The Expression of Human Papillomavirus Type 18 E6 Protein in Bacteria and the Production of Anti-E6 Antibodies

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(Accepted 6 June 1986)

SUMMARY

Human papillomavirus type 18 (HPV-18) has recently been closely linked with human malignant cervical lesions. The early region of the genome of the related bovine papillomavirus (BPV) has been shown to be important for the production of the transformed phenotype. BPV E6 has been shown to be a transforming protein. We report the primary structure of the HPV-18 E6 open reading frame and its predicted amino acid sequence. Both E6 protein and E6-β-galactosidase fusion protein were synthesized in bacteria. Antisera were raised against the E6-β-galactosidase fusion protein and against an E6 N-terminal peptide which was 14 amino acids long. We show that these antisera reacted on Western blots against E6 synthesized in bacteria. The HPV E6 antigen and antibodies described here will be useful in understanding HPV expression and its association with human malignancies and may also be diagnostically useful.

INTRODUCTION

Papillomaviruses are small DNA viruses which have been identified in a number of higher organisms including man (zur Hausen, 1985). A number of distinct human papillomavirus (HPV) types have been identified and some of these have been closely linked with specific human cancers. These include HPV-6, HPV-11, HPV-16 and HPV-18 which have been associated with tumours of the genital tract. HPV-6 and HPV-11 DNAs have been identified in benign lesions whereas HPV-16 and HPV-18 DNAs have been found in malignant lesions (Gissmann et al., 1983; Ikenberg et al., 1983; Boshart et al., 1984; Crum et al., 1984). HPV-16 and HPV-18 DNAs are also present in a number of cell lines derived from human cervical carcinomas, and in addition these DNAs are transcribed (Schwarz et al., 1985; Yee et al., 1985). For example, HPV-18 early open reading frames (ORF) E1, E6 and E7 have been shown to be transcribed in HeLa cells (Schwarz et al., 1985).

Bovine papillomavirus (BPV) has served as the prototype for the study of the molecular biology and transforming genes of papillomaviruses. The expression of BPV E6 mRNA has been shown to be necessary for efficient focus formation of mouse NIH 3T3 and C127 cells (Schiller et al., 1984; Yang et al., 1985; Androphy et al., 1985). These BPV-transformed cells demonstrate a fully transformed morphology and are tumorigenic in nude mice. Using a BPV E6-specific antiserum it has recently been established that BPV E6 is located in the nucleoplasm and membrane of BPV-transformed C127 cells (Androphy et al., 1985).

We have concentrated on the HPV-18 E6 ORF since this region may be important in the transformation of certain human cells. We report the primary structure of the HPV-18 E6 ORF and the cloning of suitable restriction enzyme fragments into prokaryotic expression vectors. Antisera were raised against both bacterially synthesized HPV-18 E6 protein and against a 14 amino acid peptide containing the N-terminal region of HPV-18 E6. These reagents should
prove to be invaluable in the understanding of HPV expression in malignant lesions containing HPV DNA and RNA and they may also prove to be useful diagnostically.

METHODS

Sequence analysis of HPV-18 DNA. Restriction enzyme fragments containing HPV-18 E6 ORF sequences were cloned into both M13mp18 and 19. Sequencing was carried out by the dideoxy chain termination method (Sanger et al., 1977).

Production of HPV-18 E6 in bacteria. HPV-18 DNA was first cloned by making a genomic library from tumour-derived DNA isolated from a cervical carcinoma biopsy (Boshart et al., 1984). The HPV-18 DNA was subcloned into plasmids and kindly made available to us by Professor H. zur Hausen.

The recombinant plasmid pUR-E6 expressing the E6-ß-galactosidase fusion protein was constructed as follows. A 766 bp Sau3AI fragment containing part of the E6 ORF was purified by gel electrophoresis and then ligated into the BamHI site of plasmid vector pUR290 (Ruther & Muller-Hill, 1983). The ligation mixture was used to transform Escherichia coli H4. 4 and ampicillin-resistant clones were grown in the presence of 100 µg/ml isopropyl-ß-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). The recombinant plasmid pAS-E6 expressing native E6 was made as follows. The 733 bp BamHI to PvuII fragment containing the E6 ORF was purified by gel electrophoresis and ligated into BamHI and NruI-cut plasmid vector pAS1 (Rosenberg et al., 1983). The ligation mixture was used to transform E. coli AR120 which contains the lambda C I gene. Ampicillin-resistant clones were grown at 37 °C to an OD55o of 1 and then in the presence of 40 µg/ml nalidixic acid for an additional 4 h at 37 °C. Induced cells were collected as described above.

Preparation of anti-E6 antibodies and analysis by Western blotting. BALB/c mice were inoculated twice subcutaneously with 2 µg gel-purified protein or 30 µg peptide coupled to thyroglobulin in incomplete Freund's adjuvant. The 14 amino acid peptide comprising the N terminus of E6 was synthesized by the solid-phase technique and then covalently coupled through its C terminus to thyroglobulin as described (Nelson et al., 1984). Mice were given an intravenous boost with a further 2 µg fusion protein or 30 µg peptide-thyroglobulin conjugate 2 weeks after the initial inoculation. One week later, sera were collected and tested for anti-E6 activity first by ELISA then by Western blotting as follows. Bacterial cells containing HPV-18 E6 protein were lysed in SDS-PAGE sample buffer (50 mM-Tris pH 6·8, 2% SDS, 5% glycerol, 0·1 M-dithiothreitol) and subjected to SDS-PAGE (Laemmli, 1970). Proteins were electrophoretically transferred onto nitrocellulose filters as previously described (Matlashewski et al., 1986). Blots were then incubated in blocking buffer (10% foetal calf serum in PBS) for 2 h at 42 °C. Anti-serum was diluted 1 : 200 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).

RESULTS

Sequence analysis of the HPV-18 ORF

Before HPV-18 E6-related proteins could be synthesized and antisera developed against these proteins it was first necessary to sequence the region surrounding the E6 ORF. A restriction map and sequencing strategy are shown in Fig. 1. The indicated BamHI and XbaI sites are consistent with the partial restriction map previously reported for HPV-18 (Schwarz et al., 1985). The nucleotide sequence is shown in Fig. 2 together with the predicted amino acid sequence. The longest ORF is capable of coding for 157 amino acids not including the initiation methionine codon, with a predicted mol. wt. of 17000 (17K). This ORF is similar to that reported for HPV-16 E6 (Seedorf et al., 1985) and HPV-16 and HPV-18 are about 60% homologous with respect to the polypeptides coded in this region. No other significant homologies were observed in the available databases. As has been found with other papillomaviruses, there is a fourfold repetition with the same spacing for the tetrapeptide sequence Cys-X-X-Cys (Fig. 2). Therefore, this characteristic is a conserved feature in the E6 proteins of papillomaviruses. This supports the view that HPV E6 protein functions in a similar way to BPV E6 and, therefore, is directly or indirectly involved in transformation.

Synthesis of E6-ß-galactosidase fusion protein

The sequence information enabled us to determine that the Sau3AI fragment which extended from nucleotides 355 to 1121 contained the coding sequence for amino acids 15 to 157 of E6.
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767 bp Sau3AI fragment was cloned into the BamHI site of the prokaryotic expression vector pUR290 (Ruther & Muller-Hill, 1983). This resulted in a construct containing the gene for β-galactosidase followed by the E6 ORF linked together (Fig. 3). The resulting plasmid (pUR-E6) was transfected into E. coli H4.4 and ampicillin-resistant clones were grown in the presence of IPTG to induce expression of the E6-β-galactosidase gene. A cell extract from one of these clones was fractionated by SDS-PAGE and stained with Coomassie Brilliant Blue. The E6-β-galactosidase fusion protein synthesized by this clone had a mol. wt. of about 130K (Fig. 4, lane 2), similar to that which would be estimated from the E6 amino acid sequence linked to β-galactosidase. Cell extracts from a clone containing pUR290 with no additional sequences produced a β-galactosidase product of 116K (Fig. 4, lane 1). No higher molecular weight fusion proteins were obtained with pUR291 or pUR292 since these vectors contained the Sau3AI E6 fragment in the wrong frames (data not shown). From these observations we concluded that the fusion protein shown in Fig. 4, lane 2 contained an E6-related peptide contributing about 14K to its molecular weight. This fusion protein was insoluble in non-ionic detergents, as commonly occurs with proteins synthesized at high levels in bacteria. The 130K band was therefore excised from SDS-PAGE gels and used with incomplete Freund’s adjuvant to immunize mice. The resulting antiserum will be discussed in a later section.

Synthesis of HPV-18 E6 15.8K protein

It was advantageous to synthesize an E6 native protein by itself so that antisera produced against the E6-β-galactosidase fusion protein could be tested for antibodies which were specific for the E6 portion of the fusion protein. For this purpose a pAS1 vector was used which contained the phage lambda P1 promoter, prokaryotic ribosome-binding site and an initiation methionine (Rosenberg et al., 1983). From the sequence information (Fig. 2) we calculated that a BamHI to PvuII 733 bp fragment would code for E6 amino acid 5 up to the C terminus at amino acid 157. This fragment was inserted into the pAS1 vector which had been cut with BamHI and NruI (Fig. 5). The resulting plasmid (pAS-E6) was transfected into E. coli AR120 which contains the lambda CI gene. In the presence of nalidixic acid the CI repressor is inactivated, allowing expression from the P1 promoter. Ampicillin-resistant clones were grown either in the presence or in the absence of nalidixic acid and the cell extracts from one of each such clones were fractionated by SDS-PAGE (Fig. 6). A protein of mol. wt. 15.8K was present in cells treated with nalidixic acid (lane 2) which was not present in cells grown in the absence of nalidixic acid (lane 1). Therefore, the 15.8K protein was under the control of the P1 promoter and is also the correct predicted size for HPV-18 E6 determined from the sequence information (Fig. 2). The HPV-18 E6 is also similar in size to the BPV E6 synthesized in bacteria (Androphy et al., 1985).

Production of antibodies against HPV-18 E6

We adopted two strategies for the production of antibodies against HPV-18 E6. First, the sequence information (Fig. 2) allowed us to predict the primary structure of an E6 N terminus-containing peptide. A peptide which was 14 amino acids long starting from the N terminus of E6
GCA TAA CTA TAT CCA TCT AAG TAA AAT AAG TGC TTT
AGG CAC ATA TTT ACT TGT TTT ACT TAA GCT AAT TGC ATA CTT GCG TOT ACA ACT
ACT TTT ATG TCC AAG ATT CTT TCT ACC CTT AAG ATG AAG ACT TAT ATG ACT AAG
CTG TGC ATA CAT AGT TTA TCG AAG CAA AAT AGG TTT GGC AGC ACA TAC TAT ACT
TTT CAT TAA TAC TTT TTA CAA TGT TAG TAT ATA AAA AAG GGA GTA ACC GAA AAG
GOT CGG GAC CGA AAA CGG TOT ATA TAA AAG ATG TGA GAA ACA CAC CAC AAT ACT

ATG GCG CCC TTT GAG GAT CCA ACA CGG GCA CCC TAC AAG CTA CTT GAT CTG TGC
MET Ala Arg Phe Glu Asp Pro Thr Arg Pro Tyr Lys Leu Pro Asp Leu Cys

ACG GAA CTC AAG ACT TCA CTT CAA GAC GTA CTA GAA GAA AAA GAA TTA TTT CAA
Thr Glu Leu Asn Thr Ser Leu Glu Asp Ile Glu Ile Thr Cys Val Tyr Cys Lys

ACA GTA TTT GAA ATT CCA GAG GTA TTT GAA TTT CAA GTT AAA GAT TTA TTT GTG
Thr Val Leu Glu Leu Thr Glu Phe Phe Ala Phe Lys Asp Leu Phe Val

GTG TAT AGA GAC AGT ATA CCC CAT GCT GCA TGC CAT AAG TTT TAG GTA TAT GGA
Val Tyr Arg Asp Ser Ser Leu Tyr Ser Leu Tyr Ser Val Tyr Gly Asp Thr Leu

GAA AAA CTA ACT AAC ACT GGG TTA TAC AAT TTA TTA ATA AGG TGC CAT AAG
Glu Lys Leu Thr Asn Thr Tyr Leu Leu Tyr Asn Leu Ile Arg Cys Val Tyr Cys Lys

CAG AAA CCG TGG AAT CCA GCA GAA AAA CTT AGA CAC CTT AAT GAA AAA CGA GCA
Gln Lys Pro Leu Asp Pro Ala Glu Lys Leu Arg His Leu Asn Glu Lys Arg Arg

TTT CAC AAC ATA GCT GGC CAC TAT AGA GGC TGC CAT TCG TGC AGC AAG CAC GCA
Phe His Asn Ile Ala Gly His Tyr Arg Gly Glu Cys Cys Ser Cys Cys Asn Arg

GCA CGA CAG GAA CCA CTC CAA CGA CCC AGA GAA ACA GTA TAA TAT TAA GTA
Ala Arg Glu Arg Leu Glu Arg Arg Glu Thr Glu Val

TGC ATG GAC CTA AGG CAA CAT TGC AAG CAA TTG TAT TGC ATT TAG ACG CCC AAA

ATG AAA GTT CCG GTT GAC CTT CTA TGT CAC GAG CTA AAG CAC TGG GAA

GAA AAG CAT GAA ATG CCA AAG GAA GTA CAT GAA ATG CTA CCA GCC CGA CGA GCC

GAA CCA CAA GCT ACC ACA ATG TGT GTA TOT CTA TGG TAA GGG TGC TGA ACC CAG AAT TGA

GCT ACT ACT ACA TGA AAC CTT ACC AGA GCA CCT TCG AGC ATT CCA GCA GCT GTT TCT

GAA CAC CCT GTC TTT TOT GTC CCG TGG TOT GCA CCC AGC ACT AAG CAA CAA TGG

CTG TTC CCC GGT CCA TAA GGT ACA GAC GGG GAG GGC AGC GGT TGT AAC GCC TGG

TTT CAT GTA CAA GCT

Fig. 2. Nucleotide sequence of HPV-18 E6 ORF. The nucleotide sequence is given in parallel with the
amino acid sequence predicted from the ORF. The boxed regions show the tetrapeptide sequence Cys-X-X-Cys. The underlined sequence shows the N-terminal 14 amino acids which were used as an immunogen to raise anti-E6 antibodies.
Fig. 3. Recombinant pUR290 plasmid (pUR-E6) coding for E6–β-galactosidase fusion protein. A 766 bp Sau3AI fragment from the E6 ORF region was cloned directly into the BamHI site of pUR290. The solid boxed area represents the E6 coding region and the open boxed area represents 3' non-coding sequences. Arrows indicate the direction of transcription. The resulting construct coded for a fusion protein containing HPV-18 E6 amino acids 15 to 157. The diagram is not to scale.

Fig. 4. SDS–PAGE of E6–β-galactosidase fusion protein synthesized in E. coli H4.4. Total protein was extracted in SDS–PAGE sample buffer, run on a gel and stained with Coomassie Brilliant Blue. Lane 1, culture containing plasmid pUR290 grown in the presence of IPTG. Lane 2, culture containing plasmid pUR-E6 grown in the presence of IPTG. Arrows indicate the position of β-galactosidase (β-gal) and E6–β-galactosidase fusion protein. The molecular weight standards were myosin (200K), phosphorylase B (97.4K), bovine serum albumin (68K) and ovalbumin (43K).

was made (Fig. 2), then linked to thyroglobulin and used to inoculate mice as described in Methods. We chose to use the N terminus instead of the C terminus because splicing events may attach E6 to other ORFs, thus replacing the C-terminal sequence (Yang et al., 1985). Also, the N-terminus of E6 was not present in the E6–β-galactosidase fusion protein which was used as an immunogen as described below and the two antigens were therefore complementary. In a second approach, E6–β-galactosidase fusion protein was gel-purified and used to inoculate mice as described in Methods. Antisera produced against both the E6 N-terminal peptide and E6–β-galactosidase fusion proteins which were positive on ELISA (data not shown) were then tested for reactivity against E6 by Western blotting with the E6 15-8K protein derived from the pAS-E6 plasmid. The results are shown in Fig. 7. Panel (a) shows the Coomassie Brilliant Blue stain of total proteins from induced and non-induced cultures. Panel (b) shows a Western blot analysis using antiserum raised against the E6–β-galactosidase fusion protein. Panel (c) shows the Western blot analysis using the anti-peptide serum and (d) shows a Western blot using preimmune serum. Both the antipeptide and anti-fusion protein sera reacted against the E6 15-8K protein (b,c) but not against any protein in the uninduced cell samples. Preimmune serum failed to react against E6 (d). This demonstrates that both the anti-peptide and anti-fusion protein sera contained antibodies which are reactive against HPV-18 E6 protein. It is important that the anti-peptide serum reacted against the 15-8K protein because this demonstrated that the reading frame shown in Fig. 2 was used by these bacteria to make E6 protein. The reactivity of the anti-peptide serum was, however, lower than the reactivity of the anti-fusion protein serum as judged by these Western blots. This may be due in part to the fact that the E6 15-8K polypeptide derived from the pAS-E6 plasmid lacked the sequences corresponding to the four
Fig. 5. Recombinant pAS1 plasmid (pAS-E6) coding for HPV-18 E6 protein. A 733 bp BamHI to PvuII fragment from the E6 ORF was ligated directly into the BamHI–PvuII sites of pASI. The solid boxed area represents the E6 coding region and the open boxed area represents 3' non-coding sequences. The solid line represents plasmid sequence. Arrows indicate the direction of transcription. The resulting construct codes for E6 amino acids 5 to 157. The diagram is not to scale.

Fig. 6. SDS–PAGE of HPV-18 E6 protein synthesized in E. coli AR120. Total protein was extracted in SDS–PAGE sample buffer, run on a gel and stained with Coomassie Brilliant Blue. Lane 1, non-induced culture containing plasmid pAS-E6 grown in the absence of nalidixic acid. Lane 2, induced culture containing plasmid pAS-E6 grown in the presence of nalidixic acid. The arrow indicates the position of E6. The molecular weight standards were myosin (200K), phosphorylase B (97.4K), bovine serum albumin (68K), ovalbumin (43K), chymotrypsinogen (25.7K), β-lactoglobulin (18.4K) and lysozyme (14.3K).

N-terminal amino acids whereas these were present in the peptide used to raise the anti-peptide serum. Therefore, the anti-peptide serum contained antibodies against E6 amino acids 5 to 14 and it was not possible to determine from this analysis whether it contained antibodies against the first four N-terminal amino acids. This serum may be more reactive against the complete E6 protein since the antigen would contain the four N-terminal amino acids. The anti-fusion protein serum presumably contained antibodies specific for a number of E6 epitopes since it showed a strong reactivity on the Western blot analysis and these mice had been inoculated with a fusion protein containing most of the E6 polypeptide.

Two different sets of polyclonal antibodies against the HPV-18 E6 protein are thus available. Both these antisera were used to analyse HPV-18-containing HeLa cells for the presence of E6-related protein. This involved both immunoprecipitation of labelled cell extracts and Western blotting using a variety of extraction procedures. These investigations have so far failed to detect any E6-related proteins within HeLa cells.

DISCUSSION

Here we have described the primary sequence of the HPV-18 E6 ORF, the synthesis in bacteria of HPV-18 E6 and the production of antisera against this protein. We have verified that the E6 ORF was translated correctly by demonstrating that an anti-peptide serum reacted
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Fig. 7. Immunoreactivity of HPV-18 E6 antisera against E6 synthesized in E. coli AR120 from plasmid pAS-E6. Cultures were grown in the presence of nalidixic acid (lanes 1) or in the absence of nalidixic acid (lanes 2). (a) Coomassie Brilliant Blue stain of total protein. (b) Western blot analysis of total protein with antiserum raised against E6-β-galactosidase fusion protein. (c) Western blot analysis of total protein with antiserum raised against E6 peptide. (d) Western blot analysis of total protein with preimmune mouse serum.

against E6 protein synthesized in bacteria. This represents a significant advance because E6 may serve an important function in malignant transformation. This view is supported by the observation that human cervical carcinoma cell lines including HeLa contain integrated copies of the early region of HPV-18 that express the corresponding E6 mRNA (Schwarz et al., 1985). Messenger RNA corresponding to the E6 ORF has also been identified in carcinomas carrying HPV-16 DNA (Yee et al., 1985). The HPV E6 antibodies described here allow a wide variety of important studies to be initiated. E6-related proteins can now be studied in human transformed cell lines such as HeLa and in human biopsy tissue containing HPV-related DNA sequences. However, we have not yet been able to detect any E6-related proteins in HeLa cells using these antisera. This may be due to these proteins being present at very low levels in HeLa cells and their being turned over rapidly. Under these circumstances the polyclonal antibodies may lack the high affinity and specificity needed. We hope that the monoclonal antibodies now being produced will overcome these difficulties.

HPV-associated carcinomas do not express sequences corresponding to viral structural proteins; therefore, the identification of early gene products such as E6 may be useful
diagnostically. In addition, the E6 antigen described here can be used to screen sera from cervical cancer patients to determine whether they produce anti-E6 antibodies. This approach has been used successfully to screen human sera with the bacterially synthesized non-structural viral pol gene product of human T-cell lymphotrophic virus (HTLV-III) synthesized in bacteria (Chang et al., 1985). We are presently widening the range of antigens to be used in this way by cloning HPV-16 E6, L1 and HPV-18 L1 in the same way as that described in this paper.

Mice instead of rabbits have been used for this work because we have generally found that mice give a faster response and that less antigen is required for immunization. More importantly, this allows the option of developing monoclonal antibodies against these viral antigens and we are now isolating such monoclonal antibodies, which should overcome the problems associated with polyclonal antibodies.

In order to appreciate the involvement of HPV in specific cancer types it is important to have HPV-specific reagents. The antibodies and antigens described here should help in understanding the role HPV plays in malignant transformation.

We wish to thank Professor H. zur Hausen for providing us with a HPV-18-containing plasmid. We are also indebted to Jonathan Rothbard for the peptide synthesis and useful discussions. We thank Clare Middlemiss and Audrey Symons for help in preparing this manuscript.

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(Received 10 April 1986)