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Human papillomavirus 16 E7 protein is associated with the nuclear matrix

(nuclear matrix protein/cervical carcinoma cells)

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ABSTRACT **The cellular localization of the human papillomavirus (HPV)-16 E7 gene product in the cell lines CaSki and SiHa has been determined by both biochemical and immunocytochemical methods. These measurements show E7 to be localized in the cell nucleus, specifically with the nonchromatin nuclear structure or nuclear matrix. This localization of E7 required an unambiguous fractionation of the nuclear constituents. This was achieved by using a gentle sequential fractionation procedure to prepare the scaffold consisting of the nuclear matrix and intermediate filaments (NM–IF). Chromatin was cleaved with nuclease and the resulting nucleosomes eluted with 0.25 M ammonium sulfate. Immunostaining of cells after this extraction procedure with monoclonal antibodies (mAbs) to E7 revealed a fine grained, punctate nuclear fluorescence in CaSki and SiHa, which was absent in normal cervical keratinocytes and the HPV-negative cell line C33.1. Western blots of cell fractions with these mAbs showed that E7 was localized in the NM–IF fraction in SiHa and CaSki but was not detected in HPV-negative cells. A second protein of slightly higher mobility is identified by these antisera in HPV-16-containing cells. The data suggest that the previous inability to directly visualize E7 by immunocytology is due to the masking of epitopes by cellular components and not to low levels of protein.**

The papillomaviruses are small DNA viruses that infect mucocutaneous surfaces where they induce benign epithelial proliferations or warts. Those viruses that infect the human anogenital mucosae are of particular interest because of persuasive evidence of an association between infection with these viruses and anogenital cancer (1, 2). The DNA sequences of human papillomavirus (HPV)-16 and to a lesser extent HPV-18, -31, -33, and -35 are found in at least 80% of cervical carcinomas and in high frequency in high-grade cervical intraepithelial lesions (3–6). HPV DNA has also been detected in cell lines derived from cervical carcinomas with SiHa and CaSki containing HPV-16 DNA and HeLa and C 41 containing HPV-18 DNA (7). Further evidence for an etiological role for these viruses in cervical carcinogenesis comes from their ability to immortalize genital keratinocytes *in vitro* (8–10).

Transcriptional analysis of infected carcinoma tissue and cell lines has revealed that transcription of the viral DNA is confined to the early region of the viral genome irrespective of whether this DNA is episomal or integrated (11, 12). The most abundant mRNA species encodes the E7 protein (11), which is the major transforming protein for HPV-16 and -18 (13–15). The E7 oncoprotein has been identified in both carcinoma biopsies and in the cell lines CaSki and SiHa by immunoprecipitation and was shown to be a 20-kDa phosphoprotein (11), apparently with a cytoplasmic locale (16). However, the functional properties of E7, which include immortalization (in association with E6) of keratinocytes (17–19), cooperation with ras in rodent cell transformation (14), and binding to the 105-kDa retinoblastoma gene product (20) suggest a nuclear location. Indeed, in cells in which E7 is expressed transiently from a simian virus 40-derived expression plasmid (21) or in cells infected with a recombinant E7-expressing vaccinia virus (22), a nuclear location for the protein can be shown by immunofluorescence. However, immunostaining has failed to detect E7 in cell lines or in biopsies in which the protein can be demonstrated by immunoprecipitation using anti-E7 monoclonal antibodies (mAbs) (23–25). The conclusion from such studies is that either the protein is present at concentrations too low for immunocytochemical recognition or the epitopes are masked by interaction with other cellular or viral components as has been shown in transient expression systems (26).

The nonchromatin structure of the cell nucleus, or nuclear matrix, is normally concealed beneath the much larger mass of chromatin. The nuclear matrix may be the scaffolding organizing that chromatin into regions of transcriptionally active euchromatin and compacted, inactive heterochromatin (27). The tenacity of chromatin attachment to the matrix resists most mild fractionation schemes. However, an *in situ* fractionation protocol has been developed (28, 29) that removes chromatin from the nucleus while preserving underlying matrix architecture. The protein composition of the matrix isolated this way varies with cell type and differentiation stage (30–32). The nuclear matrix has been identified as the site for important nuclear processes, including DNA replication (33–35) heterogeneous nuclear RNA processing (36, 37), and steroid hormone action (38–41). Several viral oncoproteins associate with the nuclear matrix such as the adenovirus E1A protein (42) and the simian virus 40 large tumor antigen (43). In addition, the trans-activating proteins of both the human T-cell leukemia virus type I and the human immunodeficiency virus type I associate with the nuclear matrix (44–46).

In view of these observations, we decided to examine the association of the E7 protein with nuclear proteins in HPV-16-containing cell lines by procedures that permit unambiguous fractionation of the nuclear constituents (29, 30). We report that both biochemical and immunocytological assays show that in the cervical carcinoma lines SiHa and CaSki HPV-16 is localized in the cell nucleus where it is tightly associated with the nuclear matrix. Furthermore, it is probable that the previous inability to demonstrate E7 by immunocytological means is due to the masking of epitopes by cellular components.

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Abbreviations: HPV, human papillomavirus; mAb, monoclonal antibody; ECL, enhanced chemiluminescence; NM–IF, nuclear matrix–intermediate filament(s).

MATERIALS AND METHODS

Cell Culture. All the cell lines used were of human origin. SiHa, CaSki, and C33.1 (American Type Culture Collection) were all derived from cervical carcinomas. SiHa and CaSki contain HPV-16 DNA; C33.1 is HPV negative. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS) (Flow Laboratories) in a humidified atmosphere of 5% CO_2/ 95% air. Normal cervical keratinocytes were cultured according to published methods (47) and grown in DMEM supplemented with 10% FBS, hydrocortisone (0.1 μg/ml), 10^{-10} M cholera toxin (Sigma), and epidermal growth factor (10 ng/ml).

Extraction Methods. These methods were essentially as described by He *et al.* (29). Cells were sequentially extracted in a series of buffers containing first the detergent Triton X-100 (Sigma) and then a mixed detergent containing Tween 20 (Sigma) and deoxycholic acid (Sigma). The intranucleosomal DNA was then digested with DNase I (Boehringer Mannheim). This was then followed by extraction with 0.25 M ammonium sulfate. The remaining pellet was finally extracted with 2 M NaCl.

Immunocytochemistry. Cells were grown to subconfluence on glass coverslips (Chance) and then sequentially extracted as described above. After extraction, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline and stained by indirect immunofluorescence. The antibodies used were Camvir 3 and 4, mouse mAbs raised against an HPV-16 E7 fusion protein (25), biotinylated anti-mouse IgG (Sera-Lab, Crawley Down, Sussex, U.K.), and streptavidin/ tetramethylrhodamine B isothiocyanate (Sera-Lab).

Electrophoresis and Western Blotting. One-dimensional 15% polyacrylamide gels were prepared according to published methods (48, 49). Proteins were transferred to nitrocellulose paper by the method of Towbin *et al.* (50). The blots underwent reaction with the primary antibody and a mixture of the mAbs Camvir 3 and 4 in equimolar concentrations and at a dilution of 1:10. The blots subsequently underwent reaction with a secondary biotinylated anti-mouse IgG (Becton Dickinson) at 1:1000 dilution, incubated with Avidin peroxidase (Dakoplatts, Glostrup, Denmark) at 1:500 dilution, and proteins were visualized after a 2-min exposure on x-ray film after reaction with enhanced chemiluminescence (ECL) reagents (Amersham).

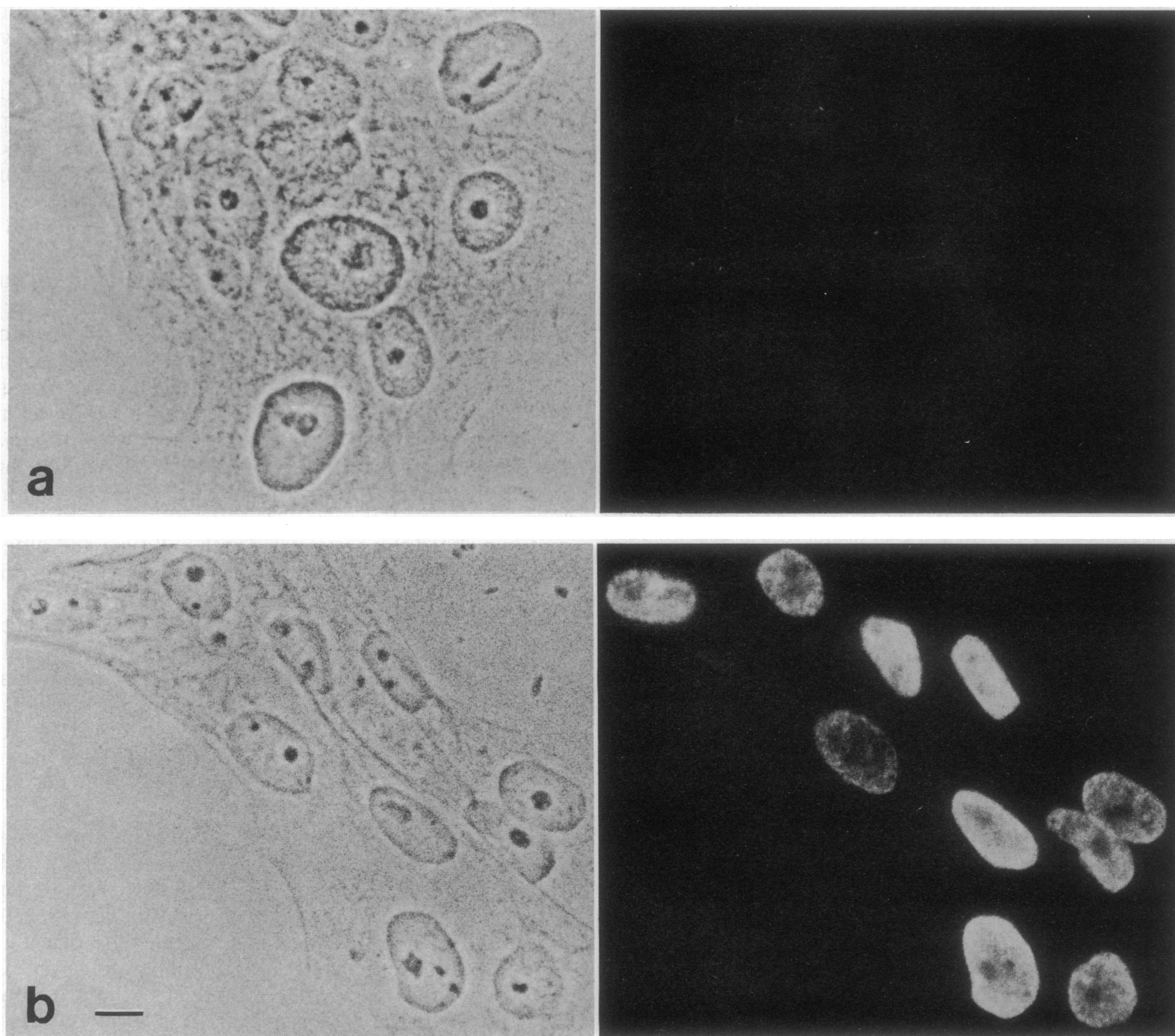

FIG. 1. (*Right*) Immunofluorescent staining of CaSki cells with a mixture of anti-E7 mAbs. CaSki cells were sequentially extracted, fixed at different stages in the procedure, and stained with a mixture of the Camvir 3 and 4 mAbs. Both immunofluorescence micrographs were photographed at the same magnification with the same exposure time and were printed identically. (*Left*) Corresponding phase-contrast micrographs. (*a*) Cells were extracted with 0.5% Triton X-100 before fixation. No staining above background was observed. (*b*) Chromatin was removed from Triton X-100-extracted cells by digestion with RNase-free DNase I and extraction with 0.25 M ammonium sulfate. A finely punctate nuclear fluorescence was seen. (Bar = 10 μm.)

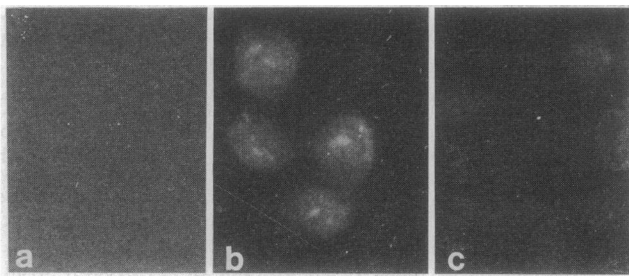

FIG. 2. Immunofluorescence micrographs of normal cervical keratinocytes (*a*), SiHa cells (*b*), and C33.1 cells (*c*) after sequential extraction with Triton X-100, Tween/deoxycholic acid, and then digestion with DNase I and extraction with 0.25 M ammonium sulfate. Positive fluorescent staining is seen only in SiHa cells (*b*), and this staining is confined to the nucleus.

RESULTS

Fig. 1 shows the results of immunofluorescent staining of CaSki cells with a mixture of the mAbs Camvir 3 and 4 using the sequential extraction protocol on cells *in situ*. Background staining only can be seen in Fig. 1*a*, but in Fig. 1*b*, which shows cells after digestion with DNase I and extraction with 0.25 M ammonium sulfate, a fine grained, punctate nuclear fluorescence is seen. This nuclear fluorescence is abolished by extraction with 2 M NaCl (data not shown), a procedure that removes most nuclear matrix proteins. In Fig. 2, the results of immunostaining SiHa, C33.1, and normal cervical keratinocytes with Camvir 3 and 4 after sequential Triton X-100, Tween/deoxycholic acid extraction, and then DNase I digestion and 0.25 M ammonium sulfate extraction are shown. No staining above background is evident in C33.1 (HPV-negative cervical carcinoma cells) or normal cervical keratinocytes, but nuclear staining is seen in SiHa. No staining above background was seen at any extraction stage when cells were stained with the mAb LP6, a mAb that recognizes the major capsid protein of herpes simplex virus type 1 (data not shown).

The results of Western blot analysis on sequential extracts of CaSki cells using a mixture of the mAbs Camvir 3 and 4 are shown in Fig. 3. The bands between 40 and 84 kDa appear to be artifacts of the ECL procedure. Reactivity with E7 is seen only in lane 4, the lane containing an extract of the pellet remaining after treatment with RNase-free DNase I and 0.25 M ammonium sulfate. In this lane, which consists predominantly of nuclear matrix proteins, a doublet, the upper band of which represents a protein of ≈20 kDa, is evident. The doublet containing the 20-kDa band is absent in lane 5, an extract of the pellet remaining after the removal of nuclear matrix proteins with 2 M NaCl. In a further set of experiments, a range of cell types, which included both HPV-16-positive and -negative lines, were sequentially extracted, the nuclear matrix and intermediate filament (NM–IF) fraction was analyzed on 15% gels and reacted with the mAbs Camvir 3 and 4, and Western blotting was performed with ECL. The results of this are shown in Fig. 4; reactivity is shown in lanes 2–4 (extracts of SiHa and CaSki), where a doublet of the appropriate molecular mass is again identified. No reactivity is found in lanes 1 and 5, which are extracts of normal cervical keratinocytes and C33.1, respectively.

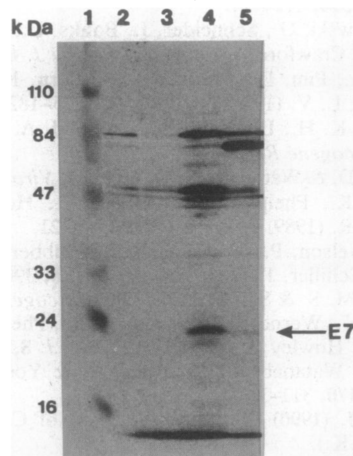

FIG. 3. Western blot analysis of CaSki cell extracts obtained after sequential extraction. Proteins were separated by PAGE on 15% acrylamide gels, transferred to nitrocellulose paper, reacted with a mixture of mAbs Camvir 3 and 4, followed by reaction with a biotinylated secondary anti-mouse IgG antibody and reaction with avidin peroxidase and visualization of proteins after reaction with ECL reagents (Amersham). The bands between 40 and 84 kDa appear to be artifacts of the ECL procedure since they appear when probing with antibodies (e.g., anti-L1) for which no antigen is present. Lanes: 1, molecular size markers; 2, proteins remaining in the pellet after extraction with Triton X-100; 3, proteins remaining in the pellet after Triton X-100 extraction followed by Tween/deoxycholic acid extraction; 4, proteins remaining in the pellet after sequential extraction as for lane 3 followed by digestion with DNase I and extraction with 0.25 M ammonium sulfate to remove chromatin; 5, proteins remaining in the pellet after Triton X-100 and Tween/deoxycholic acid extraction, digestion with DNase I, and extraction with 0.25 M ammonium sulfate to remove chromatin as in lane 4, followed by further extraction with 2 M NaCl to remove the outer matrix proteins. A protein band of ≈20 kDa is shown only in lane 4, the extract obtained after chromatin removal. This band is lost in lane 5 after extraction *with* 2 M NaCl, a treatment that leaves only the core filaments of the nuclear matrix (29).

DISCUSSION

In this study, the results of both biochemical and immunocytological measurements show the HPV-16 E7 protein in SiHa and CaSki cells to be localized in the cell nucleus, specifically with the nonchromatin nuclear structure or nuclear matrix. The allocation of a nuclear location to E7 is not surprising, since E7 is the major transforming protein (13–15) of the oncogenic HPVs and binds the 105-kDa retinoblastoma protein (20). Furthermore, in studies in which E7 is expressed by using prokaryotic expression vectors (22) or in yeast expression systems (21), it has consistently been identified as a nuclear protein. However, verification of this location in cells [other than transient expression systems (29)] or tissues

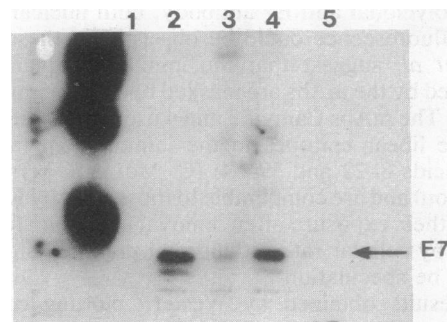

FIG. 4. Western blot analysis of the proteins remaining in cell extracts after sequential extraction with Triton X-100 and Tween/deoxycholic acid, followed by digestion with DNase I and extraction with 0.25 M ammonium sulfate. Proteins were separated by PAGE on 15% acrylamide gels, transferred to nitrocellulose paper, reacted with the mAbs Camvir 3 and 4, followed by visualization with ECL. Lanes: 1, normal cervical keratinocytes; 2, SiHa cells; 3 and 4, CaSki cells; 5, C33.1 cells. A protein band of the appropriate mobility is shown only in lanes 3–5, which are extracts of cells containing HPV-16. The HPV-negative normal keratinocytes and C33.1 carcinoma cells do not show reactivity.

has, until the present study, proved elusive. Direct visualization by immunocytochemistry using both polyclonal and monoclonal antisera has not been achieved before in cells in which the viral genome is stably integrated and expressed, even though the protein is present at 0.01% in cell lines, a level that should be detectable immunocytochemically.

The nuclear localization of E7 demonstrated in the present study required an unambiguous fractionation of the nuclear constituents and we used the method of He *et al.* (29) to prepare the scaffold consisting of NM–IF. Chromatin is first cleaved with nuclease and the resulting nucleosomes eluted with 0.25 M ammonium sulfate. This moderate ionic strength effectively removes chromatin and other contaminating constituents but appears to leave the NM–IF biochemically complete and, most important for immunostaining, morphologically intact. The NM–IF consists of the intermediate filaments, in much of their original conformation, anchored to the nuclear shell or lamina, which surrounds the interior thick polymorphic fibers of the nuclear matrix. These interior fibers contain a number of cell type-specific proteins (30). The complete nuclear matrix can be further fractionated by extraction with 2 M NaCl, a treatment that removes the outer nuclear matrix proteins uncovering a fibrogranular network of anastomosing 9–13 core filaments (29). This core filament has a simpler protein composition than the complete nuclear matrix and retains 70% of the nuclear RNA. The E7 protein, like most nuclear matrix proteins, was removed from the complete matrix by 2 M NaCl extraction and was not retained with the core filaments. Previous studies have localized the viral oncoproteins simian virus 40 large tumor antigen and adenovirus E1a (which have many properties in common with E7) to a nuclear matrix structure prepared with high salt (52, 53). In the present work, HPV-16 E7 is localized on a nuclear matrix prepared with moderate salt but removed by higher salt concentrations.

It is of considerable interest that immunocytochemical localization of E7 is achieved after the structure of chromatin is disrupted with DNase I treatment and the proteins associated with intact DNA are removed by the moderate salt treatment, which strongly suggests that the previous inability to visualize the protein was due to the masking of epitopes by cellular components. These data are supported by observations made by Kanda *et al.* (26), who showed that the E7 protein expressed transiently in COS-1 cells could not be identified as a nuclear protein by immunocytochemistry after staining with mAbs recognizing epitopes in two immunodominant regions of the protein. In contrast, after staining with a mouse polyclonal anti-E7 antibody, both nuclear and cytoplasmic fluorescence could be observed in these cells and Kanda *et al.* suggest that the immunodominant epitopes recognized by the mAbs are masked by either viral or cellular proteins. The mAbs Camvir 3 and 4 used in the present study recognize linear epitopes in the immunodominant regions (amino acids 8–22 and 39–54) (C. McLean, personal communication) and are comparable to those used by Kanda *et al.* (26) and their exposure after removal of chromatin suggests masking by cellular rather than viral proteins, although this can only be speculation.

The results obtained by Western blotting confirm the immunocytochemical data but it is of interest that a second protein of slightly higher mobility than E7 was consistently detected by the mAbs Camvir 3 and 4 in the NM–IF extract of SiHa and CaSki and has also been detected in rodent cells transformed by HPV-16 (I.G., unpublished data). The existence of a similar protein immunoprecipitated by anti-E7 antiserum has been reported in studies in which HPV-16 E7 is expressed in the fission yeast *Schizosaccharomyces pombe* (51). The identity of this protein is unknown and can only be a matter for speculation; it may be due to processing of E7 or to the presence of a protein coprecipitating with E7. Clearly, in view of the transforming properties of E7, the identity of cellular proteins, particularly nuclear proteins associating with the viral protein, is of considerable interest.

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