Identification of the Human Papillomavirus Type 6b L1 Open Reading Frame Protein in Condylomas and Corresponding Antibodies in Human Sera

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Genital warts (condylomata acuminata) are among the most frequent sexually transmitted infections. Human papillomavirus type 6 (HPV-6), which is etiologically related to a majority of these lesions, has not been propagated in tissue culture. We generated two forms of HPV-6 viral antigens: a chemically synthesized oligopeptide (referred to as the C-terminal synthetic peptide) corresponding to residues 482 to 495 of the 500-amino-acid-long L1 open reading frame (ORF), and a bacterially expressed 54-kilodalton (kDa) fusion protein containing the N-terminal 13 amino acids encoded by the lambda bacteriophage cII gene followed by one vector-insert junctional residue and 462 amino acids of the L1 ORF sequence (residues 39 to 500). The cII-L1 fusion protein was specifically recognized by an antipeptide serum directed against the N-terminal 13 amino acids derived from the cII gene, an antiserum raised against the C-terminal synthetic peptide, and a genus-specific serum prepared by immunization with disrupted viral capsids. The 54-kDa fusion protein was purified, and the sequence of its first 36 amino acids was determined and found to be as predicted by the DNA sequence. Both the genus-specific anticapsid serum and the antiserum raised against the fusion protein identified authentic L1 ORF proteins in HPV-1-induced (58 kDa) and HPV-6/11-induced (56 kDa) papillomas. The synthetic peptide antiserum recognized the 56- to 58-kDa protein in HPV-6-induced warts, but not in HPV-1- or HPV-11-infected specimens. Using the fusion protein as antigen in immunoassays, we were able to detect the corresponding antibodies in human sera.

Genital warts (condylomata acuminata) have been recognized as a sexually transmitted disease for many decades. In the United States, an estimated one million cases of genital warts occur annually (4). A large majority of these lesions are caused by two closely related human papillomaviruses (HPV), types 6 and 11. These two viruses also account for almost all cases of juvenile-onset and adult-onset respiratory papillomatosis (24, 25, 34, 37). HPV-6 and -11 are mostly associated with benign and premalignant lesions, but are also found in a small proportion of malignant lesions (24, 34). One study has shown that women with a history of condylomata acuminata had a 3.8-fold increase in the incidence of carcinoma in situ compared with the total female population residing in the same city (6). Laryngeal papillomas are benign lesions under normal conditions; however, after radiation treatment of children with the disease, malignant transformation was observed in a significant percentage of these individuals 10 to 15 years later (22). Thus, the presence of the papillomavirus (PV) is apparently a risk factor in the development of laryngeal carcinoma (34, 37).

The DNA sequence of the entire genome of HPV-6b has been published (29). Like other PVs characterized to date, HPV-6b contains the early (E) and the late (L) open reading frames (ORFs). The early ORFs are involved in viral DNA replication, plasmid maintenance, and transformation (2), and the late L1 and L2 ORFs presumably encode the major and minor capsid proteins, respectively (18, 26, 27). Although analysis of mRNA structures has shown that both

early and late ORF transcripts can be detected in HPV-6-induced condylomas (21), none of their corresponding protein products have been identified. Due to the lack of a suitable tissue culture system for virus propagation and because human genital lesions do not produce large amounts of virions, HPV-6/11 antigens have not been available for study and seroepidemiologic investigations have thus not been possible. We therefore chose to synthesize the ORF proteins of HPV-6b in *Escherichia coli* by using a high-level expression vector, pJLA16 (20). In this study we expressed the L1 ORF as a fusion protein with the cII gene product of bacteriophage λ . We also synthesized an oligopeptide corresponding to residues 482 to 495 of the putative L1 ORF protein. The antisera generated against the bacterially expressed cII-L1 fusion protein or the synthetic peptide efficiently recognized viral proteins in naturally occurring papillomas. The bacterially expressed fusion protein also reacted with antibodies in human sera.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* DC646 and MZ-1 (30) were kindly provided by D. Court and M. Zuber and were used for propagation of the expression vector and protein production, respectively.

The procaryotic expression vector used (pJLA16) (20) contains the λ p_L promoter, the cII translation initiation signal, and the first 13 amino acid codons of the cII gene. The cloned HPV-6b DNA (11) was a generous gift of L. Gissmann and H. zur Hausen (Deutsches Krebsforschungszentrum, Heidelberg).

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FIG. 1. Construction and partial sequencing of the expression plasmid pCL31. The isolated insert and vector with their partial sequences written in triplet codons are illustrated. The 18-base oligonucleotide primer used in the sequence analysis is shown for both the vector pJLA16 and the expression plasmid pCL31. A portion of the determined DNA sequence which covers the insert-vector junction is shown at the bottom of the figure. Numbers 1 to 13 in parentheses indicate the amino acid residues of the expressed cII-L1 fusion protein. Circles at position 7288 represent the nucleotides before termination codons. Numbers shown with the plasmids indicate the restriction sites and the ends of the L1 ORF (5789 to 7288).

Construction of pCL31 expression plasmid. The strategy used in constructing the expression plasmid for HPV-6b L1 ORF is outlined in Fig. 1. The plasmid pHPV6b, which contains the entire HPV-6b genome, was digested with *AccI* and *XbaI*, and the 1.9-kilobase (kb) fragment was isolated and filled in by using the Klenow fragment of DNA polymerase I in the presence of four deoxynucleotide triphosphates. The blunt-ended fragment was ligated to the 4.1-kb *NruI*-restricted vector and transformed (7) into competent *E. coli* DC646.

The ampicillin-resistant transformants were screened for HPV-6b-specific sequences by in situ colony hybridization with nick-translated ^{32}P -labeled HPV-6b-specific probe (>400 Ci/mmol; Amersham Corp.) (23). To ensure the proper orientation and correct vector-insert junction, the positive transformants were further screened by double-

stranded DNA sequence analysis (36). An oligonucleotide primer (GCAAACAAACGCAACGAG), whose sequence is identical to that of 13 to 30 nucleotides upstream from the vector-insert junction, was chemically synthesized (3) and used as a primer for DNA sequencing (Fig. 1).

Synthesis of L1 ORF proteins in *E. coli*. The plasmid with the insert in the correct orientation (pCL31) was transformed into the MZ-1 strain. The cells were induced with or without [^{35}S]methionine labeling (30), and the crude cell lysates were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography or Coomassie blue staining.

Protein purification and partial sequencing. The cII-L1 fusion protein was extracted from the induced MZ-1 cells by successive salt and detergent extractions as described (13, 19). The pellet fraction of the final potassium thiocyanate

extraction was solubilized in 50 mM Tris hydrochloride (pH 7.5)–7 M guanidine hydrochloride containing 2 mM dithiothreitol and passed through an S-300 gel filtration column (Pharmacia; 1 cm by 1 m) eluted with the same buffer. The pooled fractions were subsequently purified by high-performance liquid chromatography (HPLC) with either a C₈ or a C₁₈ reverse-phase column (Waters Associates). The finally purified protein was subjected to sequencing with an Applied Biosystems Model 470A gas-phase protein sequencer.

Peptide- and fusion protein-specific antisera. Anti-cII oligopeptide (MVRANKRNEALR) serum, which recognizes the N terminus of the fusion protein, was a generous gift of M. Zweig. An oligopeptide (PAVSKASAAPKRKR; referred to as the C-terminal synthetic peptide) corresponding to residues 482 to 495 of the 500-amino-acid-long L1 ORF protein was chemically synthesized (14) on a Vega Automated Synthesizer, purified by reverse-phase HPLC, and conjugated to keyhole limpet hemocyanin through DFDNB (1,5-difluoro-2,4-dinitrobenzene) (35). The HPLC-purified fusion protein and conjugated C-terminal synthetic peptide were used to raise polyclonal antisera (38). Antibodies to the peptide were immunoaffinity purified on a peptide-containing column (38).

Western blot analysis. To characterize the expressed fusion protein and to detect the viral antigens and immune response in HPV-infected humans, Western blot (immunoblot) analysis was carried out by the method of Chang et al. (5). The only modification adopted was to incubate the test antisera overnight at room temperature and to incubate the ¹²⁵I-labeled goat antibodies at 37°C for 90 min.

Detection of viral antigens. Two sources of viral antigens were used: purified capsids prepared from an HPV-1-infected plantar wart by the CsCl banding method (25), and proteins extracted from three condylomas kindly provided by Edward A. Sawada (Department of Health and Mental Hygiene, Baltimore, Md.). The three condylomas have been identified to be either HPV-6 induced (two cases) or HPV-11 induced (one case) by Southern transfer analysis (33). The biopsied warts were cut and minced in RIPA buffer (20 mM Tris hydrochloride, pH 7.6, 2 mM EDTA, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.25% SDS, 180 Kallikrein units of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride) and boiled for 5 to 10 min before electrophoresis. The purified HPV-1 capsid protein and the crude condyloma extracts were resolved by SDS-PAGE and subjected to Western blot analysis. The antifusion protein serum and the affinity-purified antipeptide serum were used at a dilution of 1:50. Reactivity with ¹²⁵I-labeled antibodies was detected by autoradiography.

Competition experiments were carried out as follows. The test serum was incubated with either the salt- and detergent-extracted fusion protein (30 µl of serum with 25 µg of protein) or the purified peptide (30 µl of serum with 50 µg of peptide) for 1 to 4 h at room temperature. The mixture was centrifuged, and the supernatant was diluted with milk buffer (to the final serum dilution of 1:70 to 1:100) and used for Western blot analysis.

Detection of immune responses with L1 ORF protein. The cII-L1 fusion protein eluted from the S-300 column (see above) was used as the antigen to detect antibody in human sera by Western blot. All test sera were preabsorbed with a bacterial lysate extracted from the *E. coli* cells overexpressing an HPV-6 cII-E2 fusion protein (C.-C. H. Li, R. V. Gilden, S. D. Showalter, and K. V. Shah, manuscript in preparation). About 10⁸ such cells were lysed by sonication

FIG. 2. Expression and purification of the cII-L1 fusion protein. The 54-kDa expressed protein is indicated by arrows. (A) [³⁵S]methionine labeling of uninduced (lane a) and induced (lane b) MZ-1 cells carrying pCL31. (B) Coomassie brilliant blue staining of the uninduced (lane a) and induced (lane b) cells. (C) Purification of the 54-kDa fusion protein. Lanes: a, pellet fraction after extractions with salts and detergents; b, pooled fractions after S-300 gel filtration chromatography; c, peak fraction after reverse-phase HPLC.

for 1 min, followed by boiling for 10 min in 1% SDS–0.1% β-mercaptoethanol. This bacterial extract was incubated with 20 µl of the test human serum in milk buffer containing 5% goat serum with a final SDS concentration of 0.05% for 1 to 5 h at room temperature. The incubation mixture was centrifuged, and the clarified serum was adjusted to a final dilution of 1:50. Following overnight incubation with the diluted serum and subsequent washing, the filter strips were reacted with ¹²⁵I-labeled goat anti-human immunoglobulin G (IgG) before autoradiography. Antibody to the L1 protein was detected by the presence of the 54-kilodalton (kDa) band on the autoradiogram.

The human sera were collected from two sources: 30 women attending the colposcopy clinic in Ulm, West Germany (kindly supplied by Achim Schneider), and 20 suburban children under the age of 5 who participated in a study of measles immunization in Maryland (31).

RESULTS

Construction and expression of the recombinant plasmid pCL31. As shown at the bottom of Fig. 1, ligation of the 1.9-kb insert with the 4.1-kb vector restored the correct translational reading frame. Since the L1 ORF terminates with the TAA sequence immediately after nucleotide 7288 (29) (which is within the inserted 1.9-kb fragment), the expected expressed protein will be a hybrid consisting of 13 N-terminal amino acids derived from the λ cII gene followed by a junctional residue (alanine) and 462 amino acids derived from the L1 ORF protein (residues 39 to 500 out of 500). The structure of the recombinant plasmid pCL31 was further verified by DNA sequence analysis. The sequence of about 150 nucleotides (partially shown in Fig. 1) clearly demonstrated that pCL31 contained the predicted vector-insert junction and reading frame.

After plasmid pCL31 was transformed into strain MZ-1 and induced with or without [³⁵S]methionine labeling, a

FIG. 3. Characterization of the cII-L1 fusion protein. The pooled fractions eluted from the S-300 column were electrophoresed, Western transferred, and, except for lane a, immunoassayed with test sera (specified in each lane). The reactivity was detected by ^{125}I -labeled goat anti-rabbit IgG. Arrow indicates the 54-kDa cII-L1 fusion protein. Lanes: a, amido black-stained filter; b, preimmune serum; c, anti-cII peptide serum; d, anti-C-terminal synthetic peptide serum; d', same serum preincubated with cII-L1 fusion protein; e, anti-cII-L1 fusion protein serum; e', same serum preincubated with cII-L1 fusion protein; f, genus-specific anticapsid serum; f', same serum preincubated with cII-L1 fusion protein.

54-kDa protein was detected by both fluorography (Fig. 2A, lane b) and Coomassie brilliant blue-stained SDS-PAGE (Fig. 2B, lane b). The size of the protein was consistent with the predicted molecular weight of 53,167. No corresponding protein was synthesized at 32°C (Fig. 2A, lane a; Fig. 2B, lane a). The fusion protein accounted for approximately 10% of the total labeled cellular protein.

Purification and partial sequencing of the expressed L1 ORF protein. Bacterially expressed fusion proteins have been reported to be highly insoluble (16), which frequently results in the formation of inclusion bodies due to protein aggregation (13). The cII-L1 fusion protein was also found in the pellet fraction (Fig. 2C, lane a) following successive salt and detergent extractions (19) and was about 60% pure. The protein was further purified with an S-300 column (lane b) followed by reverse-phase HPLC (lane c). About 200 μg of the purified protein was used for microsequencing. The first 36 residues from the N terminus were identified and confirmed to be as expected (data not shown).

Characterization of the bacterially expressed cII-L1 fusion protein. Both anti-cII (Fig. 3, lane c) and anti-C-terminal synthetic peptide (lane d) sera specifically recognized the 54-kDa protein in the induced cells but not in the control cells (data not shown), indicating that the expressed fusion protein contained the N-terminal cII and the C-terminal L1 amino acid sequences. Furthermore, a PV genus-specific serum (capable of reacting with capsid proteins of all PVs) raised in rabbits immunized with pooled SDS-disrupted PV virions (Accurate Biochem) also reacted with the 54-kDa protein (lane f). This showed that the synthesized L1 protein shared antigenic determinants with the PV antigens used for immunization of rabbits. The competition experiments showed that the cII-L1 fusion protein could successfully compete with the C-terminal synthetic peptide (lane d'), itself (lane e'), and the L1-like epitopes recognized by the

genus-specific antiserum (lane f'). A heterologous fusion protein (HPV-6 cII-E2) could not block the reactivity of anti-cII-L1 serum against cII-L1 protein (data not shown). This suggested that the competition data were not the result of nonspecific blocking. Furthermore, the C-terminal peptide could compete with itself but not with the fusion protein (data not shown). These observations confirmed that the reactivity detected in the Western blot analysis was immunologically L1-specific. The smaller-sized species detected in lanes d, e, and f are presumably breakdown molecules of the cII-L1 fusion protein, since competition with the fusion protein also eliminated the corresponding reactivity (lanes d', e', and f').

Identification of L1 protein encoded by HPV-6b. Antisera against the cII-L1 fusion protein (Fig. 3, lane e) and the C-terminal synthetic peptide (Fig. 3, lane d) were raised and used to identify the native gene products in purified viral capsids from plantar warts and condyloma extracts. Since condylomas contain few viral particles, no attempt was made to purify capsids from these tissues. Skin and testes tissue extracts were also used as negative controls in which no reactivity was detected with all the antisera tested (data not shown). As shown in Fig. 4, the anti-fusion protein serum (Fig. 4C, lane a) and the genus-specific antiserum (which served as a positive control, Fig. 4B, lane a) detected the same 58-kDa protein in the purified HPV-1 capsids. The observed size matched the calculated molecular weight of 57,600 for the HPV-1 L1 ORF product (8, 9). The cross-reactivity between the anti-fusion protein serum (which was made against HPV-6) and the HPV-1 L1 protein was not unexpected because there is 52% homology between the L1 proteins of HPV types 1 and 6 (8, 9, 29). Preincubation of the anti-fusion protein serum with the cII-L1 fusion protein completely blocked its reactivity with HPV-1 capsid protein (Fig. 4C, lane a'), indicating that recognition of HPV-1 is L1-specific, i.e., the anti-fusion protein serum only recognized those epitopes common to types 1 and 6 in this case. Preincubation of the genus-specific antiserum with the fusion protein, on the other hand, did not block its reactivity with the HPV-1 capsids (Fig. 4B, lane a'). This was also expected because although all the antibodies directed against the type 6 L1 epitopes were blocked by preincubation, the genus-

FIG. 4. Detection of the L1 protein in the purified HPV-1 capsid and papilloma extracts. The purified HPV-1 capsid (lanes a, a', and a'', at $\sim 10^7$ virions per lane) and proteins extracted from HPV-6b (lanes b and d) and HPV-11- (lane c) induced papillomas (~ 0.1 mg of tissue per lane) were electrophoresed, transferred to the nitrocellulose, and, except for panel A, assayed with test sera indicated below. (A) Amido black-stained filter; (B) Genus-specific anticapsid serum, except for lanes a' (same serum preincubated with cII-L1 protein) and a'' (same serum preincubated with C-terminal synthetic peptide). (C) Anti-cII-L1 fusion protein serum except for lanes a' and a'' (see above). (D) Anti-C-terminal synthetic peptide serum. The L1 proteins encoded by HPV-1 (58 kDa) and HPV-6 (56 kDa) are indicated by arrowheads and arrows, respectively.

FIG. 5. Detection of L1 antibodies in human sera. The 54-kDa cII-L1 fusion protein purified after S-300 column was used as the antigen (0.3 μ g per strip) and assayed with anti-C-terminal synthetic peptide serum (lane 1), anti-cII-L1 protein serum (lane 2), representative antibody-positive sera (lanes 3 to 9), or representative antibody-negative sera (lanes 10 and 11). Arrow indicates the 54-kDa cII-L1 protein.

specific antiserum still contained antibodies recognizing other HPV-1 epitopes which are not common between types 1 and 6. The anti-synthetic peptide serum did not recognize the HPV-1 L1 protein (Fig. 4D, lane a). This was not surprising since the C-termini of HPV-1 and HPV-6 L1 ORFs are quite dissimilar. For the same reason, preincubation of either the anti-fusion protein serum (Fig. 4C, lane a') or the genus-specific antiserum (Fig. 4B, lane a'') with this oligopeptide did not affect their reactivity with the HPV-1 capsid in Western blots.

The three condyloma extracts were derived from tissues shown to contain HPV-6 (two tissues) or HPV-11 (one tissue) by Southern transfer analysis (data not shown). In all three extracts, both the genus-specific antiserum (Fig. 4B, lanes b, c, and d) and the fusion protein antiserum (Fig. 4C, lanes b, c, and d) detected a 56-kDa protein which was probably the authentic L1 ORF gene product. The observed size agreed well with the calculated molecular weight for HPV-6 (55,601) (29) and HPV-11 (55,911) (10). A band of ~52 kDa detected in some cases was probably a degraded species. In contrast to the recognition of both HPV-6 and -11, the synthetic peptide antiserum detected the 56-kDa protein in the two HPV-6-induced condylomas (Fig. 4D, lanes b and d) but not in the HPV-11-infected tissue (Fig. 4D, lane c).

Detection of immune responses with L1 ORF protein. Eighteen of the 30 sera from the colposcopy clinic and 2 of the 20 sera from Maryland children were antibody positive (partially shown in Fig. 5) as judged by their reactivity to the 54-kDa fusion protein. The antibody titers were generally low, so that a dilution of 1:150 to 1:200 was reaching the detection limit in our system. Since all the test sera were preabsorbed with extracts of *E. coli* overexpressing the HPV-6 cII-E2 fusion protein, any positive reactivity is not likely to be the result of the recognition of the λ cII peptide or of any bacterial proteins copurified with the 54-kDa fusion protein. More detailed study is necessary to relate

serologic reactivity to specific infections; our purpose here is simply to record the feasibility of seroepidemiologic evaluation.

DISCUSSION

Although PVs were the first DNA viruses demonstrated to cause tumors, understanding of the biology of these agents has been severely impeded by the inability to propagate them in tissue culture systems. Over 45 HPV types have been identified and molecularly cloned, yet very little is known about their proteins. To circumvent the problem of not having readily available sources of HPV proteins, researchers have synthesized viral proteins based on their ORF sequences. With antibodies raised against these synthesized viral antigens, the E5, E6, L1, and L2 proteins of BPV-1 (1, 26–28), E4 and L2 proteins of HPV-1 (12, 18), and E6 and E7 proteins of HPV-16 (32) have been identified in cells and tissues. To date, similar data have not been available for HPV-6. We therefore generated two preparations of HPV-6 antigen based on the sequence of the L1 ORF, which codes for the major capsid protein (9, 26): a fusion protein containing 92% of the L1 protein (residues 39 to 500 out of 500), and a 14-amino-acid synthetic peptide corresponding to a sequence very close to the C terminus (residues 482 to 495) of the putative L1 protein. The fusion protein was obtained by using a bacterial expression vector under the control of its heat-inducible λ p_L promoter (20). The expressed fusion protein contained a 14-amino-acid-long leader peptide which was derived from the vector (13 from λ cII gene and 1 from vector-insert junction) and the entire L1 ORF sequence except its first 38 amino acids. This fusion protein specifically reacted with two anti-synthetic peptide sera, an anti-cII serum directed to its N terminus, and the antiserum raised against the C-terminal synthetic peptide. Extraction of the cell lysates with salts and detergents only yielded about 60% purity, while parallel extractions of other expressed proteins of similar construction led to about 90% purity (unpublished data), suggesting an unusual nature of the L1 fusion protein. Further purification by conventional column and HPLC indicated that the L1 fusion protein had a high tendency to form aggregates. This is probably due to the intrinsic nature of the protein sequence rather than to technical problems. The L1 protein of BPV was also reported to have similar properties (27). The fusion protein obtained after HPLC purification was subjected to partial protein sequencing, and its first 36 residues were confirmed to be as predicted from the DNA sequence.

Polyclonal antisera were raised against the C-terminal synthetic peptide and the cII-L1 fusion protein and used to identify the viral capsid antigen in purified virions and in condyloma extracts. Since the L1 ORF is highly conserved among all of the PVs, it was not surprising that the anti-cII-L1 fusion protein serum showed a reactivity similar to that of the genus-specific antiserum. On the other hand, the anti-C-terminal synthetic peptide serum was more type specific. It reacted with HPV-6-positive condyloma extracts, but did not react with the HPV-1 virion at all. In the single instance tested, the anti-synthetic peptide serum did not react with an HPV-11 condyloma extract which was reactive with both the genus-specific and the fusion protein-specific antisera. Whether this observation represents specificity of the anti-synthetic peptide serum for HPV-6 requires further analysis.

In preliminary tests, a majority of the sera from women attending a colposcopy clinic and a small proportion of the sera from children were reactive with the L1 protein. It is at

present unclear whether the reactivity of the human sera with HPV-6 L1 protein represents only past infection with HPV-6 or also represents cross-reactivity with antibodies to other HPV types. In view of the extensive sharing of nucleotide sequences between HPV types, the L1 protein very likely contains both type-specific and type-common epitopes, but it is not known which epitopes participate in the immune response in a natural infection. The antibodies were demonstrable at low serum dilutions of up to 1:200. It is not clear whether this low level of reactivity reflects a weak host immune response to a mucosal infection or that the expressed protein differs from the native protein in some ways and so binds inefficiently to antibodies formed by natural infection. These questions are currently under investigation in our laboratory by utilizing sera from patients in whom infections with different HPV types have been documented.

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