

Herpesvirus-specific RNA and protein in carcinoma of the uterine cervix

(cloned DNA/*in situ* hybridization/immunoperoxidase staining/DNA-binding protein)

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ABSTRACT Cloned probes of herpes simplex virus type 2 DNA were used in cytological hybridization experiments to detect herpesvirus RNA transcripts in the neoplastic cells of tumors of the uterine cervix. Virus-specific RNA was shown to represent transcription of limited regions of the genome, of which one is known to code for a DNA-binding protein that can be found by immunoperoxidase staining in the neoplastic cells of these tumors and has also been detected in cells transformed *in vitro* by this virus.

The thesis that the continuum of disease leading to the development of uterine cervical carcinoma is associated with prior infection by herpes simplex virus type 2 (HSV-2) (1-5) has recently been tested at the molecular level by the use of *in situ* cytological hybridization (6-9). The use of ³H-labeled viral DNAs as probes to detect virus-specific RNA (10, 11) in cervical tissue has resulted in general agreement, based on published reports, that at least 30% of all cervical intraepithelial neoplasia (CIN) and cervical carcinoma tissues contain cells that bind a HSV-2 [³H]DNA probe but not probes representing other viral DNAs, indicating the presence of HSV-specific RNA (7-9). The majority of these *in situ* hybridizations have been conducted with whole genomic DNA as the probe. In this report we summarize our studies using cloned subgenomic fragments of HSV-2 DNA as hybridization probes to define regions of the virus genome from which the detected RNA species are transcribed and correlate the findings with attempts to identify viral antigen(s) expressed in cervical carcinoma cells.

There have been numerous reports that antibodies to HSV-specific proteins are found more frequently, or at higher titer, in women with CIN or cervical carcinoma than in controls (12-14) and that HSV-specific antigens can be detected in the neoplastic cells by immunofluorescent or other methods (15-17). Among the abundance of sometimes confusing data (18, 19), two series of recent reports were of interest with respect to our *in situ* hybridization results. First, antiserum to the major HSV-2 binding protein (20) was shown to react with human cervical carcinoma cells in two separate studies (21, 22). The tumor antigens of other DNA viruses have been shown, without exception, to have DNA-binding properties (23), and a herpes simplex tumor antigen might be expected to exhibit a similar function. Second, it was demonstrated that sera from patients with cervical carcinoma precipitated two HSV-2 polypeptides (with molecular weights 38,000 and 118,000) more frequently than did sera from controls (24).

The 38,000-dalton protein was of interest because it has been shown that a protein of this size is encoded within *Bgl* II frag-

ment N of HSV-2 DNA (25, 26), which has been reported to have transforming activity in rodent cells (27, 28).

Rodent cells transformed *in vitro* by HSV-2 have been reported to express a number of HSV-specific antigens including VP143 (29), ICP10 (30), thymidine kinase (31, 32), membrane glycoproteins *gA/gB* (33, 34), and CP-1 (35). We therefore examined the HSV-2 transformants available in this laboratory in parallel with specimens of cervical carcinoma in an attempt to find a protein universally present in cells transformed *in vitro* and possibly *in vivo* by HSV-2.

MATERIALS AND METHODS

Cells and Viruses. The properties of the transformed cells used in this study have been described (11, 28, 36). Cells were cultured in an atmosphere of 10% CO₂/90% air in Dulbecco's modification of Eagle's medium (Bio-Rad) with 10% fetal calf serum (Reheis, Kankakee, IL) on plastic tissue culture plates (Falcon). HSV-2 strain 333 was propagated on monolayers of BHK-21 cells infected at low multiplicity (0.01 plaque-forming unit per cell). Viral DNA was isolated by equilibrium gradient centrifugation through sodium iodide gradients containing ethidium bromide (37).

Human Uterine Cervical Tissues. Human cervical tissue was obtained during colposcopic examination from patients who had been referred for the evaluation of abnormal Papanicolaou smears. Biopsy specimens were taken from those areas most likely to contain cervical cancer precursors as identified by colposcopy. The specimens were frozen in a cryostat, the diagnosis was confirmed by examination of frozen sections, and permanent diagnostic sections were prepared. The tissue remaining after the diagnostic evaluation was stored at -70°C until further sections were prepared. Benign cervical tissue from normal-appearing cervical transformation zones was also obtained from hysterectomy specimens of patients whose uteri were removed for other reasons. These were snap-frozen and stored at -70°C.

The cervical tissues were divided into three categories: (i) benign (including squamous metaplasia), (ii) CIN (dysplasia and carcinoma *in situ*), and (iii) invasive cancer. The tissues obtained that did not include the transformation zone were also subjected to the hybridization studies despite the fact that cervical squamous cell neoplasia generally does not arise in this region. The biopsies were interpreted as benign if the squamous epithelium matured in a normal fashion or if they contained the immature cells of squamous metaplasia at the surface or in the endocervical glands. The CIN lesions were classified by using the criteria advocated by Richart (38): CIN I, mild and moderate dysplasia;

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Abbreviations: HSV-2, herpes simplex virus type 2; CIN, cervical intraepithelial neoplasia; NaCl/Cit, standard saline citrate (0.15 M NaCl/0.015 M sodium citrate, pH 7).

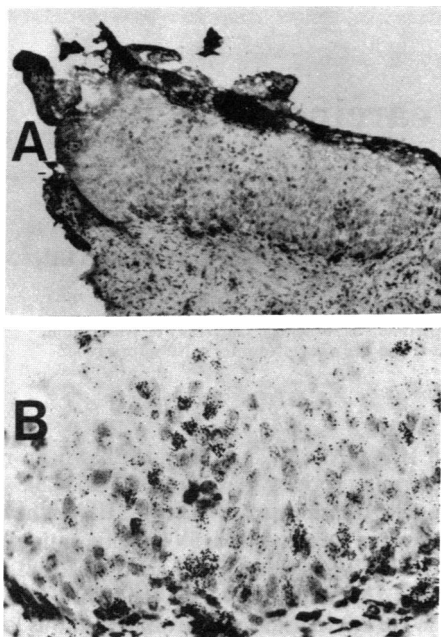


FIG. 1. *In situ* hybridization of ^3H -labeled, nick-translated pDG304 DNA to frozen section of cervical intraepithelial neoplasia. (A) After hybridization and staining with Giemsa. ($\times 50$.) (B) Higher magnification of a portion of the epithelium, showing localization of grains over cells. ($\times 300$.)

CIN II, moderate and severe dysplasia; and CIN III, severe dysplasia and carcinoma *in situ*. Invasive carcinoma was diagnosed when the neoplastic cells penetrated through the basement membrane and invaded the underlying stroma.

Hybridization. The construction and characterization of the recombinant clones of HSV-2 DNA in plasmid pBR322 have been described (39). The introduction of ^3H -labeled nucleotides into cloned DNA for use as hybridization probes was carried out as described (40).

Frozen-sectioned biopsy tissues placed on glass slides were fixed and stored in ethanol at -20°C until required for hybridization. After the preparations were dried in vacuum desiccators

Table 1. *In situ* hybridization of subgenomic HSV-2 sequences to cervical intraepithelial and invasive neoplasia tissue samples

[^3H]DNA probe*	Map coordinates	% positive for HSV-specific RNA		
		CIN (n = 50)	Invasive carcinoma (n = 25)	Non-neoplastic (n = 50)
<i>Hind</i> III B	0.07–0.26	24	20	6
305	0.25–0.33	20	12	6
510	0.30–0.33	0	0	2
304	0.33–0.40	12	20	6
301	0.40–0.43	0	0	2
18	0.43–0.58	0	0	2
<i>Xba</i> I D	0.45–0.71	0	0	0
401	0.58–0.63	8	12	2
<i>Eco</i> RI K	0.64–0.72	0	0	0
203	0.73–0.77	0	0	0
201	0.82–0.85	30	32	8
102	0.89–0.92	0	0	6
HSV-2	0.00–1.0	38	32	12
CMV	0.00–1.0	0	0	2

* See Fig. 2; CMV, cytomegalovirus.

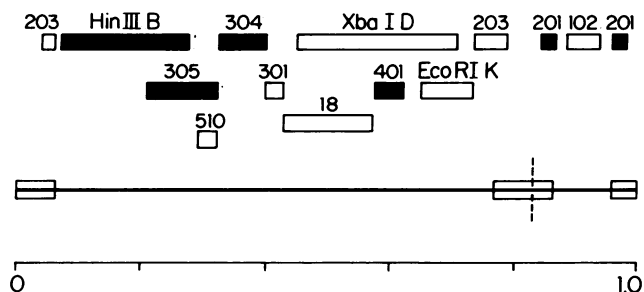


FIG. 2. Distribution of HSV-2 DNA probes used for hybridization. The solid boxes indicate positive results for HSV RNA.

to remove all traces of ethanol, 10 μl of probe was applied to each, and the drop was covered with a washed, silicone-treated coverslip. Hybridization was carried out in $6\times$ standard saline citrate (NaCl/Cit; $1\times$ NaCl/Cit = 0.15 M NaCl/0.015 M sodium citrate) at 68°C for 18 hr. After this period, the coverslips were removed and the slides were washed extensively in large volumes of $2\times$ NaCl/Cit at 4°C . Following dehydration in ethanol and under reduced pressure, the slides were dipped in Kodak NTB2 emulsion, diluted 1:1 with distilled water containing 1% glycerol, dried, and stored in light-tight boxes at 4°C for autoradiography. Preparations were developed in Kodak D19 and fixed in Kodafix. Cells and tissues were stained in 10% Giemsa, and the stained sections were examined for evidence of silver grains. Cells with 10 or more grains were considered positive when the background level was 1–3 grains per cell.

Antibodies and Immunoprecipitation. Hybridoma cell lines producing monoclonal antibodies were isolated by the method of Kohler and Milstein (41). Spleen cells from C57BL/6 mice infected with HSV-2 (333) were fused with BALB/c NSI myeloma cells by procedures described in detail elsewhere (41). Culture supernatants were assayed for anti-HSV antibodies by an antibody-binding assay (42) utilizing ^{125}I -labeled protein A against HSV-2-infected cell antigens. Three HSV-2-specific monoclonal antibodies were used in this study: one, antibody 6-A6, primarily precipitated a 140,000-dalton protein together with less preference for a 38,000-dalton protein; conversely, antibody 6-H11 primarily reacted with a 38,000-dalton protein and less so with a 140,000-dalton protein (26). Antiserum (monospecific) to ICSP 11/12 was kindly provided by K. L. Powell (University of Leeds, England); anti-VP143 was provided by R. J. Courtney (University of Tennessee).

Immunoprecipitation reaction mixtures consisted of 100 μl

Table 2. Immunoperoxidase and immunofluorescent staining with herpesvirus-specific antibodies

Antibody	CIN	Presence of herpesvirus-specific protein				
		Invasive carci-noma	Non-neo-plastic	HSV-2 infected	CMV infected	HSV-2 trans-formed
A6	0	0	0	+	0	0
H11	0	0	0	+	0	0
E12	0	0	0	+	0	0
ICSP 11/12	(+)*	(+) [†]	0	+	+	+
VP143	(+)*	(+) [†]	0	+	+	+
HSV-2	0	0	0	+	0	0

CMV, cytomegalovirus.

* Of 32 samples tested, 9 were positive (28%); of these, 6 were also positive for HSV-specific RNA after *in situ* hybridization with pDG304.

[†] Of 20 samples tested, 4 were positive (20%) and these 4 were positive with pDG304.

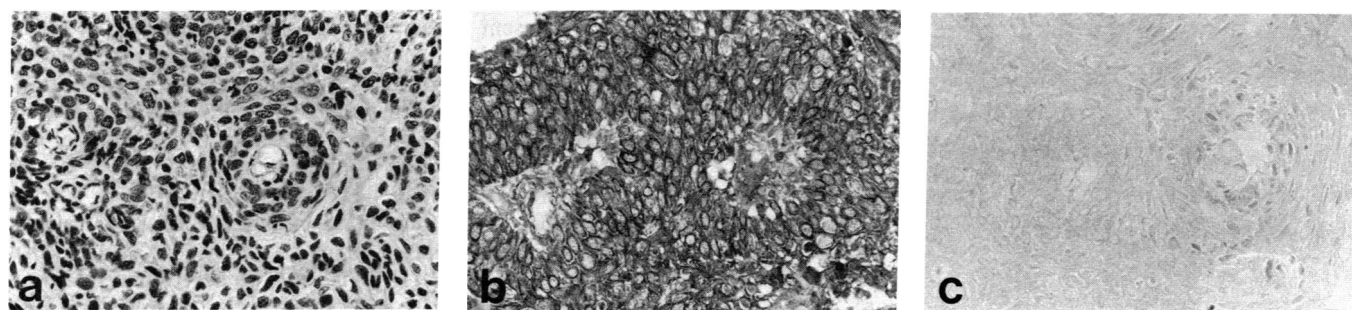


FIG. 3. Cervical intraepithelial neoplasia. Neoplastic epithelium obtained from endocervical curettage in a patient with high-grade CIN. (a) Specimen is characterized by sheets of neoplastic squamous cells with minimal discernible cytoplasmic maturation. (Hematoxylin and eosin; $\times 300$.) (b) Immunoperoxidase stain with anti-ICSP 11/12 of the same region as a illustrates extensive cytoplasmic staining of the neoplastic cells with relatively little nuclear staining. (c) Same region treated with antiserum to E12; no staining was found.

of ascites fluid used at 1:100 dilution, or a 1:2 dilution of monospecific antibodies, and 100 μ l of [35 S]methionine-labeled cell lysate incubated for 1 hr on ice. The reaction was terminated by incubation for 1 hr with 100 μ l of *Staphylococcus aureus* [formalin-fixed, 10% (vol/vol) in extraction buffer containing 0.1% ovalbumin] (43). The precipitate was collected by centrifugation in a Microfuge and the pellet was washed five times in 0.1 M Tris-HCl/0.5 M LiCl/1% 2-mercaptoethanol, pH 8.0 (44). The precipitated proteins and antibodies were solubilized in 0.05 M Tris-HCl, pH 7.0/2% (wt/vol) NaDodSO₄/5% 2-mercaptoethanol/0.055% bromophenol blue at 100°C for 2 min for analysis by electrophoresis in NaDodSO₄/9% polyacrylamide slab gels. Autoradiography was enhanced by fluorography using the commercial reagent EN³HANCE (New England Nuclear).

Immunofluorescence and Immunoperoxidase Staining. The methods used were as described (45).

RESULTS

HSV-Specific RNA in Cervical Tissues. Cloned and restriction endonuclease-derived fragments of HSV-2 DNA were hybridized to 75 frozen-sectioned biopsy specimens of neoplastic cervical tissue and carcinoma (Fig. 1). Another 50 samples of non-neoplastic cervical squamous epithelium and frozen sections from other tissues were hybridized with the HSV-2 probes as controls. Heterologous virus probes (simian virus 40, adenovirus 2, and bacteriophage λ) were hybridized to replicate sections to control for nonspecific binding of nick-translated DNA (7, 9). The results of the hybridizations with the HSV-2 probes only are shown in Table 1. Cases showing hybridization with one or more of the other ³H-labeled probes are not included.

Because these heterologous probes were never bound to specimens negative for HSV, our interpretation is that, in those specimens binding multiple heterologous probes, the result indicates a nonspecific interaction. This occurred in approximately 10% of the tissues examined. The distribution of positive hybridization results with HSV-2 DNA probes shows that the RNA species detected are limited to three regions of the virus genome: positions 0.07 to 0.4, 0.58 to 0.63, and 0.82 to 0.85 (Fig. 2). This last region is in the short repeat sequences and is therefore also represented at position 0.94 to 0.96 (46).

Herpesvirus-Specific Antigens. Transformed cells and cervical biopsy specimens were tested by immunofluorescence and immunoperoxidase methods for the presence of herpesvirus antigens. None of the three HSV-2-specific monoclonal antisera (A6, H11, and E12) gave positive staining reactions on transformed cells or with cervical tissues although they reacted with HSV-2-infected cells (Table 2). The monospecific antiserum ICSP 11/12 and antiserum VP143 were positive on virus-infected and transformed cells and on the neoplastic cells in some of the CIN and cervical carcinoma sections (Fig. 3; Table 2). The results found with the anti-VP143 and ICSP 11/12 antisera closely parallel the *in situ* hybridization results. When benign cervical tissue was examined, HSV antigens were occasionally seen in isolated mature squamous epithelial cells near the surface in the portio or transformation zone in a pattern suggesting an acute infection. In immature squamous metaplastic cells of the transformation zone, or in basal cell hyperplasia, the cells that were positive were in a basal location rather than a superficial one. Approximately 30% of the sections classified as CIN I, II, or III had detectable antigen, which was limited to the neoplastic cells, similar to the result found in vulvar intraepithelial neoplasia (Fig. 4), and approximately 20% of the invasive

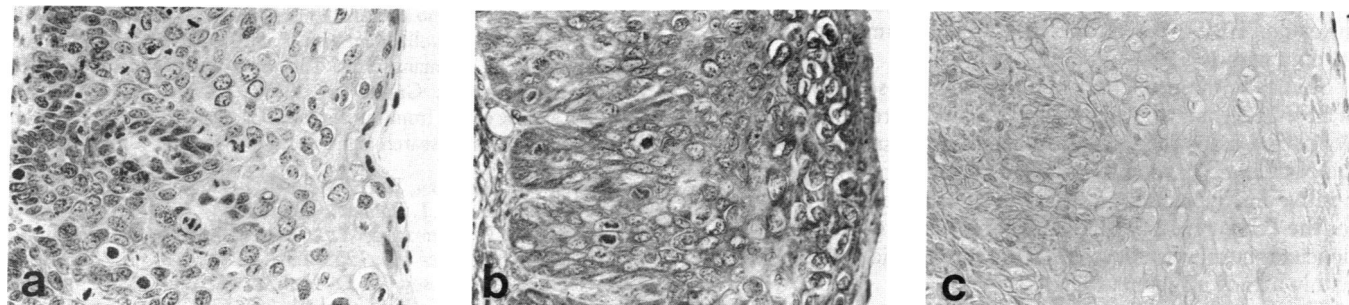


FIG. 4. Vulvar intraepithelial neoplasia. ($\times 300$.) (a) Grade II intraepithelial neoplasm characterized by numerous mitotic figures, nuclear pleomorphism, and hyperchromatism involving the entire thickness of the epithelium with discernible maturation in the upper third. There is an abnormal mitotic figure in the right mid-portion of the epithelium. (Hematoxylin and eosin.) (b) Immunoperoxidase staining with antiserum to ICSP 11/12 results in brown stain localized primarily in the cytoplasm of the cells in the neoplastic epithelium. (c) Immunoperoxidase reaction with antiserum to E12 demonstrates virtually no staining.

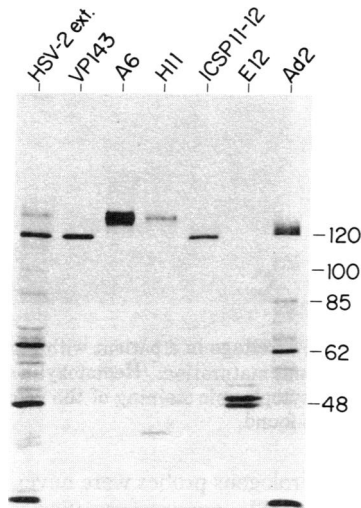


FIG. 5. Immunoprecipitation of HSV-2-infected cell proteins. A large number of infected cell-specific proteins can be seen by labeling BHK-21 cells infected with HSV-2 (333) with [35 S]methionine. This labeled extract was used in immunoprecipitation reactions with monospecific antisera made against VP143 and ICSP 11/12 and monoclonal antisera designated A6, H11, and E12. The *in vitro* translation products of mRNA from adenovirus 2-infected HeLa cells (a gift of J. Lewis) were used as molecular weight markers (shown $\times 10^{-5}$). The proteins were displayed on a 9% polyacrylamide gel with a DATD linker.

squamous cell tumors were positive. These results are in general agreement with those reported by Dreesman *et al.* (21). In each case, pre-absorption of the serum with HSV-2-infected cell lysates inhibited the staining reaction. The pattern and number of positive antiserum-antigen reactions in infected cells, *in vitro* transformed cells, and neoplastic cells indicated that the two antisera ICSP 11/12 and VP143 might be detecting the same protein. We therefore used these antisera in immunoprecipitation studies with extracts of HSV-2-infected cells. The two antisera precipitated a single band with identical electrophoretic mobility and an estimated molecular weight of 118,000 (Fig. 5), thus providing evidence for a single identity for ICSP 11/12, VP143, and ICP8 (20).

DISCUSSION

The results in this paper show that two of the three viral parameters sought in DNA virus-transformed cells have been identified in the same samples of neoplastic uterine cervical tissue. That at least in some instances the virus-specific RNA detected represents the information for a virus-specified protein can be deduced from the results after hybridization with the cloned HSV-2 DNA probe pDG304 and immunoperoxidase staining with ICSP 11/12.

Studies of the properties of a temperature-sensitive mutant of HSV-1 (47) have shown that ICP8, which corresponds to ICSP 11/12 (20), maps within the coordinates representing the HSV-2 DNA cloned in pDG304 (39). Thus, those tumor specimens positive for hybridization with the pDG304 cloned DNA and for the ICSP 11/12 (VP143; ICP8) protein provide direct evidence for herpesvirus-specific RNA and protein being encoded within a known region of DNA.

These results also provide some resolution to the difficulties presented by the use, in the literature, of different nomenclatures for the same major DNA-binding protein. The report by Flannery *et al.* (29) describing cytoplasmic and perinuclear staining with anti-VP143 antiserum in HSV-2-transformed rodent cells is now seen to be consistent with the finding that ICSP

11/12 is present in cervical carcinoma (21, 45) and in vaginal carcinoma (48) tissues and that antibodies to this protein were detected in the sera of cervical cancer patients by Anzai *et al.* (49). It may be argued that we have moved some way toward defining a role for HSV-2 in the etiology of this disease, but reflection shows that many questions remain.

The detection of ICSP 11/12 in neoplastic cells is not, by itself, sufficient to implicate HSV-2 because it has been shown that at least five different herpesviruses induce proteins that crossreact with ICSP 11/12 (50) and we have found that cytomegalovirus-infected human cells are also positive with antibody to ICSP 11/12. Cytomegalovirus RNA was not detected in the neoplastic tissues described in this paper.

The two regions of HSV-2 DNA described as containing transforming genes (27, 28, 30) map between positions 0.4 and 0.63 on the genome and do not contain the coding sequences for the major DNA-binding protein. Sequences mapping between 0.30 and 0.45 on the HSV-1 genome, which does include the ICP8 coding sequence (47), have been shown to have transforming ability (33), and DNA mapping within this region has been shown to persist in cells transformed by UV-inactivated HSV-2 (36, 51). Those transformants are positive by immunofluorescence for VP143 (ICP8; ICSP 11/12) (29). The *Bgl* II region N of HSV-2 also persists in these cells (36) but no evidence of the 38,000-dalton protein encoded by the *Bgl* II region N sequences was found in this study. Other experiments (26) have shown that monoclonal antibodies A6 and H11 react with two proteins translated *in vitro* from RNA selected with *Bgl* II fragment N of HSV-2. The coding sequences for these proteins have been shown to be partially co-linear and, for the larger protein, to be also encoded in part within *Bgl* II fragment C. Cells transformed *in vitro* with *Bgl* II N sequences alone do not appear to express the 38,000-dalton protein. It is possible that *Bgl* II region N products are present in amounts too low to be detected or that they may only be required for initiation. It is interesting that, even though cervical carcinoma cells are negative for this protein, it has been shown (24) that cervical cancer patients may have significantly increased levels of antibody to the 38,000-dalton protein.

Except for one report (52), attempts to detect HSV DNA in cervical carcinoma tissues have failed (53, 54). The demonstration that viral RNA and protein are present in about 30% of these cases has stimulated us to investigate more tumors at the DNA level by hybridization with the cloned DNA probes containing transforming sequences from HSV-1 and HSV-2. These segments of DNA provide greater sensitivity than was available for the earlier studies (55). Cloning of DNA from tumor tissues to provide libraries that can be screened (56) for herpesvirus sequences will also enable more sensitive detection of viral DNA.

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