Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4680–4684, July 1986
Biochemistry

Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein

(viral DNA integration/plasmid/virus–cell hybrid transcripts/fusion protein/early viral protein)

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Communicated by Paul D. Boyer, March 10, 1986

ABSTRACT **Human papillomavirus type 16 DNA and RNA were characterized in the cervical cancer-derived CaSki cell line, which contains only integrated DNA, and in a cervical cancer, which contains predominantly plasmid DNA. In both, a major RNA can code for the early open reading frame E7 and a minor one can code for E6. The cervical cancer, but not the CaSki cell line, contains a minor RNA that can code for an intact E2 protein, and this may relate to the continued presence of plasmid DNA. The RNA mapping data suggest that the poly(A)$^+$ RNA is transcribed from a minor fraction of the several hundred gene copies present, and in the cervical cancer these genomes appear to be integrated. The E7 protein has been identified in CaSki cells and the prevalence of its mRNA suggests a possible function in progression to, or long-term maintenance of, the malignant state.**

Papillomaviruses are widespread in nature, causing benign tumors (warts) in humans and many animal species (1, 2). Recently, new human papillomavirus (HPV) types (16 and 18) have been identified and found to be associated with cervical cancers (3, 4). It appears that a majority of cervical cancers and several cancer-derived cell lines contain integrated HPV-16 or -18 DNA (3–8). In contrast, in premalignant lesions HPV-16 DNA is usually maintained as plasmid (9). The significance of this difference in the state of the viral DNA is not known. The detection of viral RNA in at least some cancers and cancer-derived cell lines (5–7) could indicate a requirement for continued viral gene expression. The viral transcripts, however, have not been mapped to the individual open reading frames (ORFs) defined by the sequence (10) and no proteins have been identified.

We show here that, in a cervical cancer containing HPV-16 predominantly as plasmid and a cervical cancer-derived cell line (CaSki) with only integrated HPV-16 DNA, transcripts are present that could code for ORF E6 and E7. In addition, we report here identification of an early HPV protein that is expressed in malignant cells.

MATERIALS AND METHODS

Tissue Cultures, Tumors, and HPV DNA. The cervical cancer-derived cell lines C-33A, HT-3, CaSki, and SiHa were obtained from the American Type Culture Collection. Lines C-33A and HT-3 are negative for HPV-16 and -18 DNA; lines CaSki and SiHa are positive for HPV-16 DNA (5, 6). HeLa cells positive for HPV-18 (5) were obtained from A. J. Berk. Tissue cultures were grown in RPMI 1640 medium supplemented with 10% or 15% fetal calf serum. Tumor 6, a recurrence in the cervix, and tumor 4, a lung metastasis, were from a patient who previously received x-ray therapy for a cervical squamous cell carcinoma (8). Cloned HPV-6b, -16, and -18 DNA was generously provided by L. Gissman, E.-M. de Villiers, and H. zur Hausen.

Isolation of DNA and RNA. DNA and poly(A)$^+$ RNA were isolated as described (11) except that the RNA was passed over oligo(dT)-cellulose columns only once.

DNA (Southern) and RNA (Transfer) Blot Analysis. DNA electrophoresis in one or two dimensions was as described (12). Subgenomic fragments were separated on low-melting agarose and isolated by extraction with hexadecyl-trimethylammonium bromide (13). The nick-translation of probes (14) to specific activities of $0.5–2 \times 10^8$ cpm/μg and hybridization with genomic or subgenomic probes by the method of Wahl *et al.* (15) were as described (11). The RNA was electrophoresed in 1.1% agarose gels containing 2.2 M formaldehyde/0.02 M sodium phosphate buffer, pH 7.2 (16). RNA transfer to GeneScreen (New England Nuclear) and hybridization at 42°C in 50% formamide/10% dextran sulfate were as recommended by the filter manufacturer.

RNA Mapping. S1 nuclease (S1) and exonuclease VII (ExoVII) mapping was by the method of Berk and Sharp (17) with 3′ or 5′ end-labeled probes as described (11). For primer extension, 5′ labeled primers were hybridized to poly(A)$^+$ RNA under the same condition as in S1 and ExoVII mapping. RNA-bound primers were selected by oligo(dT)-cellulose chromatography and extended with 75 units of reverse transcriptase in 150 μl containing 100 mM Tris·HCl, pH 8.3/10 mM $MgCl_2$/140 mM KCl/5 mM dithiothreitol/1 mM (each) dNTP/15 μg of bovine serum albumin/75 units of RNasin (RNase inhibitor) for 30 min at 42°C. The extension product was extracted with phenol/chloroform and salts were removed by gel filtration on Sephadex G-50 and ethanol precipitation. Analysis of primer extensions was as for S1 and ExoVII digests. S1 digests were also analyzed on neutral 1% or 4% agarose gels. One percent agarose gels were fixed and dried as described (11) and nucleic acids from 4% NuSieve (FMC, Rockland, ME) gels were transferred to GeneScreen by Southern blotting for autoradiography.

Antisera to the E7 Protein. A tryp E–E7 fusion vector was constructed by inserting the *Nsi* I–*Pst* I fragment of HPV-16 that codes for 97 amino acids of E7 into the *Pst* I site of the plasmid pATH 10 (generously provided by T. J. Korner and A. Tzagoloff). This construct is similar to that described for a tryp E–reverse transcriptase gene fusion in which the similar vector pATH 1 was used (18). The tryp E–E7 fusion protein was recovered as an insoluble pellet from cell lysates and used to immunize rabbits. The calculated molecular mass

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Abbreviations: ORF, open reading frame; HPV, human papillomavirus; CRPV, cottontail rabbit papillomavirus; BPV-1, bovine papillomavirus type 1; kb, kilobase(s); ExoVII, exonuclease VII; S1, S1 nuclease.

of the fusion protein was 47 kDa; the molecular mass calculated from the mobility in $NaDodSO_4$/PAGE was 57 kDa and suggested some unusual properties for the E7 protein. Similar differences between molecular mass determined from the amino acid sequence and that determined from the mobility in $NaDodSO_4$/PAGE were observed for the E1A protein of adenovirus and its fusion protein (19).

Cell Labeling and Immunoprecipitation. Cells were labeled 2–3 hr in 5-cm dishes in cysteine- and methionine-free medium supplemented with 10% dialyzed fetal calf serum and 0.5 mCi (each) of [^{35}S]cysteine and [^{35}S]methionine (1 Ci = 37 GBq). The cells were lysed in 0.5 ml of 0.15 M NaCl/1.0% Triton X-100/1.0% deoxycholate/0.1% $NaDodSO_4$/10 mM Tris·HCl, pH 7.4/0.2 mM phenylmethylsulfonyl fluoride and the extract was briefly sonicated. Incubation with anti-E7 serum was at 4°C overnight. Antigen–antibody complexes were bound to protein A-Sepharose during a 2-hr incubation at room temperature. The Sepharose was washed four times with lysis buffer, and proteins were released by boiling 5 min in 0.062 M Tris·HCl, pH 6.8/10% glycerol/0.0015% bromphenol blue/3% $NaDodSO_4$/5% 2-mercaptoethanol and analyzed by 12% $NaDodSO_4$/PAGE (20). The fixed gels were equilibrated with EN^{3}HANCE (New England Nuclear), dried, and autoradiographed.

RESULTS

Characterization of DNA. For our analysis we chose a tumor-derived cell line (CaSki) that contained only integrated HPV-16 DNA and a cervical cancer (tumor 6) containing predominantly HPV-16 DNA plasmid. The analysis for both is shown in Fig. 1. Included in this analysis was also a lung metastasis (tumor 4) from the same patient. Tumor 6 contains almost exclusively dimeric supercoiled (form I), nicked (form II), and linear (form III) HPV-16 DNA, whereas tumor 4 contains mostly monomeric form I, II, and III DNA. However, in two-dimensional analysis in both tumors (data not shown), a small amount of viral DNA comigrated with cellular DNA. In the CaSki cell line, all DNA comigrated with cellular DNA. The intensity of the autoradiograms of the tumor and CaSki cell DNA indicated that they both contained several hundred copies of HPV-16 DNA per cell. Digestion of the DNAs with the single-cut restriction enzyme *Bam*HI resulted in a single band of about 8 kilobases (kb) for the two tumors (shown is tumor 4 only), indicating that essentially intact HPV-16 DNA was present. The *Bam*HI digestion pattern of the CaSki cell DNA is more complex.

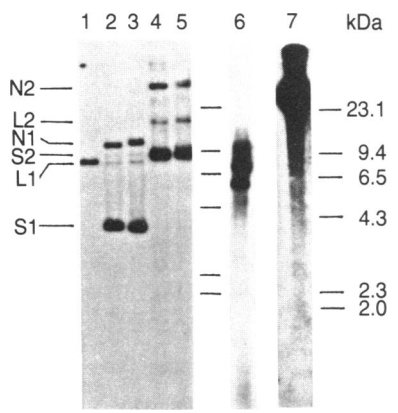

FIG. 1. Analysis of HPV-16 DNA from a cervical cancer (tumor 6) (lanes 4 and 5), a lung metastasis (tumor 4) (lanes 1–3), and CaSki cells (lanes 6 and 7). The analysis from left to right represents tumor 4 DNA *Bam*HI (single cutting) (1.0 μg) (lane 1) and *Hin*dIII (noncutting) (2.5 μg) digested (lane 2) or undigested (2.5 μg) (lane 3), tumor 6 DNA *Hin*dIII digested (2.5 μg) (lane 4) or undigested (2.5 μg) (lane 5), and CaSki cell DNA *Bam*HI digested (2.5 μg) (lane 6) or undigested (2.5 μg) (lane 7). The lane with undigested CaSki cell DNA was autoradiographed longer. S1, N1, and L1 indicate the positions of monomeric form I, II, and III DNA; S2, N2, and L2 indicate the positions of dimeric form I, II, and III DNA. The positions of linear DNA molecular mass markers are indicated between panels and on the right.

RNA Analysis. Poly(A)$^+$ RNA isolated from whole CaSki cells could be resolved into three HPV-16-specific major bands corresponding to sizes of 1.5, 2.3, and 4.5 kb (Fig. 2). The pattern of the tumor 6 RNA indicates that some degradation did occur. There is evidence for one major band at about 2.0 kb and a second one at about 3.0 kb. Hybridization with an early-region (10) probe (probe A, Fig. 2) showed strong hybridization with the same relative intensity with all three major bands of the CaSki cell line. The late region contains the L1 and L2 ORFs and transcripts homologous to this region are found in virus-producing papillomas in addition to early-region transcripts (11, 21). The tumor 6 RNA also hybridizes strongly to the early-region probe (Fig. 2, probe B, lanes T6). Only weak hybridization was observed with late-region probes (Fig. 2, probe B, lanes T6 and Ca; probe C, lanes Ca) and no distinct bands were detectable. These data indicate that in the CaSki cell line and tumor 6 most poly(A)$^+$ viral transcripts originate from the early region.

Mapping of individual exons to determine the coding capacity of viral transcripts was accomplished by S1, ExoVII digestion, and primer extension. The strategy of the experiments, the results, and their interpretation are detailed in Figs. 3 and 4. Three different exons were identified in the

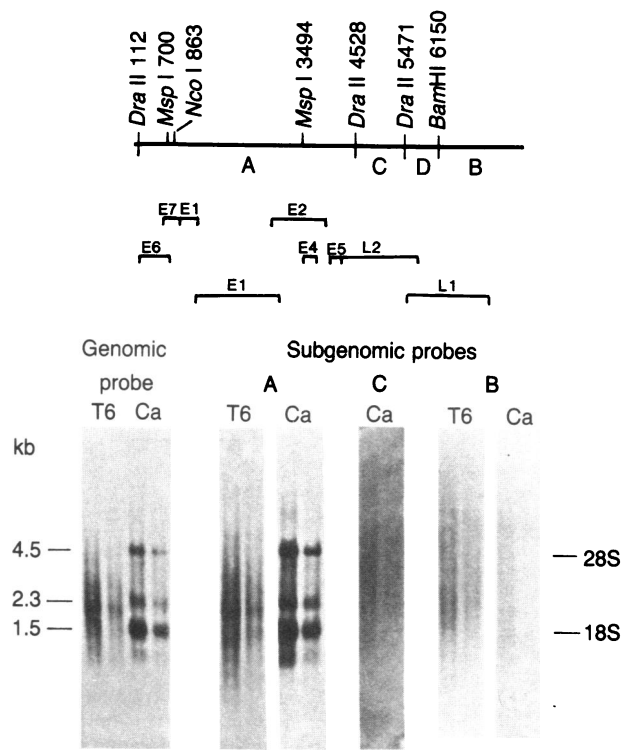

FIG. 2. Blot analysis of oligo(dT)-selected whole-cell RNA from a cervical cancer (tumor 6) and from CaSki cells hybridized with genomic or subgenomic probes. T6, tumor 6 RNA: the first lane of each pair contains ≈2.6 μg of poly(A)$^+$ RNA and the second lane contains ≈1.3 μg. Ca, CaSki cell RNA: the first lane of each pair contains ≈1.3 μg of poly(A)$^+$ RNA and the second lane contains ≈0.6 μg. Hybridization was with genomic probe or subgenomic probes as indicated. The positions and sizes of the major CaSki cell RNA bands in kb are given as are the positions of rRNA markers (18S and 28S). A HPV-16 map with the location of the subgenomic probes and the ORFs are shown at the top.

CaSki cells and two were identified in the tumor (Fig. 3A). The 5′ end of the major exon common to both and that of a minor one unique to the CaSki cells map upstream of the 5′ end of E7. The 5′ end of a common minor exon maps close to the beginning of E6 between the first and the second ATG of E6 and 32 nucleotides downstream of a "TATA" box. The ExoVII digests of the CaSki cell RNA show that the three RNA species containing these exons have a common cap site at nucleotide 97. This result together with the primer extension indicates that the major and minor RNAs with a coding potential for E7 have short leaders upstream of their coding exons. The difference in size between S1 and primer-extension band represents the size of the leader exons of 135 and 160 nucleotides for the major and minor band, respectively. The third primer-extension band lines up with the largest S1 band and thus this RNA does not have a leader. There are two 3′ ends of the exons, both downstream of E7 (Fig. 3B).

The transcripts mapping to the E6–E7 region are spliced to exons in the E2–E4 region (Fig. 4). CaSki cells and the tumor have a major 5′ exon end close to the beginning, but within E4, and a minor one upstream of the end of E2. In the tumor the minor S1 band was weak and did not reproduce in the exposure shown. Downstream the viral RNAs do not extend to the poly(A) site. The 3′ end of the S1 band of the

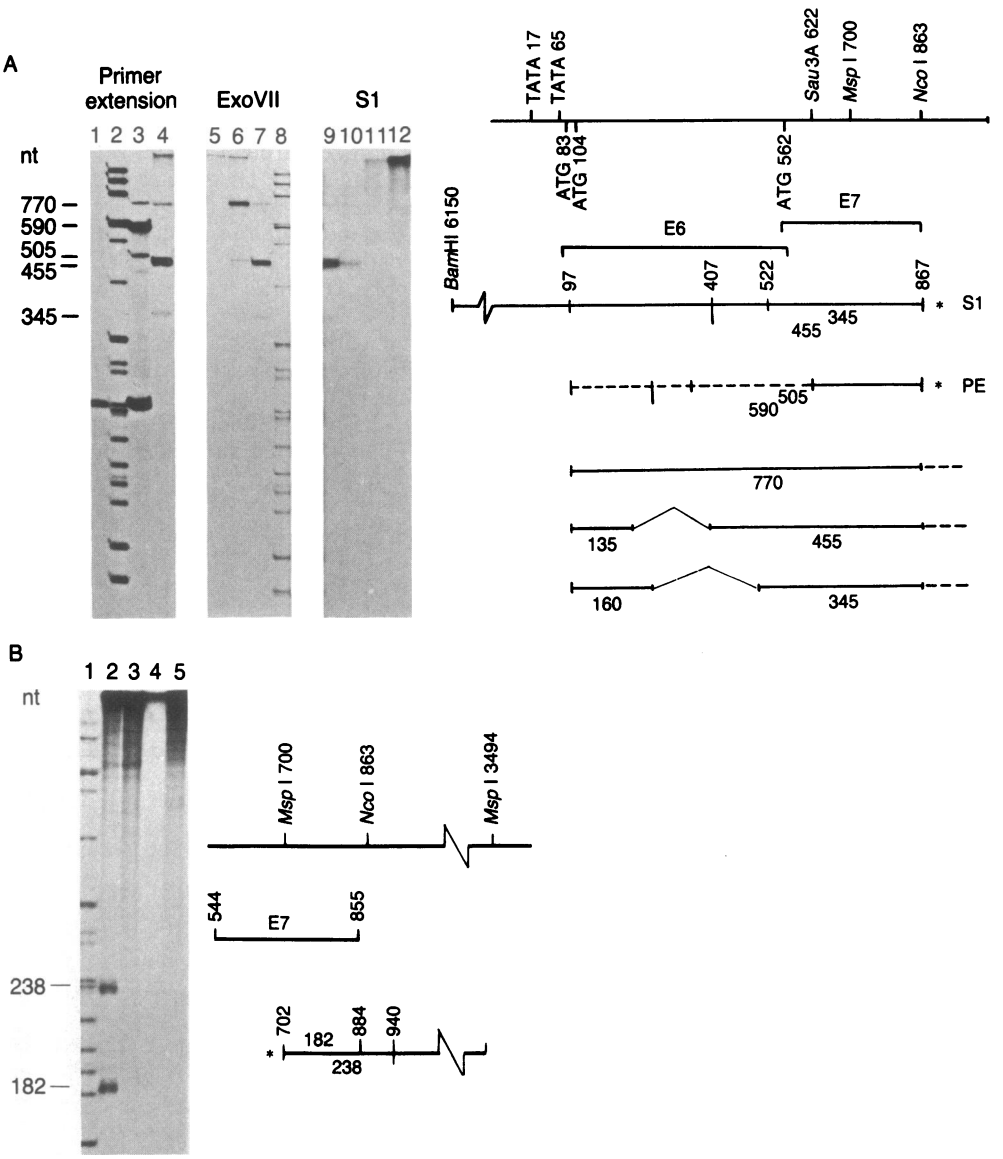

FIG. 3. Mapping of E6 and E7 coding exons. (A) Probes labeled 5′ at the *Nco* I site and extending to the *Bam*HI site were employed in S1 and ExoVII digestions, and the primer for the extension ended at the *Sau*3A site. Primer-extension panel: primer (lane 1), molecular mass markers (lane 2), primer-extension products (lane 3), and S1 digest of CaSki cell RNA (lane 4). The positions and sizes of primer-extension bands of 505, 590, and 770 nucleotides (nt) and of the S1 digestion bands of 345, 455, and 770 nucleotides are indicated. ExoVII panel: ExoVII digests with tRNA (lane 5) and with CaSki cell RNA (lane 6) and S1 digest of CaSki cell RNA (lane 7) and molecular mass markers (lane 8). S1 panel: S1 digests with CaSki cell RNA (lane 9), tumor 6 RNA (lane 10) and tRNA (lane 11), and S1 DNA probe (lane 12). The molecular mass markers are *Hae* III fragments of ϕX174 replicative form and of pBR322 DNA digested with *Msp* I. The sizes in nucleotides from top to bottom are 1353, 1078, 872, 622/603, 527, 403, 310/309, 281, 271, 243, 238, 217, 201, 190, 180, 160, and 147. The interpretation of the results is shown on the right: top, partial map of the HPV-16 DNA and location of E6 and E7; middle, S1 digestion and primer-extension products; and bottom, mapping of the 5′ end portion of the transcripts. (B) Probes labeled 3′ at the *Msp* I site (map position 700) and extending to the *Msp* I site map position 3494 were employed to map the 3′ end of the E6–E7 exons. The sizes in nucleotides and positions of the S1 bands are indicated on the left. Lane 1, molecular mass markers as indicated under A except that the first three bands are weak and the 147-nucleotide marker is not shown; lanes 2 and 3, S1 digests with CaSki cell RNA (lane 2) and tumor 6 RNA (lane 3); lane 4, 3′ labeled probe; lane 5, S1 digest with tRNA. A segment of the HPV-16 map and the location of E7 are shown on the top right and the interpretation of the S1 digests is shown on the bottom right.

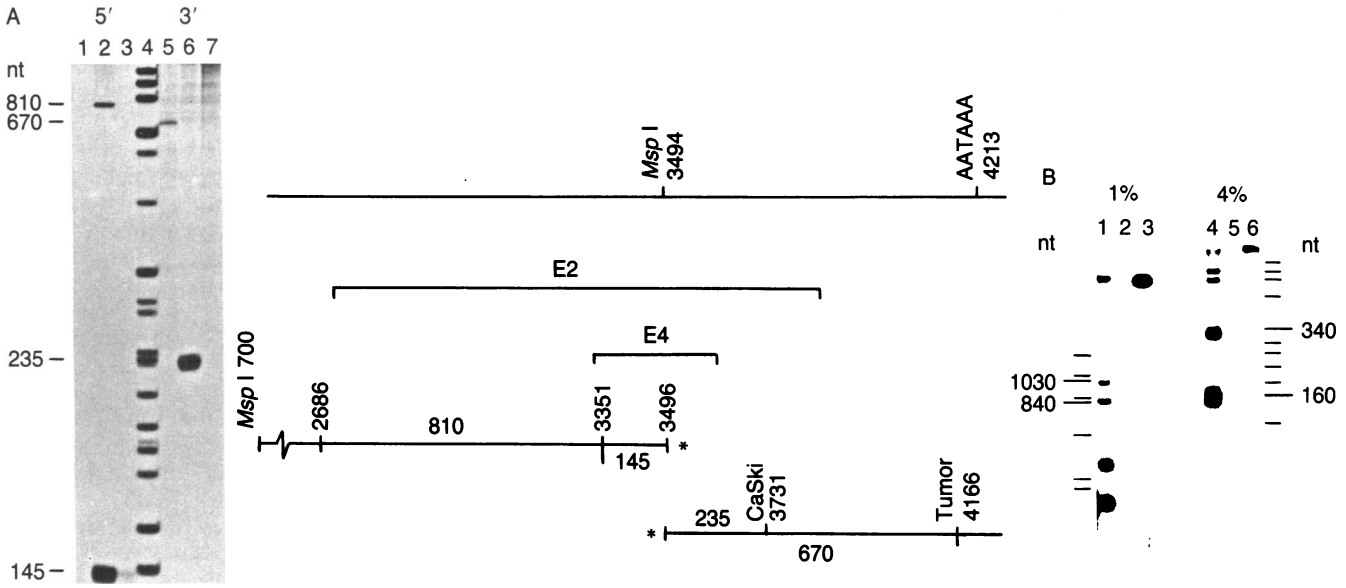

FIG. 4. Mapping of the noncoding and E2-coding downstream exons and linkage to E6–E7-coding exons. (*A*) PAGE analyses are shown on the left. The 5′ labeled probe: S1 digests with tRNA (lane 1), CaSki cell RNA (lane 2), and tumor 6 RNA (lane 3). Molecular mass markers are as described in the legend to Fig. 3*A* (lane 4). The 3′ labeled probe: S1 digests with tumor 6 RNA (lane 5), CaSki cell RNA (lane 6), and tRNA (lane 7). The positions and sizes in nucleotides of the S1 bands are given on the left. Shown on the right, from the top, are a segment of the HPV-16 map, positions of E2 and E4, and interpretation of the S1 digests with 5′ and 3′ labeled probes. (*B*) One percent and 4% neutral agarose gel analysis of S1 digests of 5′ labeled probe hybridized to CaSki cell RNA (lanes 1 and 4) and tRNA (lanes 2 and 5) and of DNA probe (lanes 3 and 6). The numbers indicate the positions and sizes of the S1 bands. The positions of molecular mass markers (ϕX174 replicative form digested with *Hae* III) are indicated on the left and right; the sizes in nucleotides from top to bottom on both sides are 1353, 1078, 872, 603, 310, and 281/271; in addition, the positions of markers at 234, 194, and 118 nucleotides are given on the right.

CaSki cells maps to the end of the E4, but within E2, and in the tumor it maps downstream of E2. Thus, the transcripts in the tumor but not in the CaSki cells could code for an intact E2 protein. As shown by neutral agarose gel electrophoresis of S1 digests for the CaSki cells (Fig. 4*B*), both exons are linked to exons in the E6–E7 region. The bands of 160 and 840 nucleotides are of similar size as the S1 bands in denaturing gels (Fig. 4*A*) and are probably generated by a cut through at the intron site. The size differences between the bands in the two double bands of 160 and 340 and 840 and 1030 nucleotides are 180 and 190 nucleotides, respectively. This size is in good agreement with the expected size of the fragment from the *Msp* I site at nucleotide 700 to the end of the E6–E7 exons. The relatively broad bands may indicate that exons ending at nucleotides 884 and 940 are involved.

The mapping experiments thus have identified in CaSki cells and in the tumor a major RNA with a coding capacity for E7 and a minor one with a coding capacity for E6. In the tumor there is also a minor RNA that could code for a complete E2.

Identification of the E7 Protein. High-titer antisera to E7 obtained by immunizing rabbits with the tryp E–E7 protein were employed in immunoprecipitations of protein from labeled extracts of HPV-16-positive and -negative cells (Fig. 5). A protein band with an apparent molecular mass of about 20 kDa was precipitated with the immune serum (Fig. 5, lane 7) but not with the preimmune serum (Fig. 5, lanes 3 and 6) or serum blocked with the tryp E–E7 fusion protein (Fig. 5, lanes 2 and 5). The protein was also precipitated from extracts of the HPV-16-positive SiHa cells (Fig. 5, lane 7) but not from the HPV-16- and HPV-18-negative cervical cancer-derived cell lines HT-3 (Fig. 5, lane 16) and G-33A (not shown) and the HPV-18-positive HeLa cells (not shown). Furthermore, the protein is not present in the tissue culture supernatant (Fig. 5, lane 8). These data indicate that the protein migrating at 20 kDa represents the E7 product. Its mobility is lower than anticipated from its size of 11 kDa (10), but this is not unexpected since the fusion protein also exhibited a lower than expected mobility.

DISCUSSION

We have mapped RNAs coding for HPV-16 early-region ORFs in the cervical cancer-derived CaSki cell line and in a recurrent cervical cancer and identified the E7 protein in the CaSki cells.

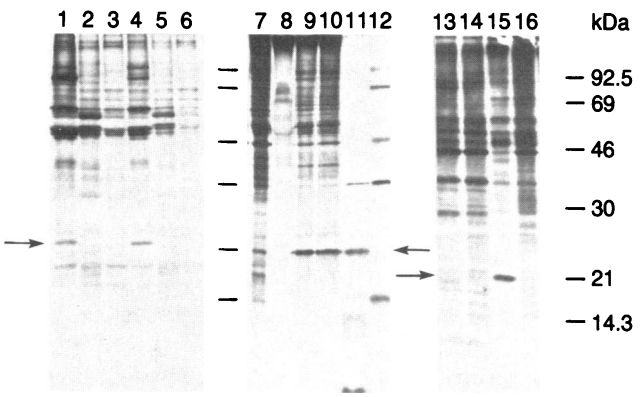

FIG. 5. Identification of the E7 protein in immunoprecipitates of ^{35}S-labeled CaSki cells. Twelve percent NaDodSO$_4$/PAGE of whole cell extracts from CaSki cells (lane 13) and HT-3 cells (lane 14); immunoprecipitates of CaSki cell extracts (lanes 1–6, 9, 10, and 15), of SiHa cells (HPV-16 positive) (lane 7), and of HT-3 cells (HPV-16 negative) (lane 16), and tissue culture supernatant of CaSki cells (lane 8). ^{14}C-labeled molecular mass markers were present in lanes 11 and 12. The antisera employed in the immunoprecipitations were as follows: 1:17 dilutions of immune serum, of immune serum blocked with E7 fusion protein, and preimmune serum, lanes 1, 2, and 3, respectively; the same sera at 1:50 dilutions, lanes 4, 5, and 6, respectively; 1:100 dilutions of immune serum, lanes 7, 8, 9, 15, and 16; and a 1:200 dilution, lane 10. The positions of the molecular mass markers are indicated for the left and middle panels between the two panels and for the right panel on the right, where the size of the marker is indicated in kDa. The arrows point to the position of the E7 protein.

The CaSki cells contain several hundred gene copies of integrated HPV-16 DNA, a state of the DNA characteristic for cervical cancers and different from that of precancerous lesions, in which HPV-16 DNA is maintained as a plasmid (9). In this regard, the recurrent cervical cancer analyzed and a lung metastasis from the same patient were unusual since they contain predominantly plasmid DNA. With cottontail rabbit papillomavirus (CRPV), where papillomas frequently progress to malignancies within about 1 year, integrated CRPV DNA is only present in a minority of malignant tumors (12). However, in the two CRPV-induced transplantable carcinomas Vx2 and Vx7 maintained for >30 years by serial propagation, only integrated viral DNA is present (22, 23). Since the latent period for the appearance of malignant tumors in humans is much longer than in rabbits, these results could indicate that, in general over a period of many years, maintenance of the viral genome may only be ascertained if the DNA is integrated. Alternatively, DNA integration *per se* could be causally connected to tumor progression with HPV-16.

In CaSki cells and tumor, poly(A)$^+$ RNA predominantly maps to the early region and it is clear that the major RNAs in CaSki cells contain only early-region sequences; these findings are similar to those obtained with cervical cancer-derived HPV-18-containing cell lines (5). Our data indicate that the longest exon of the E6–E7 region mapped would code for a slightly truncated E6 protein and this is similar to the situation in CRPV (11, 24). In CaSki cells the major and one minor transcript have introns within E6. Assuming that the splice donors and acceptors utilized are those close to the mapped exon ends, it can be determined that translation from the first AUG (the second in E6) could yield two small proteins.

Surprisingly, the 3′ ends of the downstream exons, noncoding or coding for E2, terminate upstream of the poly(A) site. Analyses of restriction enzyme-digested DNA revealed in CaSki cell and tumor a major normal-sized *Msp* I–*Dra* II fragment of 1034 base pairs containing the poly(A) site (data not shown; see Fig. 2 for the map). This finding, together with the fact that the 3′ ends do not map to splice donors, suggests that the viral transcripts continue into cellular sequences. In agreement with this notion are also the sizes of transcripts; the sizes of the viral sequences of the major transcript mapped add up to 0.97 kb for the CaSki cell and 1.4 kb for the cervical cancer. This is shorter than the sizes determined on RNA transfer blots of 1.5 and 2.0 kb, respectively (Fig. 2) even if a correction is made for the poly(A) tails. This interpretation would then also imply that the viral genomes giving rise to the defined poly(A)$^+$ transcripts represent a minority of the genomes present; particularly, in the tumor they appear to be represented by the small fraction of integrated genomes. The presence of a sequence disparity at the presumed 3′ exon end between probe and transcript appears unlikely since ExoVII digests gave the same signal (data not shown) as the S1 digests (Fig. 4*A*). One possible explanation could be that with HPV-16 but not CRPV at some stage of the cells' pathway to malignancy HPV-16 regulatory elements may become inoperable and only viral genes under control of cellular control elements may be transcribed or processed.

The potential for the synthesis of an intact E2 protein in the cancer may be causally related to the maintenance of HPV-16 plasmids. As shown with BPV-1 (25), the E2 product appears to be required in *trans* to activate transcription from a viral promoter, and this may be crucial for the synthesis of E1 required for plasmid maintenance (26). In agreement with this are findings with HPV-18-containing cancer-derived cell lines that have only integrated DNA and in which integration appears to interrupt E2 (5).

The identification of early papillomavirus proteins has been unsuccessful so far with the exception of the recent identification of E6 in BPV-1-transformed cells (27). Our data show that the most common transcript in CaSki cells and a cervical cancer codes for E7. This is surprising since genetic studies of E7 in BPV-1 indicate that its gene product is required to maintain plasmids at a high copy number (26) and E6 and E5 are involved in transformation of cells in culture (28–30). In CaSki cells, as well as in the CRPV-induced transplantable carcinoma Vx2, all viral DNA is integrated and in both the most abundant transcript codes for E7 (24, 31). This could suggest that E7 expression may have a pleiotropic effect, one directed toward maintaining a high plasmid copy number and another less-defined one related to progression toward malignancy and long-term maintenance of the malignant state.

Excellent technical and graphic assistance was provided by M. M. Tayag and we thank J. Martinez for preparing the manuscript. This investigation was supported by a grant from the Margaret E. Early Medical Research Trust and by Public Health Service Grant CA 18151 awarded by the National Cancer Institute.

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