 ng of β-galactosidase and 6 µg of L2-β-galactosidase fusion protein were electrophoresed through 8% polyacrylamide gel containing 0.1% SDS (Laemmli, 1970). Polypeptides separated by SDS–PAGE were transferred electrophoretically to nitrocellulose membranes (Towbin et al., 1979). Blots were washed for 15 min in 0.4% Tween-20, 0.15 M NaCl, 0.02 M Tris–HCl pH 7.5 (TBS) and incubated for 1 h at room temperature with MAbs, polyclonal antibodies or mouse sera diluted with TBS. The blots were washed three times with TBS and incubated with alkaline phosphatase-conjugated goat (IgG) anti-mouse IgG diluted in TBS for 1 h at room temperature. The reaction was developed by immersing the membrane in a solution containing 0.1% naphthol AS-BI phosphoric acid, 0.1% fast violet B salt, 0.001 M MgCl₂, 0.1 M Tris–HCl pH 9.5, followed by three washes with TBS. To estimate the Mr of detectable polypeptides, parallel blots of protein standards were stained by amido black (Kaplan & Pedersen, 1985).

**RESULTS**

**Selection of hybridomas for cloning**

Tissue culture supernatants from 10% (70 of approx. 700) of wells containing hybridomas were positive by ELISA for reactivity with L2-β-galactosidase fusion proteins. The 70 positive supernatants were then tested by ELISA on intact and disrupted BPV-1 particles; 32 of 70 culture well fluids were reactive only with disrupted virus particles by ELISA (Table 1). When tested in Western blots, 15 of the 32 supernatants were reactive with polypeptides having Mr values of 76K, 68K and 55K; the remaining 17 supernatants appeared to bind non-specifically with a 55K protein. Four of the 15 supernatants that were reactive with both fusion proteins and disrupted BPV-1 particles were reactive with BPV-1 productively infected fibropapillomas by IF. These four hybridoma cell lines were then cloned twice and the corresponding MAbs were retested for reactivity and specificity, which remained unchanged.

All MAbs were IgG with kappa light chains; two were of the G1 subclass and two were G2a (Table 2).

**Identification of BPV-1 L2 gene products**

By Western blot analysis, all anti-BPV-1 L2 MAbs detected 76K, 68K and, possibly, 55K minor structural proteins in purified BPV-1 virions (Fig. 1). Rabbit antisera raised against L2-β-galactosidase fusion proteins gave identical results. To confirm the specificity of anti-BPV-1 L2

**Table 1. ELISA reactivity of MAbs with intact and disrupted BPV-1 measured in optical density units**

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>Normal mouse serum</th>
<th>13D6*</th>
<th>1H8*</th>
<th>3A10</th>
<th>3E8</th>
<th>3F8</th>
<th>6A8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact BPV-1</td>
<td>0.018</td>
<td>0.002</td>
<td>1.631</td>
<td>0.000</td>
<td>0.004</td>
<td>0.006</td>
<td>0.007</td>
<td>0.008</td>
</tr>
<tr>
<td>Disrupted BPV-1</td>
<td>0.009</td>
<td>0.020</td>
<td>0.000</td>
<td>1.954</td>
<td>0.103</td>
<td>0.152</td>
<td>0.160</td>
<td>0.269</td>
</tr>
</tbody>
</table>

* Monoclonal antibody 13D6 recognizes an external conformational epitope; MAb 1H8 recognizes an internal linear epitope (Cowsert et al., 1987).

**Table 2. Characterization of MAbs prepared against BPV-1 L2-β-galactosidase fusion proteins by ELISA for immunoglobulin class and IF for distribution of reactive epitopes in BPV-1, BPV-2 and HPV-1 productive infections**

<table>
<thead>
<tr>
<th></th>
<th>Control*</th>
<th>MAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMS 1H8 (IgG1 x)</td>
<td>3A10 (IgG2a x)</td>
<td>3E8 (IgG1 x)</td>
</tr>
<tr>
<td>BPV-1 fibropapilloma</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>BPV-2 fibropapilloma</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Human plantar wart</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

* NMS, Normal mouse serum; 1H8, MAb that reacts with internal, linear, broadly cross-reactive epitope of PV major capsid protein (Cowsert et al., 1987).
Fig. 1. Comparison of reactivity of anti-L1 and anti-L2 MAbs and anti-L2-β-galactosidase polyclonal antibodies with BPV-1 capsid proteins by Western blot. Lane 1, Mr standards (amido black stain); lane 2, anti-BPV1 L1 MAb (1H8) reactivity with BPV-1; lane 3, anti-BPV-1 L2 MAb (3A10) reactivity with BPV-1; lane 4, anti-BPV-1 L2 MAb (3E8) reactivity with BPV-1; lane 5, rabbit anti-L2-β-galactosidase polyclonal antibody reactivity with BPV-1. Anti-BPV-1 L2 MAbs that are type-specific (3E8) and BPV-1/BPV-2 cross-reactive (3A10) and the L2-β-galactosidase polyclonal antibody detected epitope(s) on BPV-1 structural polypeptides (76K, 68K and 55K). Anti-BPV-1 L1 MAb (1H8) detects major capsid protein (55K).

MAbs, Western blot analysis of β-galactosidase and L2-β-galactosidase fusion proteins was also carried out. All four MAbs failed to react with purified β-galactosidase (and BPV-1 L1-β-galactosidase by ELISA); these MAbs detected a 137K protein in a partially purified L2-β-galactosidase fusion protein preparation. Several lower Mr polypeptides were also detected and may represent degradation products of the higher Mr protein. (Fig. 2, 3.)

Topography of the L2 gene products in the BPV-1 capsid

The four anti-BPV-1 L2 MAbs only reacted with disrupted BPV-1 particles by ELISA. Occasionally, however, reactivity was seen with intact particles. This reactivity appeared to correspond to partial denaturation of the virus particle, probably caused by prolonged physical contact with plastic surfaces. For this reason, intact particles were coated on plastic wells for only 1 h before incubation with appropriate antibodies. In addition, the integrity of the intact particles was determined by lack of reactivity with an anti-BPV-1 L1 MAb that recognizes only internal major capsid epitopes. The presence of reactivity with an anti-BPV-1 L1 MAb that recognized only external conformational capsid epitopes was used as a positive control for capsid integrity.

All anti-BPV-1 L2 MAbs were reactive with L2-β-galactosidase fusion proteins as well as disrupted BPV-1 particles by ELISA (Table 1). ELISA values were greater with the fusion protein preparations (> 2000) than with disrupted virions (0.100 to 0.270), perhaps reflecting an increased concentration of epitopes and/or increased affinity of the MAbs for the configuration of the epitope in the L2-β-galactosidase fusion protein preparation used as the immunogen. These MAbs did not react with either β-galactosidase or L1-β-galactosidase fusion protein by ELISA.
Identification of BPV-1 L2 ORF gene products

Fig. 2. Reactivity of anti-L2-β-galactosidase polyclonal antibodies (lane 2), anti-β-galactosidase MAb (lane 3), anti-BPV-1 L2 MAb 3A10 (lane 4) and anti-BPV-1 L2 MAb 3E8 (lane 5) with L2-β-galactosidase fusion protein by Western blot. Mr standards (amido black stain) are shown in lane 1. All antibodies react with a 137K polypeptide. Several lower Mr polypeptides are visible and probably represent degradation products.

Fig. 3. Western blot analysis of β-galactosidase. Lane 1, anti-β-galactosidase MAb; lane 2, anti-BPV-1 L2 MAb (3A10); lane 3, anti-BPV-1 L2 MAb (3E8). Only the anti-β-galactosidase MAb is reactive with β-galactosidase and associated degradation products.

**Detection of L2 epitopes in productively infected fibropapillomas**

All four MAbs (3A10, 3E8, 3F8, 6A8) were reactive with BPV-1-induced fibropapillomas by IF. Two MAbs (3A10, 3F8) were reactive with both BPV-1- and BPV-2-induced fibropapillomas by IF. The anti-L2-β-galactosidase polyclonal antisera reacted with both BPV-1- and BPV-2-induced fibropapillomas. Both MAbs and polyclonal antisera showed specific intranuclear reactivity with acetone-fixed frozen sections of the bovine fibropapillomas by IF (Fig. 4). This was identical to the staining pattern seen when consecutive sections of bovine fibropapillomas were reacted with a MAb that recognized type- and genus-specific epitopes carried on the BPV-1 major capsid protein. Neither the MAbs nor the polyclonal antisera were reactive with human papillomavirus type 1 (HPV-1)-induced plantar warts shown to be positive for viral particles by electron microscopy (Table 2). We have previously shown that serotyping of PV can be accomplished as accurately by IF of productive infections as by ELISA and Western blotting of partially purified intact and disrupted PV particles (Cowsert *et al.*, 1987).
Fig. 4. BPV-1 type-specific L2 antigenic epitopes are identified in the nuclei of the outer granular layer of squamous epithelium in acetone-fixed frozen sections of BPV-1-induced bovine fibropapilloma reacted with anti-BPV-1 L2 MAb 3E8 and visualized by IF.

DISCUSSION

Four MAbs generated against BPV-1 truncated L2-β-galactosidase fusion proteins were used to identify BPV-1 type-specific and BPV-1/BPV-2 cross-reactive non-conformational (linear) minor structural protein epitopes by their immunological reactivity with (i) denatured BPV-1 particles and L2-β-galactosidase fusion proteins by ELISA, (ii) BPV-1 structural proteins of Mr 76K, 68K and possibly 55K, and an L2-β-galactosidase fusion protein of approx. Mr 137K in Western blots, and (iii) BPV-2 and/or BPV-1 productively infected fibropapillomas by IF. The specificity of these MAbs for BPV-1 minor capsid epitopes was further shown by their lack of reactivity with L1-β-galactosidase by ELISA and β-galactosidase in both ELISA and Western blots. These data provide definitive evidence that the BPV-1 L2 ORF encodes the minor capsid protein.

All anti-BPV-1 L2 MAbs reacted with 76K, 68K and 55K structural polypeptides of BPV-1 by Western blot analysis. Several possibilities exist for the presence of these immunologically related polypeptides. It is possible that one or more of the polypeptides represent the gene products of complex RNA splicing patterns (Engel et al., 1983), post-translational modification of the minor capsid protein, or degradation products of the 76K protein.

Polyclonal antisera have been raised against HPV-1 and HPV-6 L2-β-galactosidase fusion proteins (Doorbar & Gallimore, 1987; Tomita et al., 1987). The HPV-1 L2-β-galactosidase antisera were considered to be HPV-1 type-specific since they were reactive only with productively infected HPV-1-induced plantar warts and not warts infected by other HPVs by IF. The HPV-6 L2-β-galactosidase antisera were reactive with PV structural antigens in condylomata acuminata, although these venereal warts were apparently not typed for HPV-6 or HPV-11. In the present study, BPV-1 L2 type-specific and BPV-1/BPV-2 L2 cross-reactive MAbs raised against BPV-1 L2-β-galactosidase fusion proteins were capable of distinguishing
between BPV-1 and BPV-2 productive infections by IF. However, the anti-BPV-1 L2-β-galactosidase polyclonal antibodies were reactive with both BPV-1- and BPV-2-induced fibropapillomas by IF. Based on studies by Doorbar & Gallimore (1987) and Komly et al. (1986), and the results of this study, it seems that polyclonal antisera prepared against L2 fusion proteins may be capable of distinguishing between distantly related PV types by IF, but MAbs will probably be necessary to distinguish between closely related viral types such as BPV-1/BPV-2 and possibly HPV-6/HPV-11. It will be particularly important to determine whether serotyping of PV in tissue by using MAbs raised against L2-β-galactosidase fusion proteins will parallel the genotyping of the same PV by using DNA hybridization.

HPV-1 L2–β-galactosidase polyclonal antisera did not react with intact HPV-1 particles in immunodiffusion studies, suggesting that the minor capsid protein is not present on the surface of the capsid (Komly et al., 1986). In the present study, intact BPV-1 particles were not reactive with anti-BPV-1 L2 MAbs by ELISA. However, PV particles appear to be easily denatured by physical contact, particularly with plastic surfaces, and epitopes normally inside intact particles are readily exposed to reactive antibodies. Therefore, the integrity of intact BPV-1 particles was monitored by positive ELISA reactivity with a MAb that recognizes only type-specific, conformational epitopes on the surface of BPV-1 capsids, and negative ELISA reactivity with a MAb that recognizes genus-specific epitopes which are internal to the intact virus particle. On the basis of the present study and that of Komly et al. (1986), it appears that genetically engineered PV minor capsid proteins would not provide the antigenic determinants necessary for development of protective immunity in the host against PV infection.

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