NOTES

Human Papillomavirus Type 18 E6*, E6, and E7 Protein Synthesis in Cell-Free Translation Systems and Comparison of E6 and E7 In Vitro Translation Products to Proteins Immunoprecipitated from Human Epithelial Cells

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Expression of the E6 and E7 transforming genes of human papillomavirus type 18 (HPV18) occurs via structurally bicistronic mRNAs in which the downstream open reading frame (ORF) E7 is preceded either by the full-length ORF E6 or by a spliced ORF, E6*. We have used in vitro transcription and translation of HPV18 cDNAs in order to analyze the synthesis of E6*, E6, and E7 proteins and to compare the E6 and E7 in vitro translation products with the authentic proteins immunoprecipitated from cervical cancer cells. In wheat germ extract, in vitro translation resulted in the production of all three proteins, E6*, E6, and E7. In rabbit reticulocyte lysate, however, only the E6 and E7 proteins were produced. The lack of E6* protein was due neither to template RNA degradation nor to an inhibitory influence of the RNA 5' leader sequences, thus indicating the possibility of either inhibition of synthesis or degradation of E6* protein in reticulocyte lysate. The E7 protein was synthesized from both E6*-E7 and E6-E7 RNAs. In vitro-synthesized and authentic HPV18 E7 proteins revealed identical electrophoretic mobilities in two-dimensional gel electrophoresis, thus indicating similar modifications. By using a monoclonal antibody against the N terminus of HPV18 E6* and E6, an 18-kDa protein was detected not only in HPV18-positive but also in HPV18-negative epithelial cells. The 18-kDa proteins and the in vitro-synthesized HPV18 E6 protein exhibited comparable electrophoretic characteristics in two-dimensional gels. These results suggest the possible existence of a cellular protein related to HPV18 E6.

Cancer of the uterine cervix is linked to infections with certain types of human papillomaviruses (HPVs), in particular HPV type 16 (HPV16) and HPV18 (for reviews, see references 44 and 46). The oncogenic and cell growth-stimulating potential of HPV16 and HPV18 seems to reside primarily in two early viral genes, E6 and E7. The importance of these genes for malignant cell proliferation has been deduced from the selective retention and expression of the E6 and E7 genes in cervical carcinomas and in carcinoma-derived cell lines which carry HPV16 or HPV18 DNA integrated into the cellular genome (2, 13, 36, 37, 40). In addition, it has been shown that expression of the E6 and E7 genes is essential for in vitro proliferation of established cervical carcinoma cells (43). Transfection experiments have indicated a role in an early step of carcinogenesis, since the E6 and E7 genes are able to immortalize primary rodent and human cells (5, 6, 10, 19, 20, 26). Complex formation of the E6 and E7 proteins with cellular tumor suppressor gene products (E7 and Rb, E6 and p53) has been recognized as one possible pathway by which the viral genes interfere with cellular regulatory genes and thereby disturb normal control of cell proliferation (12, 27, 45).

Analysis of HPV16 and HPV18 transcripts in cervical cancer cell lines has revealed that the E6 and E7 genes are

expressed via structurally bicistronic, differentially spliced RNAs. The mRNAs contain as a 5' cistron either the full-length open reading frame (ORF) E6 or a spliced variant, E6*, followed downstream by ORF E7 (Fig. 1). E6* splicing seems to be characteristic for the cancer-associated genital HPVs, whereas the HPV types associated mainly with benign genital tumors (HPV6 and HPV11) lack E6* splicing sites (9, 34, 36). This peculiar difference has raised the question of whether and by which mechanism E6* expression might contribute to the oncogenic potential of the cancer-associated genital HPVs. It has been suspected that either the E6* protein exerts a function (36) or E6* splicing generates the templates for high-level translation of ORF E7 (39). Whereas the E6 and E7 proteins have been identified in cervical carcinomas and cancer-derived cell lines (1, 3, 38, 40), attempts to detect an HPV16 or HPV18 E6* protein in cells cultured in vitro have been unsuccessful so far (1, 3, 35). Only in one HPV18-positive human cervical carcinoma passaged in nude mice could a candidate for the E6* protein be detected (35). In this study, we have analyzed the synthesis of proteins E6, E6*, and E7 of HPV18 by in vitro transcription and translation of HPV18 cDNAs. Furthermore, two-dimensional (2D) gel electrophoresis was used for a biochemical characterization of the in vitro-synthesized E6 and E7 proteins in comparison with the proteins immunoprecipitated from cultured cervical cancer cells.

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FIG. 1. Structures of HPV18 cDNAs used for construction of expression plasmids. Shown are HPV18 cDNA clone 7-23, derived from an mRNA containing ORFs E6 and E7 (A), and cDNA clone H4, derived from a spliced mRNA containing ORFs E6* and E7 (B). The nucleotide positions are numbered according to the numbering scheme in reference 36. HPV18 ORFs are indicated by open boxes, and cotranscribed flanking cellular sequences are indicated by zigzag lines. Excised E6* intron sequences are indicated by thin slanted lines. Restriction sites used for construction of HPV18 expression plasmids (Table 1) are indicated by R (*EcoRI*), N (*NcoI*), and S (*SspI*). The proteins encoded by the ORFs are E6* (57 amino acids of which the N-terminal 43 residues are identical to those of E6; calculated molecular weight, 6,505), E6 (158 amino acids; molecular weight, 18,875), and E7 (105 amino acids; molecular weight, 11,997).

For the *in vitro* transcription and translation studies, different HPV18 cDNA expression plasmids were constructed (Table 1), and the linearized DNAs were used as templates for production of cRNAs by *in vitro* transcription (33). The cRNAs were structurally either monocistronic (containing ORF E6*, E6, or E7) or bicistronic with 5' ORF E6* or E6 followed 3' by ORF E7 (E6*-E7 and E6-E7, respectively). The bicistronic cRNAs resembled the natural HPV18 mRNAs found in cervical carcinoma cell lines (Fig. 1). Two cell-free systems, reticulocyte lysate (RTL) and wheat germ extract (WGE), were used for *in vitro* translation. *In vitro* translation in WGE resulted in the production of all three HPV18 proteins, E6*, E6 and E7 (Fig. 2A). In contrast, in RTL only the E6 and E7 proteins were produced, but no detectable amounts of the E6* protein were produced (Fig. 2B). The HPV18 E7 protein was synthesized from both types of bicistronic cRNAs, E6*-E7 and E6-E7, in both RTL and WGE (Fig. 2, lanes 2 and 4). The authenticity of the *in vitro*-synthesized proteins was confirmed by immunoprecipitation with specific antibodies (Fig. 2C through E). The following antibodies were used: a polyclonal antiserum directed against HPV18 E7 (E7 PAb [38]), a mixture of monoclonal antibodies (MAbs) E6*/E6-18-1 and E6*/E6-18-2 (E6*/E6 MAb) directed against an epitope in the N terminus common to HPV18 E6 and E6* (7, 35), and a mixture of

MAbs E6*18a and E6*18b (E6* MAb) raised against a synthetic oligopeptide of the C-terminal 23 amino acids of the E6* protein (35).

The lack of E6* protein in RTL was not caused by a pronounced degradation of the input RNAs, since neither in the absence nor in the presence of the RNase inhibitor RNasin could the E6* protein be detected. An inhibitory influence of the 5'-nontranslated sequences of the cRNAs could also be ruled out. RTL-directed *in vitro* translation always failed to produce E6* protein, irrespective of the length and primary structure of the 5' leader sequences of the cRNAs used (plasmids N3, E3, 3.5, and 4.1 [Table 1]). Therefore, it seems likely that the lack of E6* protein is due to either inhibition of synthesis or degradation of the E6* protein occurring specifically in RTL but not in WGE. The relevant mechanism, as well as its role in HPV18 early gene expression, has yet to be determined. On the assumption that the vertebrate-derived RTL *in vitro* translation system mimics the intracellular conditions for translation of HPV mRNAs more faithfully than the plant-derived WGE system, a similar block to E6* protein synthesis or accumulation might also be active in human cells. This would provide an explanation for the consistent failure to detect E6* proteins in cervical carcinoma cells cultured *in vitro* (1, 3, 35). There is, however, the exception of a candidate HPV18 E6* protein detected by immunoprecipitation with the E6*/E6 MAbs in a cervical carcinoma passaged in nude mice (35). The two results of *in vitro* translation, the lack of detectable E6* protein in RTL and the synthesis of E7 protein from bicistronic E6*-E7 cRNAs, together support the assumption that E6* splicing plays a role in translation of ORF E7 rather than in synthesis of an E6* protein.

2D gel electrophoresis was performed (according to methods described in references 14, 28, and 30) for a further biochemical characterization of the *in vitro*-synthesized proteins in comparison with the authentic proteins immunoprecipitated from cultured cervical carcinoma cells. The RTL-synthesized E6 protein gave rise to a protein streak in the pI range of about 9, in agreement with the calculated value of 8.56 (Fig. 3a) (pI values were calculated by using the HUSAR computer program [17]). The WGE-synthesized HPV18 E6* protein was present as one main spot (Fig. 3d) with a pI of about 4.6 (calculated pI = 4.55). The *in vitro*-synthesized E7 protein appeared as three or four spots which differed in their pI but not in their molecular weight (Fig. 3e). A very similar pattern was obtained for the authentic E7 protein (Fig. 3f). Such a pattern is typical for phosphorylated proteins (28, 31), and the E7 proteins of

TABLE 1. Features of HPV18 expression plasmids

Plasmid			cDNA insert features			cRNA features	
Clone	Vector ^a	Promoter	HPV18 ORFs contained	cDNA clone from which derived ^b	Restriction sites used for cloning ^b	Length (nucleotides)	No. of 5'-nontranslated nucleotides upstream from first AUG ^c
E3	pSPT18	T7	E6*, E7	H4	<i>EcoRI-EcoRI</i>	1,323	25 (16/9)
N3	pSPT18	T7	E6*, E7	H4	<i>NcoI-EcoRI</i>	1,313	15 (14/1)
7.3	pSPT18	T7	E6, E7	7-23	<i>EcoRI-EcoRI</i>	1,405	54 (16/38)
3.5	pGEM3	T7	E6*	H4	<i>EcoRI-SspI</i>	347	25 (16/9)
4.1	pGEM4	SP6	E6*	H4	<i>EcoRI-SspI</i>	395	73 (64/9)
9.1	pGEM3	SP6	E7	7-23	<i>SspI-EcoRI</i>	885	34 (27/7)
9.3	pGEM3	T7	E6	7-23	<i>EcoRI-SspI</i>	558	53 (16/37)

^a Vector plasmids were obtained from Pharmacia and Promega.

^b See reference 36 and Fig. 1.

^c Numbers in brackets indicate ratio of nucleotides from vector to nucleotides from cDNA insert.

FIG. 2. In vitro synthesis of HPV18 proteins E6*, E6, and E7. cRNAs of the following HPV18 clones (Table 1) were used as templates for in vitro translation in WGE (A, C), and RTL (B, D, and E): lanes 1, clone 3.5 (E6*); lanes 2, clone N3 (E6* plus E7); lanes 3, clone 9.1 (E7); lanes 4, clone 7.3 (E6 plus E7); and lanes 5, clone 9.3 (E6). Lanes 6, no RNA was added to the in vitro translation reaction mixture. In vitro translation in nuclease-treated RTL (Promega) was performed at 37°C for 60 min in a reaction mixture (50 μ l) containing 1 μ g of cRNA, 1 μ l of amino acid mixture minus cysteine (1 mM each amino acid), 50 μ Ci of [35 S]cysteine, and 40 U of RNasin. In vitro translation in WGE (Amersham) was performed at 25°C for 60 min in a 50- μ l reaction mixture containing 25 μ l of WGE, 1 μ g of RNA, 3.5 μ l of amino acid mixture minus cysteine (1 mM each amino acid), 50 μ Ci of [35 S]cysteine, and 40 U of RNasin. The internal K^+ concentration was raised to 140 mM with potassium acetate. Immunoprecipitations (see legend to Fig. 3) of in vitro-synthesized proteins were performed with E6* MAb (C), E7 PAb (D), and E6*/E6 MAb (E). The labelled proteins were separated on 15% polyacrylamide gels as described in reference 22. The bars indicate the positions of the molecular weight marker proteins (top to bottom: 30×10^3 , 21.5×10^3 , 14.3×10^3 , and 6.5×10^3). The 14-kDa band present in all lanes in panel B represents the hemoglobin of the RTL.

FIG. 3. 2D gel analysis of in vitro-synthesized HPV18 proteins E6, E6*, and E7 and of authentic E7 and 18-kDa protein obtained by immunoprecipitation from cells. (Top) Schematic representation of the 2D gels. The numbered square boxes indicate the sections of autoradiographs shown in the bottom part of the figure (box 1, panels a through c; box 2, panels e and f; box 3, panel d). (Bottom) HPV18 E6 protein synthesized from 9.3 cRNA in RTL (a); 18-kDa protein immunoprecipitated with E6*/E6 MAb from HeLa cells (b) and from SK30 cells (c); HPV18 E6* protein synthesized from 4.1 cRNA in WGE (d); HPV18 E7 protein synthesized from 7.3 cRNA in RTL (e) and immunoprecipitated from HeLa cells (f). For protein labelling, cells were incubated in Dulbecco modified Eagle medium without cysteine (select amine kit; GIBCO) with 1 mCi of [35 S]cys-

teine per ml for 4 h. Immunoprecipitations of ultrasonicated lysates (18) were performed with E6*/E6 MAb in buffer A as described in reference 35. For immunoprecipitation with E7 PAb, cells were resuspended in 1 ml of buffer B (20 mM Tris [pH 6.5], 1 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μ g of aprotinin per ml) and were lysed by freeze-thawing. After addition of antibody and protein A-Sepharose (Pharmacia), immunoprecipitation was performed overnight at 4°C. After centrifugation, the immunocomplexes were washed three times in the corresponding buffer, A or B, three times in buffer A or B containing 500 mM NaCl, and once in phosphate-buffered saline before the pellet was resuspended in lysis buffer (28). Protein samples were analyzed by isoelectric focusing (IEF [28]) and non-equilibrium pH gradient electrophoresis (NEPHGE [14, 30]). The first-dimension gels were 3.8% acrylamide–0.2% bisacrylamide, and the pH gradient was stabilized by ampholytes in the following ranges: IEF, pH 3.5 to 10 and pH 5 to 7 (Pharmacia-LKB, Bromma, Sweden, and Serva, Heidelberg, Germany, respectively), and NEPHGE, pH 7 to 9 and pH 8 to 9.5 (Pharmacia-LKB and Serva, respectively). The second-dimension gels were run on 15% acrylamide–0.075% bisacrylamide gels. The gels were processed for fluorography as described in reference 24 or with Amplify (Amersham International, Little Chalfont, United Kingdom). Molecular weights and isoelectric points (pIs) of the analyzed proteins were interpolated by using standard proteins of known molecular weight and pI (Amersham and Pharmacia-LKB).

FIG. 4. Immunoprecipitation with E6*/E6 MAb and E7 PAB from lysates of HPV18-positive and -negative tissue culture cells. Cells were metabolically labelled with [35 S]cysteine, and proteins were immunoprecipitated with E6*/E6 MAb (A) or E7 PAB (B) from ultrasonicated lysates of the following cells: HeLa (HPV18 positive; lanes 1), HaCaT (HPV negative; lanes 2), SK30 (HPV negative; lanes 3), CaSki (HPV16 positive; lanes 4), and human embryonic fibroblasts (lanes 5). The proteins were separated on 15% polyacrylamide gels. Molecular weight marker proteins are indicated by bars (from top to bottom: 92×10^3 , 69×10^3 , 45×10^3 , 30×10^3 , 21.5×10^3 , 14.3×10^3 , and 6.5×10^3). The 18-kDa protein was the only protein specifically immunoprecipitated by the E6*/E6 MABs, since all other proteins of higher molecular mass, including a 32-kDa protein, were also precipitated by the C-terminal E6* MAB.

To obtain the authentic HPV18 E6 protein, immunoprecipitation experiments with the N-terminal E6*/E6 MAB were performed. As expected, a protein with a molecular weight of 18,000 (the size of the HPV18 E6 protein) was immunoprecipitated from protein extracts of HPV18-positive cells. Surprisingly, however, the E6*/E6 MAB also immunoprecipitated an 18-kDa protein from cells of the spontaneously transformed keratinocyte line HaCaT, which is not known to harbor any HPV DNA (8), from the HaCaT-pSV2neo derivative SK30 (11), and from HPV16-positive cells, whereas human embryonic fibroblasts were negative (Fig. 4). For the HPV18-negative cell lines, Southern and Northern (RNA) blot analysis and immunoprecipitation with HPV18 E7 PAB showed that neither HPV18-specific DNA nor HPV18-specific RNA nor the HPV18 E7 protein was present, thus excluding the possibility of contamination with HPV18. Therefore, the 18-kDa protein immunoprecipitated from the HPV18-negative cells could not be the HPV18 E6 protein. Furthermore, since the HPV18 E6*/E6 MABs do not cross-react with HPV16 E6 (35), the 18-kDa protein in the HPV16-positive cells is unlikely to be the HPV16 E6 protein. We determined whether the 18-kDa proteins of HPV18-positive (HeLa) cells and HPV-negative (SK30) cells can be distinguished by 2D gel electrophoresis. However, the electrophoretic characteristics (molecular weight, isoelectric point, and focusing features) of the two 18-kDa proteins were very similar and, furthermore, resembled those of the HPV18 E6 protein synthesized in RTL (Fig. 3a through c). We conclude from these data that the 18-kDa protein immunoprecipitated from HPV18-negative epithelial cells may be a cell-encoded protein cross-reacting with the N-terminal HPV18 E6*/E6 MABs. At present, however, we cannot fully exclude the possibility of an E6 protein of yet unidentified HPV types. With regard to the HPV18-positive cell lines, it is impossible to distinguish whether the 18-kDa

protein immunoprecipitated with the E6*/E6 MABs is the HPV18 E6 protein or the putative cellular 18-kDa protein or a mixture of both. Our results thus illustrate problems inherent in the detection of HPV early proteins in human cells by immunoprecipitation analysis (see also reference 35). Cross-reactivity of cellular proteins with antibodies directed against viral oncoproteins has also been observed in other cases, e.g., HPV16 E6 (1) and the simian virus 40 large T antigen, for which the related cellular protein has been isolated and functionally characterized (21, 23). Further characterization of the 18-kDa protein found in the HPV-negative cells will be necessary to clarify its cellular or viral origin and to determine its relationship to HPV18 E6. It is an intriguing possibility that a cellular gene related to HPV18 E6 is expressed in human cells and that the viral E6 protein might interfere with the function of the cellular gene, thereby promoting deregulation of cell growth or differentiation.

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