Identification of the Human Papillomavirus Type 18 E6* and E6 Proteins in Nuclear Protein Fractions from Human Cervical Carcinoma Cells Grown in the Nude Mouse or in Vitro

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ABSTRACT

We recently reported the transcription patterns of human papillomavirus (HPV) type 18 sequences in human cervical carcinoma cell lines. The open reading frames (ORFs) E6* and E6 represent the 5' -terminal cistrons in HPV18 mRNAs. ORF E6* was assumed to be specific for HPV types associated with genital carcinomas. To identify the predicted gene product, ORF E6* from a HeLa cDNA clone was expressed as an MS2 fusion protein in Escherichia coli. The C-terminal 23 amino acid residues were chemically synthesized. A panel of monoclonal antibodies was generated, recognizing E6* and E6* plus E6, respectively. In human cervical carcinoma cell lines grown in vitro these monoclonal antibodies specifically immunoprecipitate the putative M, 17,000 and 18,000 HPV18 E6 proteins in nuclear protein fractions. In a HPV18 DNA containing human cervical carcinoma established in nude mice, these monoclonal antibodies specifically immunoprecipitate a polypeptide with a molecular weight of 6500 as predicted for the HPV18 ORF E6* gene product in a nuclear protein fraction.

INTRODUCTION

HPVs³ are closely associated with benign and malignant genital tumors (1). A distinct subset of HPV types can be identified in >90% of all human cervical carcinomas, HPV16 (2) and HPV18 (3) being the most prevalent types.

HPV DNA found in cervical carcinoma cells usually is integrated into the host genome. In cervical carcinoma cell lines, the integration event interrupts or deletes specific regions of the viral genome, but leaves intact the ORFs E6 and E7 (4). Human cervical carcinoma cell lines show typical patterns of HPV18 transcription, ORFs E6* and E6 representing the 5' -terminal cistrons of HPV18 mRNAs; ORF E6* is generated by a splicing event in E6 (5). It was shown previously by DNA sequence analysis that identical splice sites which are utilized in HPV18 mRNAs to generate ORF E6* are also present in HPV16 but in contrast are absent in HPV6 and 11 (5). These observations prompted us to speculate that ORF E6* expression is specific for HPV types preferentially associated with malignant genital tumors and precursor lesions and therefore is correlated with the oncogenic properties of certain HPV types. mRNA mapping data confirmed that ORF E6* containing mRNAs are transcribed from the HPV16 genome (6) but not from the HPV6 and 11 genome (7).

Recently, E7 proteins of HPV16 (6) and E6 proteins of HPV16 (8) and HPV18 (9) could be identified in human cervical carcinoma cell lines. In this communication our main interest focused on the question, if the spliced E6* mRNA is translated into a corresponding polypeptide in human cervical carcinoma cells.

Here we report the expression of HPV18 E6* in bacteria as a fusion protein and the generation of MAbS reacting with E6* plus E6 and MAbS recognizing the C-terminal part of the E6* molecule, which is unique for E6*. With these reagents we were able to detect the putative E6 protein in human cervical carcinoma cell lines and the putative E6* polypeptide in a HPV18-positive human cervical carcinoma established in nude mice.

MATERIALS AND METHODS

Cell Lines. Human cervical carcinoma cell lines HeLa, SW756, and HT-3 were originally obtained from the American Type Culture Collection and were grown in monolayer cultures in Dulbecco's minimum essential medium containing 10% FCS. HEF were kindly supplied by G. Lenoir (International Agency for Research on Cancer, Lyon, France) and grown as suspension culture in RPMI 1640 containing 10% FCS. The Burkitt lymphoma-derived cell line BL-2 was kindly provided by G. Lenoir (International Agency for Research on Cancer, Lyon, France) and grown as suspension culture in RPMI 1640 containing 10% FCS.

Tumor Growth inAthymic Mice and Detection of HPV Sequences. 4-6-week-old female NMRI nude mice were purchased from Central-institut für Versuchstierzucht, Hannover, FRG, and were maintained in a pathogen-free environment. Small pieces of five human cervical carcinomas removed at surgery were directly transplanted in nude mice s.c. at the lateral thoracic region. The histological classification was done according to established guidelines (10). Tumor S18 (see below) was a poorly differentiated invasive squamous-cell carcinoma of the cervix of a 51-year-old patient. All tumors were established in the nude mouse before initiation of the experiments in successive passages ranging from 4 to 6. The average tumor size at the time of animal sacrifice was about 1 g.

For Southern blot analysis DNA of the tumors was digested with BamHI plus EcoRI and transferred to Gene Screen Plus filters (NEN) after electrophoretic separation in 0.8% agarose gels. Hybridizations with 32P-nick-translated restriction fragments (lacking the noncoding region) of HPV16 (2) and HPV18 (6) were performed in 50% formamide, 5 × SSC at 42°C. The filters were washed in 2 × SSC, 0.1% SDS at 68°C and exposed to X-ray films with an intensifier screen at ~70°C for 1-10 days.

Expression Vector and Bacterial Strain. The original expression vector pLC24 (11, 12) was modified by W. Röwekamp by deletion of the EcoRI site and introduction of multiple cloning sites as described elsewhere. The vector allows the expression of fusion proteins encoded by the insert fused to the first 98 amino acids of the MS2 polymerase under the control of the λ P1 promoter. The bacterial host strain Escherichia coli C600/537 harbors a temperature-sensitive CI repressor gene of phage λ on a kanamycin resistance multicopy plasmid.

Expression and Purification of MS2-E6* Fusion Protein. A NcoI/EcoRI restriction fragment of cDNA clone H4 derived from HeLa cells encoding E6* of HPV18 (5) was prepared and the expression vector

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3 The abbreviations used are: HPV, human papillomavirus; ORF, open reading frames; MAb, monoclonal antibody; MEM, minimum essential medium; FCS, fetal calf serum; HEF, human embryonic fibroblasts; SSC, standard saline citrate (0.15 M sodium chloride: 0.015 M sodium citrate, pH 7.4); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbsent assay; NBP, nucleic acid binding protein; BPV, bovine papillomavirus.

4 Kratt et al., manuscript in preparation.
digested with BamiHI. After filling recessed 3' ends of DNA with Klenow fragment of *E. coli* DNA polymerase, the fragment was cloned by blunt end ligation and a clone representing the proper orientation was selected. This expression plasmid was transferred into *E. coli* C600/537 cells which were grown under selective pressure at 28°C to high density. Cells were diluted with three volumes of prewarmed medium without antibiotics and incubated for 3 h at 42°C. Cells of 11 cultures were collected and suspended in 20 ml of 8% sucrose, 50 mM EDTA, 50 mM Tris-Cl pH 8.0, lysosyme 200 µg/ml, and incubated for 15 min at 37°C. Cells were lysed by addition of 0.1% Triton X-100, sonification, and stirring for 15 min at 37°C. After centrifugation at 40,000 × g for 15 min the pellet was first extracted with 20 ml 1 M urea by sonification, stirring and centrifugation as described above. The fusion protein was subsequently purified from the 1 M urea fraction by 7 M urea extraction. The 7 M urea preparation contained about 80% fusion protein with an overall yield of about 20 mg protein from 1 liter of culture.

Preparation of Synthetic Peptide. A 23-amino acid peptide comprising the C-terminus of E6* (CKTVELTLETPAVPTESSRTK) was synthesized in a continuous-flow instrument constructed and operated as described (13). Peptide chain assembly was performed by the solid-phase method on a 1% cross-linked polystyrene support using fluoronylmethoxycarbonyl amino acids and in situ activation with benzotriazole-yl-oy-ox-tris-(dimethylamino)phosphonium hexafluorophosphate. The synthetic peptide was purified by reversed-phase high-pressure liquid chromatography.

For immunizations, the peptide was coupled to keyhole limpet hemocyanin (Calbiochem, San Diego, CA) by the glutaraldehyde method as described (14).

Production of Monoclonal Antibodies. BALB/c mice were inoculated s.c. three times with 50 µg MS2-E6* fusion protein from the bacterial 7 M urea fraction dialyzed against PBS, 50 µg MS2-E6* after partial purification by SDS-PAGE, or 30 µg of the 23 amino acid peptide coupled to keyhole limpet hemocyanin. Two weeks later mice were given an i.p. boost with 50 µg fusion protein or 30 µg peptide. Five days later mouse spleenocytes were fused with 8-azaazaguanine-resistant nonsecreting mouse myeloma cell line X63-Ag8.653 at a ratio of 2:1 using 50% polyethylene glycol, M, 6000 (15, with modifications). About 2000 hybridoma cell clones were obtained from the fusion of spleenocytes of one mouse with myeloma cells. Hybridoma supernatants were screened in parallel ELISA tests (16) for reactivity with MS2-E6* fusion protein, MS2 protein (including the amino acids encoded by the multiple cloning site of the expression vector), and the synthetic peptide. About 150 clones showed reactivity with MS2-E6* but not with MS2, about 50 of these clones also recognized the synthetic peptide.

Total Cell Extracts and Western Blot Screening. 2 × 10⁶ HEK cells (HPV18 negative) were trypsinized, washed twice with PBS, resuspended in 0.5 ml PBS, and lysed by adding 0.5 ml twofold concentrated Laemmli sample buffer. 50 µg of these total cell proteins were separated per lane on 12.5% SDS-PAGE (17) and transferred to nitrocellulose filters by Western blotting (18). Nitrocellulose filters were preincubated in 20 mM Tris-Cl (pH 7.8), 7.5% milk powder (Merek), 140 mM NaCl, 0.02% Tween 20 for 1 h and then incubated with 0.5 ml hybridoma supernatants for 1 h. Filters were washed three times with 20 mM Tris-Cl (pH 7.8), 140 mM NaCl, 0.05% Tween 20. Reaction with horse anti-mouse IgG (Vector) for 30 min in 20 mM Tris, 140 mM NaCl, 7.5% milk powder and horseradish peroxidase Avidin D (Vector) were both followed by the described washing procedure. Filters were then stained with substrate. Hybridoma clones producing bands or high background Western blot screening of these HEF total cell extracts or MS2 proteins were discarded because of assumed cross-reactivity and unspecificity.

Immunoperoxidase Staining of Cells. Cells grown in Terasaki plates were fixed using the methanol or Bouin method and stained by the avidin-biotin complex immunoperoxidase staining procedure (Vector).

Purification and IgG Subclasses of M Abs. Hybridomas showing no unspecific binding in Western blot screening or Immunoperoxidase staining of HEF cells were cloned. Ascites was produced in Pristane (Sigma) pretreated mice as described (19). IgGs were purified using the Affi-Gel Protein A MAPS Kit (BioRad) according to the manufacturers instructions. IgG subclasses were determined by Ouchterlony immunoelectrophoresis using specific antisera (Miles). MAb E6*18a was IgG2a, MAb E6*/E6-18-1 was IgG1, MAb E6*18b and E6*/E6-18-2 were both IgG2b.

Differential Cell Extracts and Western Blot Analysis using 125I-Labeled Protein A. 5 × 10⁶ HeLa and HT-3 cells, respectively, were harvested and washed in PBS. Cyttoplasmic plus membrane proteins and nuclear extracts with increasing NaCl concentrations (100, 300, and 530 mM NaCl) were prepared as described (20, with minor modifications). 50 µg of the protein preparations per lane were subject to SDS-PAGE, Western blotting and preincubation as described above. Filters were incubated with 10 µg purified MAb per lane in 20 mM Tris-Cl (pH 7.8), 140 mM NaCl, 7.5% milk powder at 4°C overnight and washed 4 times with 20 mM Tris-Cl (pH 7.8), 140 mM NaCl, 0.1% Tween 20 and once with the same buffer containing 500 mM NaCl. Filters were then stained with 125I-labeled protein A (Amersham, 0.5 µCl/ml) for 2 h, followed by the washing procedures mentioned above. Filters were then exposed to X-ray films using an intensifier screen for 1–5 days.

Labeling of Asynchronous Cells with a Mixture of 1²C-Amino Acids and Immunoprecipitation. 2 × 10⁶ cells grown in a 35-mm Petri dish were labeled with Dulbecco’s DME lacking amino acids, 10% dialyzed FCS, and 0.5 mM/ml of ³²C-amino acids (CFB 104, Amersharm) as described (21). Nude mice tumors were extirpated, cut in pieces, and disrupted with five strokes in a glass dounce homogenizer prior to labeling with ¹²C-amino acids. Cyttoplasmic/membrane proteins and nuclear extracts were prepared as described above. Immunoprecipitations were carried out under the following conditions: 140 mM NaCl, 50 mM Tris-Cl (pH 7.8), 0.6% NP-40, 2 µg/ml Aprotinin (Bochnering Mannheim), 1 mM PMSE, 0.5 mM dithiothreitol, 1 mM EDTA, 0.25 mM EGTA at 4°C. Protein preparations were preincubated with 5 µg rabbit anti-mouse IgG (BioRad) (in case of MAB IgG1) for 30 min and then with 40 µl 50% Protein A-Sepharose (Pharmacia). The supernatant was removed and incubated with 6 µg of the appropriate MAb overnight. In the case of IgG1 Mabs, 10 µg rabbit anti-mouse IgG was added and incubated for 30 min, then 40 µl 50% Protein A-Sepharose were added and again allowed to react for 30 min. Protein A-Sepharose was pelleted, washed three times with buffer A (140 mM NaCl, 50 mM Tris-Cl (pH 7.8), 0.6% NP-40, 2 µg/ml Aprotinin, 1 mM phenylmethylsulfonyl fluoride), and three times with buffer B (A, 500 mM NaCl).

The pellet was resuspended in sample buffer containing 5% mercaptoethanol and boiled for 5 min. After pelleting the Protein A-Sepharose the supernatant was analyzed by 10-60 % SDS-PAGE and fluorography with exposure times of 10–60 days.

Lentil Lectin-Sepharose Affinity Chromatography. Prior to immunoprecipitation ¹²C-amino acid labeled HeLa protein extracts were subject to Lentil Lectin-Sepharose 4B (Pharmacia) affinity chromatography as described (22). To prevent removal of Lentil Lectin-bound manganese, chelating agents were excluded from the buffers used for protein preparations in this experiment.

RESULTS

E6* Splice Pattern in Other HPV Types. Recently, we showed by DNA sequence comparison, that the E6* splice pattern observed in HPV18 mRNAs expressed in cervical cancer cell lines is also possible in HPV16, but most unlikely in HPV types associated with benign lesions (5). The arrangement of splice donor and acceptor sites seen in HPV18 was found in the sequence of HPV315 and HPV33 (23) (Table 1).

Thus HPV types associated with malignant genital tumors (HPV16, 18, 31, 33) contain splice sites needed for the generation of ORF E6*, whereas in HPV types associated with benign lesions (HPV1, 6, 11) the corresponding sequences do not match with the essential dinucleotides of the splice consensus sequences.

³A. Lörincz, personal communication.
Table 1 E6* splice sites in the HPV18 DNA sequence and corresponding sequences in other HPV types

<table>
<thead>
<tr>
<th>Consensus sequence</th>
<th>Nucleotide positions</th>
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<tr>
<td><strong>5' splice (donor) site</strong></td>
<td></td>
</tr>
<tr>
<td>- exon AAGT AAGT-intron-</td>
<td></td>
</tr>
<tr>
<td>HPV 1</td>
<td>GAGAAGCTGG 212-220</td>
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<tr>
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<td>HPV 33</td>
<td>GAGATTATTT 222-237</td>
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<tr>
<td><strong>3' splice (acceptor) site</strong></td>
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<td>-intron-Py Py Py Py XCA GG G-exon</td>
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<td>HPV 33</td>
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Fig. 1. Transcription pattern of HPV18 sequences in cDNA clones from HeLa, SW756, and C4-1 cells. Top, HPV18 ORFs (boxes). Line, integrated HPV18 DNA including a TATA box element (T), splice donor sites (do), and splice acceptor sites (ac), zig zag lines, flanking cellular sequences. Bottom, three types of cDNAs can be distinguished. Thin, slanted lines, excised intron sequences; splice acceptor sites (ac), zig zag lines, flanking cellular sequences. Bottom, three types of cDNAs can be distinguished. Thin, slanted lines, excised intron sequences; heav, the polylinker sequence (LeuAsnSerGlyGlySer) and 57 amino acids of MS2 polymerase, six amino acids encoded by ORF E6*, starting with the methionine of the E6* start codon. Upon induction, this plasmid led to the synthesis of large amounts of the predicted MS2-E6* fusion protein (Fig. 2).

In addition, a peptide corresponding to the C-terminal 23 amino acid residues encoded by ORF E6* was chemically synthesized.

Production of MAbs against E6*. Mice were immunized using the MS2-E6* fusion protein and the synthetic peptide coupled to keyhole limpet hemocyanin, respectively. Hybridoma supernatants which were positive on ELISA and Western blot screening, (a) with MS2-E6* but not with MS2 or (b) with the synthetic peptide, were assumed to recognize E6* epitopes. Preimmune mouse sera did not react with MS2-E6* in ELISA or Western blot screening.

In order to detect clones with high specificity, cross-reactivity of these hybridomas was tested in screening Western blots of MS2/bacterial proteins, HEF proteins and by immunoperoxidase staining of HEF cells. A large majority of clones showed cross-reactivity and was discarded.

The remaining clones were tested for reactivity with MS2-E6 of HPV16 and HPV18 (kindly provided by K. Seedorf). Although the amino acid sequence homology of the HPV16 and HPV18 E6 proteins is rather high (54%), none of the clones reacted with MS2-E6 of HPV16. The majority of clones reacted with MS2-E6 of HPV18 and therefore recognized epitopes within the N-terminal 43 amino acids which are shared between E6* and E6. As expected, all clones not reacting with this N-terminal part of the molecule recognized the C-terminal synthetic peptide. The large number of clones specifically recognizing the MS2-E6* protein as well as the synthetic peptide demonstrates, that the correct reading frame was used for translation of the MS2-E6* fusion protein in bacteria.

Two hybridomas specifically recognizing epitopes within the N-terminal part of E6* and thus also recognizing E6 were termed E6*/E6-18-1 and E6*/E6-18-2, respectively (Fig. 3).

Two other hybridomas specifically recognizing epitopes within the C-terminal, E6* specific domain of the E6* molecule, were termed E6*18a and E6*18b. All four MAbs were stable after two rounds of cloning by limiting dilution.

Fig. 2. SDS-PAGE of MS2-E6* fusion protein synthesized in E. coli C600/537. After 3 h of induction, cells were lysed and extracted as described in "Materials and Methods." Aliquots were run on a gel and stained with Coomassie brilliant blue. Lane 1, supernatant after lysis; lane 2, Triton X-100 extraction; lane 3, 1 M urea extraction; lane 4, 7 M urea extraction; lane 5, molecular weight marker. MS2-E6*, about 80% of overall proteins in the 7 M urea fraction.

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Construction of HPV18 E6* Fusion Protein. cDNA sequence analysis and primer extension studies of RNA from HeLa cells indicated that HPV18 mRNAs are initiated at a viral promoter and that the 5'-terminal ends map a few nucleotides 5' or directly at the A residue of the common start codon of ORFs E6* and E6; they encode putative polypeptides of 57 (E6*) and 158 amino acid residues (E6) with calculated molecular weights of 6,500 and 18,900, respectively; the N-terminal 43 amino acids which are shared between E6* and E6 whereas the C-terminal 14 amino acids of E6* are unique to this putative gene product (5) (Fig. 1).

In the DNA sequence of HPV18 integrated in HeLa cell DNA, an NcoI restriction site is located at the start codon (ATG-Met) common to ORFs E6* and E6 (5). We used this restriction site for the construction of a recombinant expression plasmid that encodes a fusion protein composed of the first 98 amino acids of MS2 polymerase, six amino acids encoded by the polylinker sequence (LeuAsnSerGlyGlySer) and 57 amino acids encoded by HPV18 ORF E6*, starting with the methionine of the E6* start codon. Upon induction, this plasmid led to the synthesis of large amounts of the predicted MS2-E6* fusion protein (Fig. 2).
These data strongly suggest that the immunoprecipitated M, was not detected in competition experiments with unlabeled MS2-E6* (Fig. 4B, lane 4) or by MAb W6/32 (Fig. 4B, lane 5). As in HeLa cells, this band could be detected in the 300 HIM NaCl nuclear protein fraction by the MAb mixture and by MAb E6*/E6-18-2 alone (Fig. 4A, lane 3). These proteins were not detected in HEF (Fig. 4A, lane 4), BL-2 (Fig. 4B, lanes 6-8), and furthermore the proteins were not detected in competition experiments using unlabeled MS2-E6* (Fig. 4C, lane 6) and was absent in 14C-amino acid-labeled protein extracts from nude mouse epidermis (Fig. 4C, lane 9). Furthermore, MAb E6*/E6-16-1* recognizing an epitope within the N-terminal part of the HPV18 E6 protein (which is shared with HPV16 E6* polypeptide) did not show cross-reactivity (Fig. 4C, lane 5).

These results strongly suggest that the M, 6,500 polypeptide represents the predicted M, 6,500 HPV18 E6*. However, further experiments are necessary to demonstrate that this M, 6,500 polypeptide is also detectable in other HPV18-positive cervical carcinomas and is regularly absent in HPV unrelated tumors grown in nude mice. A protein with a molecular weight of 50,000 of the 300 mM nuclear fraction is also specifically precipitated by the E6* MAbs (Fig. 4C, lane 4). mRNA analysis will have to clarify, whether fusion proteins involving E6* or E6 sequences are encoded by RNAs in this tumor. A high molecular weight protein precipitated in the cytoplasm/membrane fraction (Fig. 4C, lane 1) might be due to cross-reactivity of a similar epitope, since the HPV16 E6 MAb reacted with this protein even stronger (Fig. 4C, lane 2).

DISCUSSION

This is the first report of expression of the HPV18 E6* polypeptide, the production of MAbs against E6*, and detection of the presumptive E6* polypeptide in cells derived from a human carcinoma.

HeLa and SW756 cells contain M, 17,000/18,000 and 18,000 proteins, respectively, that are specifically precipitated by MAbs directed against a MS2-E6* fusion protein. Cells from an HPV18-positive human cervical carcinoma, established in nude mice, contain an M, 6,500 polypeptide that is specifically precipitated by these MAbs. The molecular weights of the presumptive E6 and E6* polypeptides are in good agreement with those expected for a glycosylated protein was observed (Fig. 4A, lanes 7 and 8).

The immunoprecipitations failed to detect any E6*-related polypeptide in protein extracts of cervical carcinoma cell lines. This observation is in agreement with the inability to detect E6* in HeLa cells by other investigators using potentially cross-reacting HPV18 E6 polyclonal antisera (8, 9).

We used human cervical carcinomas established in nude mice as an alternative model to study HPV18 E6*/E6 gene expression. One out of five cervical carcinomas maintained in nude mice was shown by Southern blot analysis to contain HPV18 sequences. Cells of this tumor S18 harbor about 10-20 copies of the HPV18 genome integrated into the host chromosome (data not shown).

Immunoprecipitations of 14C-amino acid labeled proteins from nu/nu tumor S18 detected a polypeptide with a molecular weight of 6,500 in the 300 mM NaCl nuclear protein fraction (Fig. 4C, lane 4). This M, 6,500 polypeptide was not precipitated by MAb W6/32 (Fig. 4C, lane 7), was undetectable in competition experiments with unlabeled MS2-E6* (Fig. 4C, lane 6) and was absent in 14C-amino acid-labeled protein extracts from nude mouse epidermis (Fig. 4C, lane 9). Furthermore, MAb E6*/E6-16-1* recognizing an epitope within the N-terminal part of the HPV16 E6 protein (which is shared with HPV16 E6* polypeptide) did not show cross-reactivity (Fig. 4C, lane 5).

Identification of the HPV18 E6 and E6* Polypeptides in Cells derived from Human Cervical Carcinomas. MAbs E6*/E6-18-1 and 2 and E6*18a and b were tested for the ability to detect the E6* and E6 polypeptides in human cervical carcinoma cell lines HeLa and SW756, that had been shown to transcribe HPV18 E6* and E6 encoding mRNAs (5).

First attempts of Western blot analysis using 125I-labeled protein A and immunoprecipitations of [35S]methionine-labeled cells failed to detect any peptides of the predicted molecular weights of 6,500 (E6*) and 18,900 (E6). In order to increase sensitivity, we then used a 125I-amino acid mixture for protein labeling of the cells and a mixture of all four MAbs for immunoprecipitation. Cells that do not harbor known HPV sequences (cervical carcinoma cell line HT-3, lymphoblastoid cell line BL-2, and HEF) were also analyzed.

In HeLa cells, proteins of molecular weights 17,000 and 18,000 were specifically immunoprecipitated in the 300 mM NaCl nuclear protein fraction by the MAb mixture and by MAb E6*/E6-18-2 alone (Fig. 4A, lane 3). These proteins were not detected in competition experiments using unlabeled MS2-E6* (Fig. 4A, lane 6), and furthermore the proteins were not detected (Fig. 4A, lane 5) by the unrelated MAb W6/32 which recognizes gene products of the major histocompatibility complex (24). No proteins of molecular weights 17,000 and 18,000 were detected in HEF (Fig. 4A, lane 4), BL-2 (Fig. 4B, lanes 6-8), and HT-3 cells (data not shown). The M, 18,000 protein also could be detected in the 300 mM NaCl nuclear protein fraction of SW756 cells (Fig. 4B, lane 3). As in HeLa cells, this band was not detected in competition experiments with unlabeled MS2-E6* (Fig. 4B, lane 4) or by MAb W6/32 (Fig. 4B, lane 5).

These data strongly suggest that the immunoprecipitated M, 6,500 polypeptide represents the predicted M, 6,500 HPV18 E6*. However, further analyses are necessary to clarify, whether fusion proteins involving E6* or E6 sequences are encoded by RNAs in this tumor. A high molecular weight protein precipitated in the cytoplasm/membrane fraction (Fig. 4C, lane 1) might be due to cross-reactivity of a similar epitope, since the HPV16 E6 MAb reacted with this protein even stronger (Fig. 4C, lane 2).

* Schneider-Gadicke et al., manuscript in preparation.
presumptive E6 and E6* polypeptides in the 300 mM NaCl nuclear protein fraction, which contains most of the NBP (20). In mouse cells transfected with plasmids encoding the BPV E6 ORF driven by a retroviral long terminal repeat, the putative BPV E6 protein was identified in the nuclear and membrane fractions (8). The detection of HPV18 E6 protein exclusively in a nuclear protein fraction might either demonstrate the different localization of E6 proteins expressed at low levels in human carcinoma cells or at high levels in mouse cells transfected with a high expressing plasmid construct or this difference might be due to different properties of HPV and BPV E6 proteins.

Two E6 proteins with slightly different electrophoretic mobilities (M, 17,000 and 18,000) were detected in HeLa cells (Fig. 4A, lane 3). It remains to be determined whether these differences are due to posttranslational modifications. The lack of N-glycosylation sites in the amino acid sequences, the nuclear localization and the lack of binding in lentil-lectin affinity chromatography exclude the possibility of glycosylation. Interestingly, pulse-chase experiments carried out in SiHa cells with HPV16 E6 polyclonal antisera suggest the existence of two components of HPV16 E6 with different half-lives of <30 min and about 4 h (8). This finding might be correlated with the existence of two slightly different E6 proteins in HeLa cells. The detection of only one component (M, 18,000) in SW756 cells (Fig. 4B, lane 3) and CaSki and SiHa cells (8) might be due to the inability to detect the short half-life component under certain experimental conditions.

Whereas in general only a very small percentage of antiviral MAbs react with normal human tissue (26), most of MS2-E6* hybridoma supernatants stained HEF cells and were therefore discarded. In this context it is of interest that a DNA sequence highly conserved between E6 ORFs (corresponding to HPV18 nucleotides 226–272, numbering as in Reference 5) of HPV types associated with malignant tumors is also present in normal human DNA and transcribed in HEF cells [a 47mer oligonucleotide corresponding to this HPV18 E6 exon/E6* intron sequence detects about six copies of an (almost) identical sequence at a Tm — 5°C in all no HPV-containing human DNAs tested; the amino acid sequence (EVFEFAFKD) encoded by the 5'-terminal half of this highly conserved DNA sequence is almost identical to an aa sequence of insulin C-peptide highly conserved in fishes (EVAFAFKD)]. These observations might indicate that cellular genes with similar functions as HPV early genes might exist. Therefore all reports describing the detection of early papillomavirus proteins in human cells should be taken with criticism unless the identity of the proteins is proven by peptide mapping or sequencing. To some extent this reservation is also valid for E6* MAbs described here, although they specifically detect proteins of the predicted molecular weights in the predicted cellular localization in HPV18-positive but not in HPV18-negative cells.

The ability to detect the presumptive HPV18 E6* and E6 proteins by MAbs has basic and potential clinical applications. These MAbs should facilitate the characterization of the biochemical properties and functions of the E6* and E6 proteins. The identification of an M, 6,500 E6* polypeptide in a cervical carcinoma grown in nude mice (Fig. 4C, lane 4) and, on the contrary, the inability to detect any such polypeptide in HeLa cells (although the majority of HPV18 mRNAs in this cell line contains ORF E6*) suggests the possibility that translation of the viral mRNAs is different in tissue culture cells in vitro and tumor cells in vivo. Experiments are now in progress to determine whether the E6* polypeptide is expressed in tumors of HeLa cells grown in nude mice.

Since most HPV-associated carcinomas do not produce viron structural proteins, E6* MAbs have a potential application...
in detecting HPV18 in clinical material and might constitute an alternative to nucleic acid hybridizations.

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