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Detection of Herpes Simplex Virus Type 2 Glycoproteins Expressed in Virus-Transformed Rat Cells

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Rat embryo fibroblasts transformed by herpes simplex virus type 2 (HSV-2) were assayed for the expression of certain virus-specific glycoproteins on the surface membranes. Monospecific antisera to HSV-2–specific glycoproteins, designated gAgB, gC, and gX, were used in membrane immunofluorescence studies with HSV-2–transformed cell lines tREF-G-1, tREF-G-2, and a tumor-derived rat fibrosarcoma cell line produced in syngeneic rats inoculated with tREF-G-1 cells. Analysis of the three HSV-2–transformed cell lines showed that antisera to the gAgB and gX glycoproteins were reactive with these cells. In contrast, no significant reactivity was observed when anti-gC serum was reacted with the HSV-2-transformed cell lines. All three antiglycoprotein sera reacted positively with rat cells productively infected with HSV-2. Additionally, the HSV-2–transformed and tumor-derived cell lines showed positive internal immunofluorescence after reaction with antiserum to an early, nonstructural viral protein designated VP143 (molecular weight, 143,000). Infectivity of HSV-2 in standard plaque assays was neutralized by hyperimmune rat antisera to tREF-G-2 or rat fibrosarcoma cells and to HSV-2 virions and by sera from rats bearing the fibrosarcoma. Adsorption of rat anti–HSV-2 serum with tREF-G-2 or rat fibrosarcoma cells reduced neutralizing activity to 10 and 12%, respectively, compared with 90% neutralization by antiserum adsorbed with nontransformed rat embryo fibroblast cells and 100% neutralization with unadsorbed antiserum. In summary, HSV-2–transformed rat cells retained and expressed genetic information necessary for the production of HSV-2 glycoproteins and a nonstructural protein after high passage in tissue culture or in the syngeneic host.

Five herpes simplex virus (HSV)-specific glycoproteins, designated gA, gB, gC, gD, and gE (1, 27), have been detected on the viral envelope and the plasma membrane of HSV-infected cells. The presence of virus-specific antigens on the surface membranes of HSV-transformed cells has also been detected by immunofluorescence tests, using hyperimmune antisera to HSV (5, 6, 16, 18, 19, 21, 28). Also, the observation that sera of animals bearing tumors produced by HSV-transformed cells contain HSV-neutralizing antibodies (2, 5, 16) suggests that such cells synthesize HSV-specific glycoproteins. Reed et al. (24) have shown that HSV-transformed hamster cells express an antigen, designated CP1, which is probably equivalent to gD. Other glycoproteins expressed in transformed hamster cells may also be virus specific but have not yet been fully characterized (13, 14).

In this investigation, we attempted to determine whether certain rat cell lines transformed by HSV type 2 (HSV-2) express the high-molecular-weight, HSV-2–specific glycoproteins on the surface of the transformed cells. The unique approach employed in this study involved the use of monospecific antisera prepared to HSV-2 glycoproteins gAgB, gC, and gX. In addition, these transformed cells were also tested for reactivity to an antiserum to the nonstructural protein designated VP143 (molecular weight, 143,000).

MATERIALS AND METHODS

Cells and media. All cells were grown at 37°C in Eagle medium (7) supplemented with 10% fetal calf serum, 100 IU of penicillin and 100 μg of streptomycin per ml, 10% tryptose phosphate broth, and 0.075% $NaHCO_3$ (growth medium). For immunofluorescence experiments, cells were grown on cover slips in plastic petri dishes at 37°C in a humidified atmosphere of 5% CO_2 in growth medium.

Secondary fibroblasts obtained from 18-day-old rat embryos (syngeneic White Buffalo strain), two clones of HSV-2–transformed cell lines, designated tREF-G-1 (passage 50) and tREF-G-2 (passage 24), and a tumor-derived cell line rat fibrosarcoma (RFS; passage 40) were used in the membrane immunofluorescence stud-

ies. Transformation of the secondary rat embryo fibroblasts (REF; passage 7) was accomplished by infecting cells with photoinactivated HSV-2 (strain ANG) as described previously (17, 18).

Rabbit antisera. Antiserum against VP143, an early, nonstructural protein, was prepared as described previously (9). Hyperimmune rabbit antisera to HSV-2 glycoprotein components were prepared as described for the HSV-1 glycoproteins (8). Cells were infected at a multiplicity of 10 PFU per cell with the 186 strain of HSV-2 and harvested at 24 h postinfection, and glycoproteins were solubilized with 1% sodium deoxycholate and 1% Tween 40 for 1 h at 37°C. This solubilized extract was centrifuged at 100,000 × *g* for 1 h. The glycoprotein-containing supernatant was removed, and the high-molecular-weight glycoproteins (designated VP119; average molecular weight, 119,000) were purified, using preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8). The glycoproteins which migrated within the VP119 region were further fractionated by sodium dodecyl sulfate-hydroxylapatite chromatography (23) into three glycoprotein components, designated gC, gX, and gA/gB

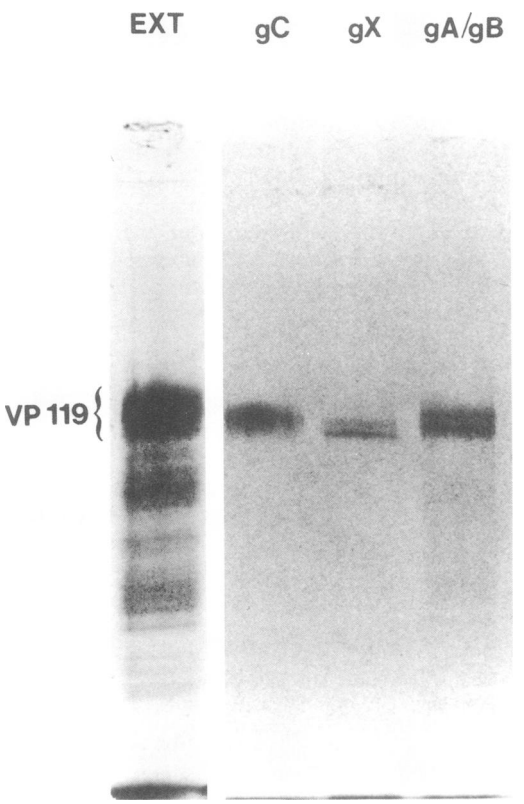

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel analysis of [^{14}C]glucosamine-labeled HSV-2 VP119 components resolved by sodium dodecyl sulfate-hydroxylapatite chromatography. The procedure used for purification of HSV-2 glycoproteins gC, gX, and gA/gB was essentially as described previously (8) and is briefly outlined in the text. EXT, Detergent extract of infected cells.

(Fig. 1). Gel sections containing these individual glycoprotein components were then cut from the gel and emulsified in Freund adjuvant. These emulsions were used to immunize rabbits (8). The specificity and characteristics of the antisera to each of these glycoproteins has been determined (R. Eberle and R. J. Courtney, submitted for publication). In immunoprecipitation tests, anti-gA/gB immunoprecipitates only gA/gB, and anti-gC immunoprecipitates only gC. In contrast, anti-gX immunoprecipitates both gX and gC. The relationship of gX to gC is currently under study.

Immunofluorescence. All antisera used in immunofluorescence tests were adsorbed before use. Each milliliter of antiserum was adsorbed with 0.1 g of rabbit liver powder for 2 h in a 37°C water bath, followed by centrifugation at 100,000 × *g* for 15 min. Antisera were then further adsorbed with approximately 10^7 HEp-2 (human epithelial) cells per ml of antiserum for 2 h at 37°C and then centrifuged at 100,000 × *g* for 15 min to remove particulate matter. Adsorbed antisera were stored at −20°C. Antisera to polypeptides were thawed and diluted either 1:2 or 1:4 in 0.01 M phosphate-buffered saline, pH 7.0 (PBS) immediately before use; a hyperimmune rabbit antiserum to HSV-2 was diluted 1:10 in PBS. The fluorescein isothiocyanate-conjugated goat anti-rabbit gamma globulin (Hyland Laboratories, Costa Mesa, Calif.) was diluted 1:20 in PBS immediately before use.

For both membrane and internal immunofluorescence studies, approximately 5×10^5 cells were seeded on cover slips. Immunofluorescence staining of the cover slips was conducted essentially as previously described (9). For membrane immunofluorescence, the cells were first reacted with normal goat serum, followed by the addition of the specific rabbit sera. The reaction with normal goat sera helped to eliminate nonspecific staining of the cells with the fluorescein isothiocyanate-conjugated goat anti-rabbit gamma globulin. For internal immunofluorescence, the cells were fixed with methanol before the addition of the specific rabbit sera. All cover slips were then stained with fluorescein isothiocyanate-conjugated goat anti-rabbit gamma globulin.

Preparation of rat antisera. Two types of antisera were assayed for virus-neutralizing activity. Hyperimmune rat antisera to HSV-2 or HSV-2–transformed cells were prepared by nine weekly subcutaneous injections of syngeneic White Buffalo rats with virions grown in REF cells overlaid with Eagle medium supplemented with 2% normal rat serum or with transformed cell homogenates in distilled water, respectively. Primary injections were made after mixing equal volumes of virus suspension (10^7 PFU) or cell homogenate from 10^7 cells with complete Freund adjuvant. All booster injections were identical to the primary injection, except incomplete Freund adjuvant was used.

The second type of antisera was prepared by harvesting and pooling sera from syngeneic White Buffalo rats bearing tumors produced by a single subcutaneous injection of 10^6 viable HSV-2–transformed cells. The sera were collected 3 weeks to 9 months after injection of the transformed cells, when the tumors were at least 2.5 cm in diameter.

Virus neutralization by antisera. All antisera were heat inactivated at 56°C for 30 min and adsorbed with nontransformed REF cells (10^7 intact cells per ml of antiserum) before use (17, 18). For virus neutraliza-

tion, 0.1 ml of the adsorbed antiserum (diluted in Eagle medium supplemented with 2% fetal calf serum), 0.1 ml of guinea pig complement (diluted 1:5 in Eagle medium with 2% fetal calf serum), and 0.2 ml of virus (2,000 PFU/ml) were mixed. After 1 h of incubation at 25°C, a 0.2-ml sample of the mixture was inoculated onto a monolayer of REF cells. Controls consisted of virus incubated with PBS or with preimmune rat serum. After a 1-h adsorption period, the infected cell monolayers were overlaid with Eagle medium supplemented with 2% fetal calf serum and 0.5% methyl cellulose. Residual PFU were measured after 48 h of incubation at 35°C in a humidified atmosphere of 5% CO_2 and staining the cell monolayers with 1% crystal violet in 95% ethanol.

RESULTS

Reactivity of antisera to glycoprotein with rat cells productively infected with HSV-2. Antisera prepared against the HSV-2 gAgB, gX, and gC glycoproteins were initially used to determine whether nontransformed REF would express antigenically similar glycoproteins after productive infection with HSV-2 strain 333. For membrane immunofluorescence tests, REF cells seeded onto glass cover slips were infected at a multiplicity of 10 PFU per cell. Cells were harvested at 6 h postinfection and reacted with the antisera.

When the HSV-2 productively infected REF cells were treated with the various antisera, distinct membrane immunofluorescence was observed. The anti-gAgB, anti-gX, and anti-gC sera all showed reactivity with the HSV-2–infected REF cells (Fig. 2). A preimmune serum gave no detectable fluorescence. A similar negative reaction was observed when mock-infected REF cells were stained with anti-gAgB, anti-gX, anti-gC, or preimmune sera (data not shown).

Reactivity of antisera to glycoprotein with rat cells transformed by HSV-2. Having determined that nontransformed REF cells infected with HSV-2 express the viral gAgB, gX, and gC glycoproteins, studies were undertaken to identify which, if any, of these virus-specific glycoproteins were expressed in the HSV-2–transformed rat cell lines.

When HSV-2–transformed cell lines tREF-G-1 and tREF-G-2 and a tumor-derived RFS cell line, were stained with antisera to the gAgB and gX glycoprotein components, specific membrane immunofluorescence was observed (Fig. 3 and Table 1). Notably, the tumor-derived RFS cell line reacted more strongly than either the tREF-G-1 or tREF-G-2 cell lines. Staining the three HSV-2–transformed cell lines with the anti-gC serum yielded contrasting results; i.e., there was no detectable immunofluorescence in tREF-G-1, tREF-G-2, or RFS.

A rabbit antiserum to HSV-2 produced distinct membrane immunofluorescence in all three transformed cell lines; however, this immunofluorescence was not normally as intense as that seen with the anti-gAgB serum (Fig. 3D). No specific reactivity resulted when the transformed cell lines were stained with a preimmune rabbit serum (Fig. 3F and Table 1).

Reactivity of anti-VP143 serum with HSV-2–transformed rat cells. Parallel studies were conducted, using fixed HSV-2 tREF-G-1 cells which were reacted with anti-VP143 serum. The tREF-

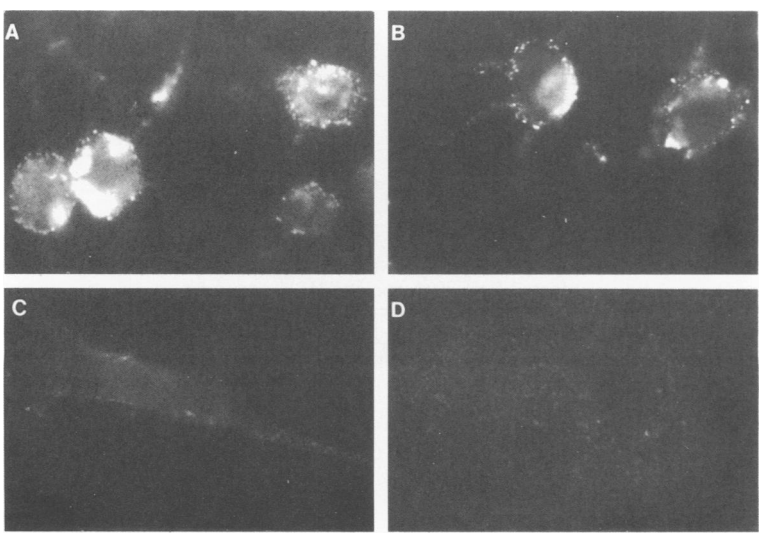

FIG. 2. Patterns of membrane immunofluorescence staining observed in HSV-2 productively infected and mock-infected REF after treatment with anti-gAgB, anti-gX, anti-gC, and preimmune sera. HSV-2–infected REF cells were stained with anti-gAgB serum (A), anti-gX serum (B), anti-gC serum (C), and preimmune serum (D).

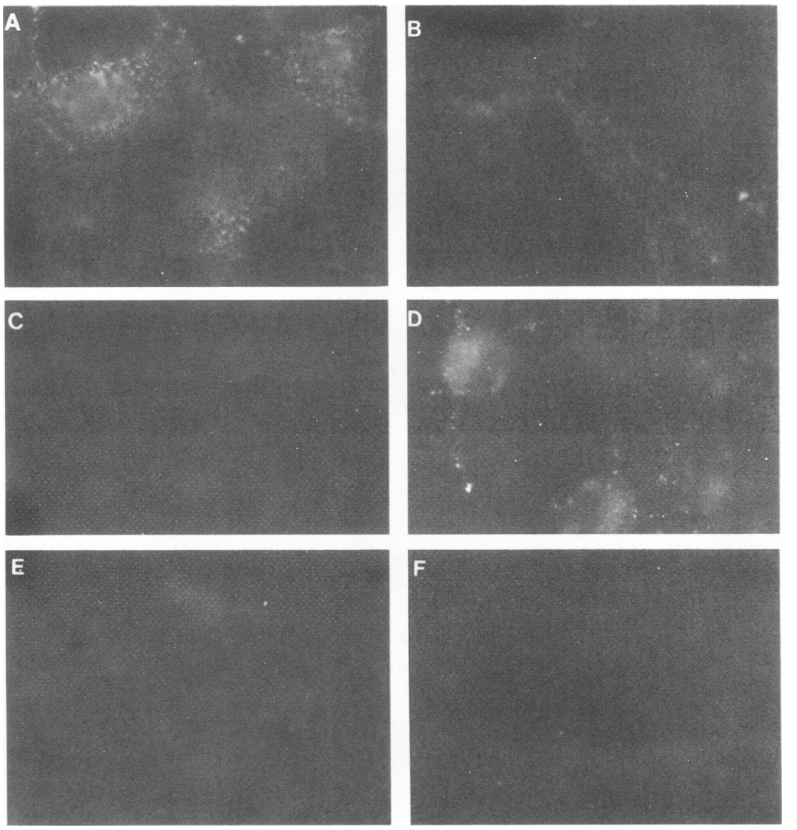

FIG. 3. Membrane immunofluorescence of the HSV-2–transformed cell line tREF-G-1 stained with antisera to individual HSV-2 glycoproteins. Cell line tREF-G-1 was stained with anti-gAgB serum (A), anti-gX serum (B), anti-gC serum (C), and anti-HSV-2 serum (D). Nontransformed REF cells were stained with anti-gC serum (E), and tREF-G-1 cells were stained with preimmune serum (F).

G-1 cells showed a strong positive perinuclear fluorescent staining reaction (Fig. 4A). Similarly, reactivity of tREF-G-2 and RFS cells with anti-VP143 serum showed positive perinuclear fluorescent staining (data not shown). These reactions were similar to the reaction reported previously with HSV-1– and HSV-2–transformed hamster cells (9). The tREF-G-1 cells showed only background fluorescence with preimmune serum (Fig. 4B). Fixed, nontransformed REF cells exhibited no detectable internal fluorescence when treated with anti-VP143

TABLE 1. Immunofluorescence reactivity of HSV-2–transformed, nontransformed, and HSV-2 productively infected REF with specific HSV-2 antisera

Cells	Reactivity with[a]:				
	Antiserum to glycoproteins				Nonstructural VP143
	gC	gX	gAB	Preimmune	
Transformed					
tREF-G-1	−	+	+	−	+
tREF-G-2	−	+	+	−	+
Tumor (RFS)	−	+	+	−	+
Nontransformed (REF)	−	−	−	−	−
Productive infection (REF)	+	+	+	−	+

[a] +, Positive; negative −.

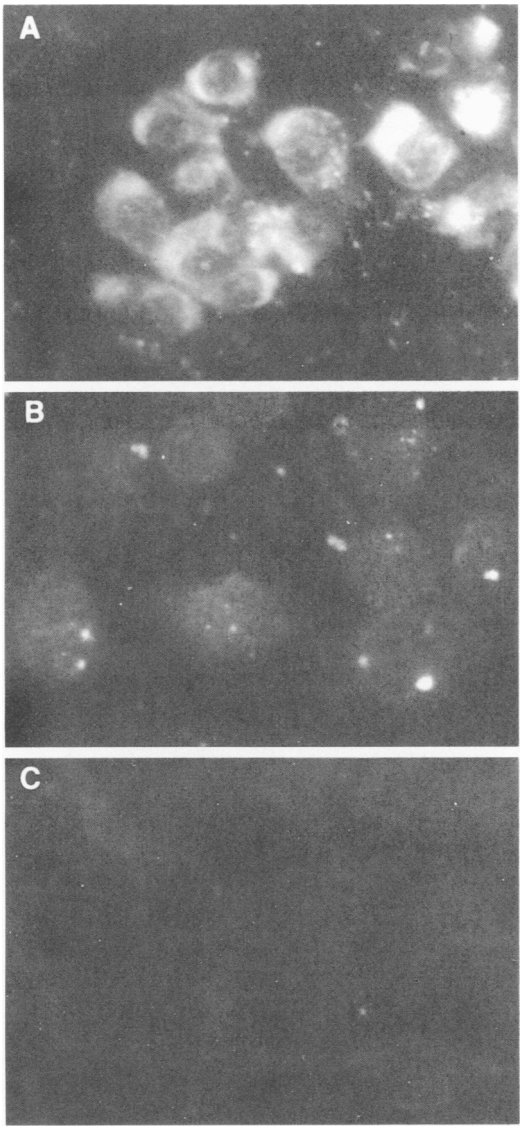

FIG. 4. Internal immunofluorescence of fixed HSV-2–transformed cell line tREF-G-1 treated with antiserum to an early nonstructural HSV polypeptide (VP143). Cell line tREF-G-1 was treated with anti-VP143 serum (A) and preimmune serum (B); fixed, nontransformed REF cells were treated with anti-VP143 serum (C).

serum (Fig. 4C). The immunofluorescence results are summarized in Table 1.

Neutralization of HSV-2 infectivity by hyperimmune rat sera and tumor-bearing rat sera. Since a specific antiviral glycoprotein serum (anti-gAgB) gave positive membrane fluorescence with HSV-2–transformed cells and a tumor-derived cell line, experiments were designed to determine whether tumor-bearing rats or rats hyperimmunized with transformed cells generated HSV-2–neutralizing antibodies. Antisera raised against virions, HSV-2–transformed tREF-G-2 cells, tumor-derived RFS cells, or antisera from rats bearing tumors produced by tREF-G-1 or RFS cells possessed virus-neutralizing activity in the presence of active complement (Table 2). Only the anti–HSV-2 serum neutralized virus in the absence of active complement. PBS or preimmune serum did not reduce virus infectivity in the presence of active complement (Table 2).

Experiments were designed to determine whether neutralizing antibodies present in rat anti–HSV-2 serum could be adsorbed by viable HSV-2–transformed or tumor-derived cells. When samples of dilute (1:40) anti–HSV-2 serum were adsorbed with tREF-G-2 or RFS cells, virus neutralization activity was markedly reduced (Table 3). Anti–HSV-2 serum adsorbed with nontransformed REF cells had virus-neutralizing activity comparable to that of unadsorbed antiserum (Table 3). These data suggest that HSV-2–transformed cells and tumor-derived cells induce virus-neutralizing antibodies in rats. The neutralizing activity binds specifically to HSV-2–transformed cells and tumor-derived cells but not to nontransformed REF cells.

DISCUSSION

After serial passage in tissue culture, HSV-2–transformed rat cells retain genetic information necessary for the production of the HSV-2 gAgB antigen and the nonstructural protein VP143. Correspondingly, HSV-2–neutralizing antibodies in sera of RFS-bearing rats may be ascribed to antibody evoked to viral antigens (e.g., gA/gB) on the membranes of the tumor cells.

Perhaps the most salient point regarding the apparent absence of gC in the HSV-2–transformed rat cell lines is that the segment of the HSV genome coding for gC (0.65 to 0.70 map units) may be outside the transforming region. Its absence suggests that gC may play no role in initiation or maintenance of the transformed state. These results are further supported by a recent report (20) implying that strong negative selection pressure exists against incorporation of HSV-1 DNA sequences between 0.57 to 0.82 map units (which includes the region coding for the gC glycoprotein) into HSV-1 biochemically transformed host cells. It should be noted that, in other studies in which HSV-2 morphologically transformed hamster cells and specific antisera to gAgB and gC are used, the expression of the HSV-2 gC, as well as gAgB glycoproteins, has been detected (J. Lewis and R. J. Courtney, unpublished data).

Results obtained with an antiserum to the

TABLE 2. Neutralization of HSV-2 PFU by hyperimmune rat anti-HSV-2, anti-tREF-G-2, and anti-RFS sera or tumor-bearing rat serum[a]

Serum	Active complement	% Virus neutralized at final serum dilution of[b]:					
		1:4	1:8	1:16	1:32	1:64	1:128
Rat antiserum to:							
HSV-2	+	100	100	100	100	100	100
	−	100	100	100	100	100	100
tREF-G-2	+	83	78	68	50	13	ND[c]
	−	0	0	0	0	0	0
RFS	+	99	87	73	64	20	ND
	−	0	0	0	0	0	0
Tumor-bearing rats injected with:							
tREF-G-1	+	99	96	85	53	40	25
	−	0	0	0	0	0	0
RFS	+	96	84	71	58	36	31
	−	0	0	0	0	0	0
Control							
PBS	+	0	0	0	0	0	0
Rat preimmune serum	+	0	0	0	0	0	0

[a] See text.
[b] Percent virus neutralized was calculated by dividing the residual PFU in the antiserum-virus mixture by the residual PFU in the preimmune serum mixture and multiplying by 100.
[c] ND, Not determined.

early nonstructural β-group polypeptide VP143 showed that all three transformed cell lines examined reacted with this serum. Similar findings have been reported for HSV-2–transformed hamster cells (9). Also, VP143 was detected within cervical tumor cells from patients (4; G. A. Cabral, F. Marciano-Cabral, D. Fry, H. L. Tomlin, D. Hall, and D. R. Goplerud, Program Abstr. Int. Conf. Human Herpesviruses, Atlanta, Ga., abstr. no. Y32, 17–21 March 1980). Expression of VP143 may be correlated with expression of the gAgB glycoproteins, which were also invariably expressed in HSV-2–transformed hamster (J. Lewis and R. J. Courtney, unpublished data) and rat (Table 1) cells. The relationship of this polypeptide to those which have been mapped in the region of the HSV genome responsible for HSV transformation is currently being studied.

TABLE 3. Removal of neutralizing antibodies from hyperimmune rat antiserum to HSV-2 by adsorption with intact tREF-G-2 and RFS cells[a]

Antiserum to HSV-2 adsorbed with:	% Virus neutralized at final serum dilution of:			
	1:80	1:160	1:320	1:640
Transformed tREF-G-2	12	0	0	0
Tumor RFS	10	0	0	0
HSV-2 virions	0	0	0	0
Nontransformed REF	90	72	31	26
Unadsorbed control	100	92	69	53

[a] Growing cells were scraped from plastic flask cultures, washed twice with PBS, and counted. To 0.5 ml of hyperimmune rat anti–HSV-2 serum diluted 1:40 with PBS were added 5×10^7 viable cells (t-REF-G-2, RFS) or HSV-2 (5×10^7 PFU). After 2 h of incubation with constant shaking at 37°C, the mixture was centrifuged (100,000 × *g*, 1 h, 4°C). The supernatants containing the adsorbed antiserum were harvested and assayed for virus-neutralizing antibodies as described in the text. Virus was partially purified by differential centrifugation.

The detection of the gAgB glycoproteins in hamster cells transformed by the *Xba*I restriction fragment (0.30 to 0.45 map units on the HSV-1 genome) suggested that this region of the virus genome is continuous or overlapping with the region which codes for information sufficient to cause morphological transformation (3).

Of note is that the HSV-2 gAgB glycoproteins identified in transformed cells have molecular weights similar to that of the 118,000-dalton glycoprotein shown to be more frequently immunoprecipitated by sera from cervical carcino-

ma patients than by matched sera from controls (12). Further support for association of the gAgB glycoproteins in transformation comes from hybridization data showing that HSV-2–transformed cells (11) and cervical carcinoma cells (22) retained DNA sequences in the region 0.21 to 0.33. This region overlaps the location of viral DNA (0.30 to 0.43 map units) which codes for the gAgB glycoproteins (26).

The location of the putative oncogene for HSV needs further discussion. With transfection experiments, Jariwalla and co-workers (15) found that oncogenic transformation was contained in the *Bgl*II/*Hpa*I double-digested fragment (mapping between coordinates 0.43 and 0.58), and Reyes and co-workers (25) reported morphological transformation with a *Blg*II N fragment located at 0.58 to 0.62 map units but not at 0.30 to 0.43 map units in the HSV-2 genome. The apparent differences between these results may be due to the map location of the oncogene among different viral strains or the nature of the transforming event under study. Reyes and co-workers (25) used morphological transformation as the endpoint in their experiments, whereas Jariwalla and co-workers (15) used oncogenic transformation as the endpoint.

Although the sequence complexity of HSV-2 DNA appeared to vary in transformed hamster cells passaged in parallel (10), two blocks of sequences located between 0.21 to 0.33 and 0.60 and 0.65 map units on the HSV-2 genome were retained (11) and expressed by hamster cells transformed by whole virus (22). Similar blocks of DNA sequences were detected in human cervical tumor cells (22). The fact that sequences around the region of 0.21 and 0.33 were retained by virus-transformed cells suggests that this region may play some role in the initiation or maintenance of cell transformation by whole-virus particles, whereas in transfection experiments with isolated viral DNA fragments, the sequence around the region of 0.43 to 0.58 (15) or 0.60 to 0.65 (25) is sufficient to cause cell transformation.

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