Expression of Human Papillomavirus Type 6 and Type 16 Capsid Proteins in Bacteria and Their Antigenic Characterization

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SUMMARY

The L1 and L2 capsid proteins encoded by human papillomavirus types 6 and 16 (HPV-6 and HPV-16) have been synthesized in bacteria. Antisera were raised against the HPV-6 L1- and L2-β-galactosidase fusion proteins and against an HPV-16 L1 C-terminal peptide which was 14 amino acids long. The HPV-16 L1 peptide antibodies have been shown to be highly reactive with the HPV-16 L1-β-galactosidase fusion protein but not against the equivalent HPV-6 L1-β-galactosidase fusion protein. The effectiveness of these antibodies was compared with commercially available anti-bovine papillomavirus type 1 (BPV-1) antibodies and the results demonstrated that the anti-BPV-1 antibodies reacted well against HPV-6 L1-β-galactosidase but not against HPV-16 L1-β-galactosidase. In addition, the L2 portion of the HPV-6 L2-β-galactosidase fusion protein appeared particularly immunogenic, since antibodies raised against this fusion protein were predominantly reactive with the L2 moiety. The HPV-16 L1 peptide antibodies described here will be preferred reagents for the specific detection of HPV-16 capsid antigens, which may be particularly important in early diagnosis of HPV-16 infection.

INTRODUCTION

Papillomaviruses are found in a number of higher organisms including man, in which over 40 different types have now been identified. Of these many types, most cause only mild if inconvenient lesions of skin and mucosa (zur Hausen, 1977). Recently however, a small number of these viruses have been shown to be probable aetiological agents in a number of human cancers of which cervical cancer is by far the most prevalent. Over 90% of cervical cancer biopsies contain human papillomavirus (HPV) DNA of types 16, 18, 31, 33 or 35, of which types 16 and 18 are most common (zur Hausen, 1985). Cell lines derived from these tumours have been shown to contain HPV DNA (Dürst et al., 1983; Boshart et al., 1984), RNA (Schwarz et al., 1985; Schneider-Gadicke & Schwarz, 1986) and a number of HPV early proteins (Smotkin & Wettstein, 1986; Banks et al., 1987; Androphy et al., 1987). In contrast, HPV-6 and HPV-11 infections tend to be associated with benign lesions of the genital tract. Hence early identification of the HPV type in an infection is particularly important, and immunologically based assays seem to offer the most convenient means of diagnosis.

To date most studies on HPV capsid antigens in infected tissue have relied upon commercial anti-bovine papillomavirus type 1 (BPV-1) antisera (Dako, Copenhagen, Denmark) raised
against detergent-disrupted BPV-1 virions (Jenson et al., 1980; McDougall et al., 1986; Fey et al., 1986). Recently however it has been shown that the L2 open reading frame (ORF) of HPV-1a encodes a type-specific viral capsid protein easily detectable in HPV-1-induced warts (Komly et al., 1986). Thus viral capsid proteins appear to be the most likely targets for immunological diagnosis. Here we report the expression of the viral capsid proteins in *Escherichia coli* from the L1 and L2 ORFs of both HPV-6 and HPV-16. Antisera have been raised to these proteins and these have been used to investigate their antigenic properties.

**METHODS**

*Production of HPV-6 capsid proteins in E. coli.* From sequence information of HPV-6 (Schwarz et al., 1983) we located the L1 and L2 ORFs, and identified suitable restriction fragments for cloning into the pUR vectors (Rüther & Müller-Hill, 1983). For HPV-6 L1, a *XbaI* to *HindIII* fragment extending from nucleotides 5903 through 7902 to 2307, coding for amino acids 39 to 501, was purified by gel electrophoresis and then ligated into *XbaI/HindIII*-cut pUR288. For HPV-6 L2, a *BamHI* to *XbaI* fragment extending from nucleotides 4722 to 5903, coding for amino acids 94 to 460, was purified by gel electrophoresis and then ligated into *BamHI/XbaI*-cut pUR278. The resulting plasmids were used to transform *E. coli* H4.4 and ampicillin-resistant clones were grown in the presence of 100 µg/ml isopropyl-β-D-thiogalactoside (IPTG). Cells were grown to saturation at 37 °C, collected by centrifugation then washed in PBS (150 mM-NaCl, 10 mM-sodium phosphate, pH 7.2). Bacterial cells were then lysed in SDS-PAGE sample buffer (50 mM-Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 0.1 M-dithiothreitol), subjected to SDS-PAGE (Laemmli, 1970) and stained with Coomassie Brilliant Blue.

The recombinant plasmid pAS-6L2 expressing HPV-6 L2 alone was made as follows. The *BamHI* to *XbaI* fragment extending from nucleotides 4722 to 5903, was made blunt-ended at the *XbaI* site with T4 DNA polymerase and purified by gel electrophoresis. It was then ligated into *BamHI*- and *SalI*-cut plasmid vector pAS1 (Rosenberg et al., 1983) which was also made blunt-ended at the *SalI* site. The ligation mixture was used to transform *E. coli* AR120 which contains the *λCl* gene. Ampicillin-resistant clones were grown at 37 °C to an *A550* of 1 and then in the presence of 40 µg/ml nalidixic acid for an additional 4 h at 37 °C. Cells were collected and analysed as described above.

*Production of HPV-16 capsid proteins.* From sequence information of HPV-16 (Seedorf et al., 1985) the L1 and L2 ORFs were located. For HPV-16 L1, a *BamHI* to *DdeI* fragment extending from nucleotides 6150 through 7904 to 24 coding for amino acids 196 to 531 was made blunt-ended with T4 DNA polymerase, a *BamHI* linker was inserted, the fragment was purified by gel electrophoresis and then ligated into the *BamHI* site of pUR289. For HPV-16 L2 a *StuI* to *BamHI* fragment extending from nucleotides 4466 to 6150, coding for amino acids 78 to 473, was made blunt-ended with T4 DNA polymerase, a *BamHI* linker was inserted, the fragment was purified by gel electrophoresis and then ligated into the *BamHI* site of pUR278. The resulting plasmids were then used to transform *E. coli* H4.4 and resulting clones were analysed by SDS–PAGE as described above.

In addition, HPV-16 L1 sequences were also cloned into pEX expression vectors (Stanley & Luzio, 1984). Two constructs coding for HPV-16 L1-β-galactosidase fusion proteins from the pEX vectors were produced: pHX-1 containing a *HphI* to *BamHI* fragment coding for HPV-16 L1 amino acids 1 to 195 and pHX-2 containing a *BamHI* to *SphI* fragment coding for amino acids 196 to 531. These were induced by growing bacterial cultures overnight at 30 °C followed by temperature shift to 42 °C for 2 h. Proteins were then analysed as described above. The differences between pHX-2 and pUR-16 L1 are additional 3' non-coding sequences in pUR-16 L1 and pHX-2 is induced by temperature shift whereas pUR-16 L1 is induced with IPTG.

*Production of anti-HPV-16 L1 antibodies and analysis by Western blotting.* BALB/c mice were inoculated twice subcutaneously with 30 µg peptide coupled to thyroglobulin in incomplete Freund's adjuvant. The 14 amino acid peptide from the carboxy-terminal region of L1 was synthesized by the solid-phase technique and then coupled through its N terminus to thyroglobulin as described (Nelson et al., 1984). Mice were given an intravenous boost with a further 30 µg peptide-thyroglobulin conjugate 2 weeks after the initial inoculation. One week later, sera were collected and tested for anti-L1 activity by Western blotting as follows. Bacterial cells containing HPV-6 or HPV-16 L1-β-galactosidase fusion proteins were lysed in SDS-PAGE sample buffer and subjected to SDS–PAGE. Proteins were electrophoretically transferred onto nitrocellulose filters as described previously (Banks et al., 1985). Blots were then incubated in blocking buffer (10% calf serum in PBS) for 2 h at 42 °C. Antiserum was then diluted in blocking buffer and the blots were incubated at room temperature for 3 h. Blots were washed three times in PBS then reacted with goat anti-mouse-biotin conjugate (Sigma) followed by avidin-peroxidase conjugate (Sigma). Blots were developed in the presence of 0.1% hydrogen peroxide and 1.5 mg/ml Hanker-Yates reagent (Polysciences).

*Production of anti-HPV-6 capsid antibodies.* Mice were inoculated as described above with 3 µg per inoculation of gel-purified HPV-6 L1-β-galactosidase or HPV-6 L2-β-galactosidase fusion proteins. Sera were collected and tested for either anti-L1 or anti-L2 activity by Western blotting as described above.
RESULTS

Expression of HPV-6 capsid proteins in E. coli

For HPV-6 L1 the XbaI to HindIII fragment containing coding sequence for amino acids 39 to 501 was cloned into the prokaryotic expression vector pUR288 as described in Methods. This resulted in a construct coding for the HPV-6 L1 ORF linked to the C terminus of the β-galactosidase gene (Fig. 1a). Similarly, for HPV-6 L2 a BamHI to XbaI fragment containing coding sequence for amino acids 94 to 460 was cloned into pUR278. This resulted in a construct coding for the HPV-6 L2 ORF linked to the C terminus of the β-galactosidase gene (Fig. 1b). Upon IPTG induction the HPV-6 L1-β-galactosidase fusion protein synthesized had a mol.wt. of about 153K (Fig. 2a, lane 2), which was somewhat smaller than would have been estimated from the L1 amino acid sequence linked to β-galactosidase, although it was apparent that there were a number of degradation products. The HPV-6 L2-β-galactosidase fusion protein had a mol.wt. of about 160K (Fig. 2a, lane 3), which was similar to that which would be estimated from the L2 amino acid sequence linked to β-galactosidase.

Expression of HPV-16 capsid proteins in E. coli

For HPV-16 L1 a BamHI to DdeI fragment containing coding sequence for amino acids 196 to 531 was cloned into pUR289. This construct coded for the HPV-16 L1 ORF linked to the C terminus of the β-galactosidase gene (Fig. 1d). Finally, for HPV-16 L2 a StuI to BamHI...
fragment containing coding sequence for amino acids 78 to 473 was cloned into pUR278 as described in Methods. This construct coded for the HPV-16 L2 ORF linked to the C terminus of \( \beta \)-galactosidase (Fig. 1 e). Upon IPTG induction the HPV-16 L1–\( \beta \)-galactosidase fusion protein synthesized had a mol.wt. of about 125K (Fig. 2b, lane 2) which was considerably smaller than would be estimated from the L1 amino acid sequence, suggesting that a large amount of degradation was taking place. The HPV-16 L2–\( \beta \)-galactosidase fusion protein synthesized had a mol.wt. of about 130K (Fig. 2b, lane 3) which was again somewhat smaller than the estimate of 160K mol.wt. Both of the HPV-16 fusion proteins appeared to be unstable, thus explaining the low levels of expression. However, as shown below, an HPV-16 L1–\( \beta \)-galactosidase fusion protein of the expected size was detectable by Western blotting, although the protein was not visible on a Coomassie Brilliant Blue-stained gel.

Production of antibodies against HPV-16 L1

From the HPV-16 sequence information it was possible to predict the primary structure of an L1 C-terminal peptide. This was chosen since from our previous experience, antibodies to peptides derived from the C terminus of a protein are most likely to react with the native protein. A peptide was made which was 14 amino acids long and ended eight amino acids from the C terminus of L1 (Fig. 3a). This was linked to thyroglobulin and used to inoculate mice. The anti-L1 peptide antiserum was then reacted on Western blots with the L1–\( \beta \)-galactosidase fusion proteins.
proteins. Since the L1-β-galactosidase fusion protein generated above tended to become degraded, two additional L1-β-galactosidase fusion proteins were used. These proteins consisted of the amino-terminal half of HPV-16 L1 (pHX-1 coding for amino acids 1 to 195) and the carboxy-terminal half of HPV-16 L1 (pHX-2 coding for amino acids 196 to 531) and were found to be more stable than the pUR-derived L1 fusion protein. The results obtained are shown in Fig. 3. Clearly the antiserum to the L1 peptide reacted strongly with two of the HPV-16 L1-β-galactosidase fusion proteins (Fig. 3c, lanes 4 and 5) and, as expected, no reaction was observed against the pHX-1 amino-terminal fusion protein (Fig. 3c, lane 3). No reaction was observed against either β-galactosidase alone or the HPV-6 L1-β-galactosidase fusion protein (Fig. 3c, lanes 1 and 2 respectively). This result confirms the correct translation of the HPV-16 L1-β-galactosidase fusion protein. The peptide antiserum detected a band of approximately 145K mol.wt. (Fig. 3c, lane 5) although only a weak Coomassie Brilliant Blue stained band was visible in this position (Fig. 3b, lane 5). However, we believe that this stained protein represents a background polypeptide, confirming the extensive degree of degradation of this fusion protein.

Production of antibodies against HPV-6 capsid proteins

The HPV-6 L1- and L2-β-galactosidase fusion proteins were excised from preparative polyacrylamide gels and used to inoculate mice over a number of weeks as described previously (Banks et al., 1987). The activity of the antibodies was then determined by Western blot analysis of E. coli H4.4 cells expressing the appropriate fusion protein. The results obtained are shown in Fig. 4. Anti-HPV-6 L1-β-galactosidase fusion protein antibodies clearly reacted strongly with both fusion proteins (Fig. 4a), demonstrating antibody against at least the β-galactosidase portion of the L1 fusion protein. The anti-HPV-6 L2-β-galactosidase fusion protein antibodies
reacted with the L2–β-galactosidase fusion protein and degradation products thereof, but only very weakly with the L1–β-galactosidase fusion protein (Fig. 4b, lane 1). Only a very weak reaction was observed against pUR–β-galactosidase alone (data not shown), and the strong reaction obtained against the protein of lower mol.wt. (Fig. 4b, lane 2) represented reaction against residual L2 sequences on the degraded L2–β-galactosidase fusion protein. This result was particularly surprising, since it demonstrated that virtually the entire humoral response in these mice was directed against the L2 portion of the L2–β-galactosidase fusion protein. The specificity of these antibodies was investigated further by Western blot analysis on E. coli AR120 cells expressing HPV-6 L2 not fused to β-galactosidase. This construct was generated as described in Methods (Fig. 1c), and upon nalidixic acid induction of these cells a 50K mol.wt. protein was induced (Fig. 5a). When these cells were reacted with anti HPV-6 L2–β-galactosidase fusion protein antibody, a strong reaction was obtained (Fig. 5b).

Antigenic analysis of the HPV capsid proteins

From the above data it is apparent that the anti-HPV-16 L1 peptide antiserum would be an ideal reagent for differentiating between HPV-6 and HPV-16. However, we were also interested in determining how they compared with commercially available antisera to papillomavirus capsid proteins. Hence a series of Western blots were done with the papillomavirus capsid proteins using antisera to BPV capsid antigens (Jenson et al., 1980) now marketed by Dako,
which is routinely used with biopsy samples to diagnose HPV infection. The results obtained are shown in Fig. 6, and demonstrate a number of interesting points. First, the BPV antiserum (Fig. 6d) had no reactivity against any of the L2-β-galactosidase fusion proteins (lanes 1 and 5), confirming that the L2 protein is not conserved between different papillomaviruses, and is thus an ideal target for immunological typing of an HPV infection. Second, although the anti-BPV serum reacted very strongly against the HPV-6 L1-β-galactosidase fusion protein (lane 6), very little activity was observed against the HPV-16 L1-β-galactosidase fusion proteins (lanes 2 to 4). This result indicates that this antiserum was not really effective against HPV-16, and would not be an efficient or reliable reagent for diagnosing HPV-16 infection even where capsid proteins were being produced. Finally, it is apparent that the anti-BPV sera reacted against only the pHX-2 fusion protein (lane 4) and not pHX-1 (lane 3). This indicates that most of the cross-reactive epitopes which exist on the L1 protein are contained primarily within the carboxy-terminal half of the ORF.

**DISCUSSION**

Here we have described the expression of the capsid proteins of HPV-6 and HPV-16 in *E. coli*, and the production of antisera specific for the HPV-6 L1 and L2 proteins and the HPV-16 L1 protein. These reagents should prove invaluable for early diagnosis of HPV-16 infection in
samples where this protein is being expressed. These antibodies should also permit detailed analysis of HPV capsid antigen expression in a variety of different cell systems.

Western blot analysis of these proteins has revealed a number of interesting points. Although polyclonal antibodies raised against detergent-disrupted BPV-1 detect papillomavirus genus-specific antigens shared by all papillomaviruses (Orth et al., 1978; Jenson et al., 1980) it is clear that this cross-reactivity varies to a considerable extent. Here it is apparent that antiserum raised to detergent-disrupted BPV-1 reacts very well against HPV-6 L1 protein, but very poorly against HPV-16. Hence the efficacy of using such antiserum for detection of HPV-16 antigens must be questionable. It is notable that the small reactivity observed with the anti-BPV-1 serum is against the carboxy-terminal half of the L1 protein. This is significant from the point of possible vaccine development, since sequences from the carboxy-terminal half of the L1 protein are quite highly conserved between HPV types. Because of the weakness of this reaction with HPV-16 L1, studies on genital dysplasias with anti-BPV serum could give rise to misleading results. Samples that contain HPV-16 L1 may be recorded as negative, whereas those recorded as positive are most likely to contain the relatively harmless HPV-6. The discrimination between HPV-6 and HPV-16 is extremely important in view of the very different prognoses of the two types of lesion. Numerous studies have shown that HPV-6 is associated with rather benign lesions, whereas HPV-16 is associated with malignant carcinomas and advanced premalignant lesions. The failure to detect HPV-16 could therefore be serious.

It is also apparent from the above experiments that the BPV-1 antiserum is highly specific for L1 proteins of the viral capsid, and not L2, confirming that the L2 protein is type-specific as shown previously (Komly et al., 1986). Interestingly, this protein appears to be very immunogenic since antibodies raised to the HPV-6 L2-β-galactosidase fusion protein are predominantly directed against the L2 portion of the fusion protein. Again this appears particularly significant for possible vaccine development.

The antibodies described here are currently being used to analyse HPV proteins expressed in a variety of lesions. The HPV-6 L1-β-galactosidase fusion protein antibodies react with a protein of approximately 60K mol.wt. in Western blots of plantar warts (data not shown) and some reactivity has been observed with anti-HPV-16 L1 peptide antibodies on thin sections of cervical biopsies. However, high background reactions have been observed in some cases, and for these reasons the reagents are being refined by the generation of monoclonal antibodies. Ultimately, these reagents should offer the best means of typing a given HPV infection. Also, the fusion proteins described here can also be used to screen sera from cervical cancer patients to determine whether they produce anti-HPV antibodies, and these studies are currently in progress.

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REFERENCES


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