ied (*10*). This specialized function would not apply to the heterochromatin in the secondary constriction regions or in the long arm of the Y chromosome, and other functions will undoubtedly be assigned to these regions.

The type of variation which we have described has previously been reported only as a rare phenomenon. For example, asymmetry of chromosomes No. 1, 9, and 16 and variation in the length of the Y chromosome have been observed in standard leukocyte preparations and are usually inherited (*11*). It is probable that these variations, detectable by conventional cytological methods, simply represent one end of the spectrum of heterochromatin polymorphism. The sensitivity of the denaturation-renaturation technique in detecting heterochromatin variants has, therefore, led to the significant discovery that these variants occur with a much higher frequency than was heretofore suspected. Additional studies of heterochromatin variability, both in populations and within families, may determine how much tolerance the genome has for such variants.

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5. The staining solution consists of 1.5 ml of citric acid adjusted to *p*H 6.8 with 0.2*M* Na_2HPO_4, 1.5 ml of absolute methyl alcohol, 50 ml of distilled water, and 5 ml of Giemsa stock solution (Curtin).
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Herpesvirus Type 2 Isolated from Cervical Tumor Cells Grown in Tissue Culture

Abstract. *A herpesvirus has been isolated from spontaneously degenerating cultures of cervical tumor cells grown in vitro. The virus was identified as a type 2 herpesvirus on the basis of biologic properties, including plaque morphology and microtubule formation in infected HEp-2 cells, and of immunologic specificity as determined by neutralization. Herpesvirus antigens and virus particles were not seen in duplicate cultures of viable cervical tumor cells.*

Two types of herpesvirus, biologically and antigenically distinct (*1*, *2*), have been isolated from humans: herpesvirus type 1 (HSV-1) associated with facial lesions, and herpesvirus type 2 (HSV-2) isolated from smegma and cervical lesions (*3*) and shown to be venereally transmitted (*4*). Recent data indicate that patients with invasive (*5*, *6*) and preinvasive (*6*) cervical neoplasia have a significantly higher prevalence of antibody to HSV-2 than a matched control population, and that exfoliated dyskaryotic cells from these patients possess HSV-2 antigens as determined by immunofluorescence (*7*). In this report we describe the isolation of a herpesvirus with immunologic specificity of HSV-2, from cells of carcinoma of the cervix grown in tissue culture.

A line designated S332G was established by the Maximow double cover slip technique (*8*) from a biopsy obtained from a case of intraepithelial carcinoma of the cervix. The line, characterized by atypical cells with large lobulated nuclei and occasional multiple nucleoli and approximately 10 percent multinucleated cells (Fig. 1A), was established in minimum essential medium (MEM) supplemented with fetal calf serum (20 percent) (Grand Island) under a 5 percent CO_2 atmosphere. Electron microscopy of thin sections (Fig. 2A) revealed large epitheloid cells with elaborate microvilli, a convoluted nuclear membrane, small condensations of chromatin at the nuclear periphery, and prominent membranous organelles. These consisted of an extensive rough endoplasmic reticulum with dilated cisternae, smooth endoplasmic reticulum and a large Golgi apparatus. Packed fibrils 5 to 8 nm in diameter and consistent with tonofibrils of squamous cells (*9*) were observed surrounding the membranous cytoplasmic elements.

After ten transfers (6 months) in MEM with 10 percent fetal calf serum, and again at three subsequent intervals (12th, 15th, and 18th transfer), the cells in one out of replicate culture flasks began to round up, and within 48 hours cell degeneration consisting of swollen refractile cells (Fig. 1B) became prominent. The cells were scraped, collected by centrifugation at 400*g* for 10 minutes, and resuspended in 1 ml of MEM. Duplicate cultures established from the same seeding of cells and that had not degenerated were used as controls; they were designated "viable" and studied at the time of degeneration. Cultures of viable cells were also studied (3rd, 5th, and 8th transfer) before onset of degeneration. Two series of experiments were done to test for virus in degenerated cells. In the first series, one-half of the cell suspension was frozen and thawed, and the extract was used to in-

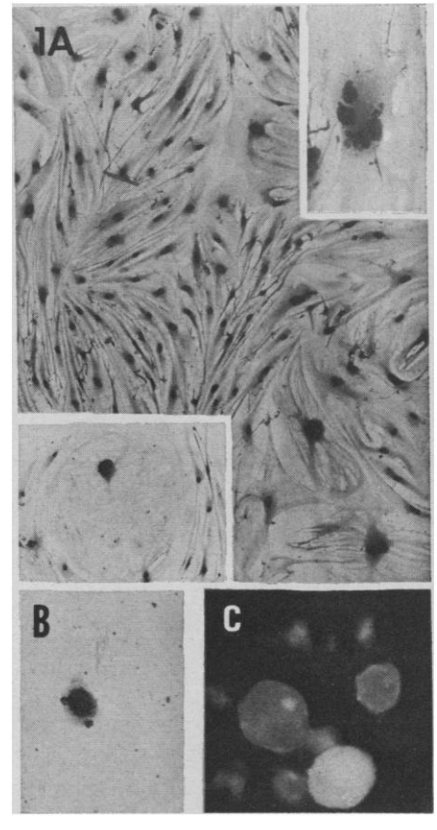

Fig. 1. (A) S332G cells stained with Giemsa display characteristic pleomorphism (× 110); upper inset, multinucleated cells (× 220); lower inset, large cells with extensive filamentous cytoplasm (× 110). (B) Degenerated S332G cell stained with Giemsa (× 220). (C) Degenerated S332G cells stained with Ra-2 serum (× 480).

fect a culture of approximately 10^5 HEp-2 cells. An extract made by freezing and thawing of approximately 10^5 viable S332G cells was used as control. Characteristic cytopathogenic changes became evident within 48 hours in the HEp-2 cell culture infected with degenerated S332G cells; these were increased by subsequent passage on HEp-2 cells of the extracts made by freezing and thawing of the infected cells. Five passages of the extracts from the viable S332G cells after the 3rd, 5th, 8th, 10th, 12th, and 15th transfer failed to induce a cytopathogenic effect.

Electron microscopic examination of thin sections of HEp-2 cells on the fourth consecutive passage after infection with the original inoculum of S332G cells revealed the presence of intranuclear particles with specific herpesvirus morphology, characteristic nuclear alterations (*10*), and enveloped cytoplasmic particles 140 to 160 nm in diameter (Fig. 2B). Of particular significance are the relatively high number of unenveloped cytoplasmic particles (Fig. 2B) and the filamentous intranuclear structures, 20 to 22 nm in diameter (Fig. 2C), both of which have been described as characteristic of HSV-2 infected cells (*11*). Complete or incomplete virus particles were not seen in viable S332G cells at the 3rd, 5th, 8th, 10th, 12th, and 15th transfer.

The cells in the second half of the degenerated S332G cell suspension were washed and resuspended in 0.1 ml of MEM with 0.5 percent bovine albumin, and one drop of cells placed on each of five spots of microscopic slides. These were dried in air and fixed in cold (−40°C) methanol before being stained for herpesvirus antigens by the indirect immunofluorescent procedure. Increasing dilutions of rabbit antiserum to HSV-2 (Ra-2) and rabbit antiserum to HSV-1 (Ra-1) were used. The preparation, absorption, and specificity of these serums for HSV-2 and HSV-1 infected HEp-2 cells have been described (*7*). Phosphate buffered saline, in place of antibody, was used as control for nonspecific fluorescence. The results indicate that both serums react with degenerated S332G cells. The exact number of cells displaying fluorescence could not be determined with accuracy because of the extensive degeneration of the cultures; however Ra-2 serum was still positive at a dilution of 1 : 20 (Fig. 1C), whereas Ra-1 serum did not react beyond a dilution of 1 : 5; the cells display immunologic specificity of type 2 herpesvirus. Similar results were reported by Nahmias *et al.* (*12*) for infected HEp-2 cells. Slides made from suspensions containing 10^4 viable S332G cells each, at the 3rd, 5th, and 8th transfers, and 10^5 viable S332G cells each at the 10th, 12th, 15th, and 20th transfer failed to react with both serums.

The immunologic specificity of the virus isolates from S332G cells that had degenerated at the 10th, 12th, 15th, and 18th transfer and respectively designated S-1, S-2, S-3, and S-4 was determined by a neutralization test based on the findings that a laboratory strain (HSV-MP) reacts in neutralization tests as having an immunologic specificity intermediate between that of HSV-1 and HSV-2 (*2*). Thus, Ra-2 serum added to a mixture of HSV-1 and HSV-MP neutralizes HSV-MP preferentially; the same serum added to a mixture of HSV-2 and HSV-MP neutralizes the homologous virus preferentially. Ra-1

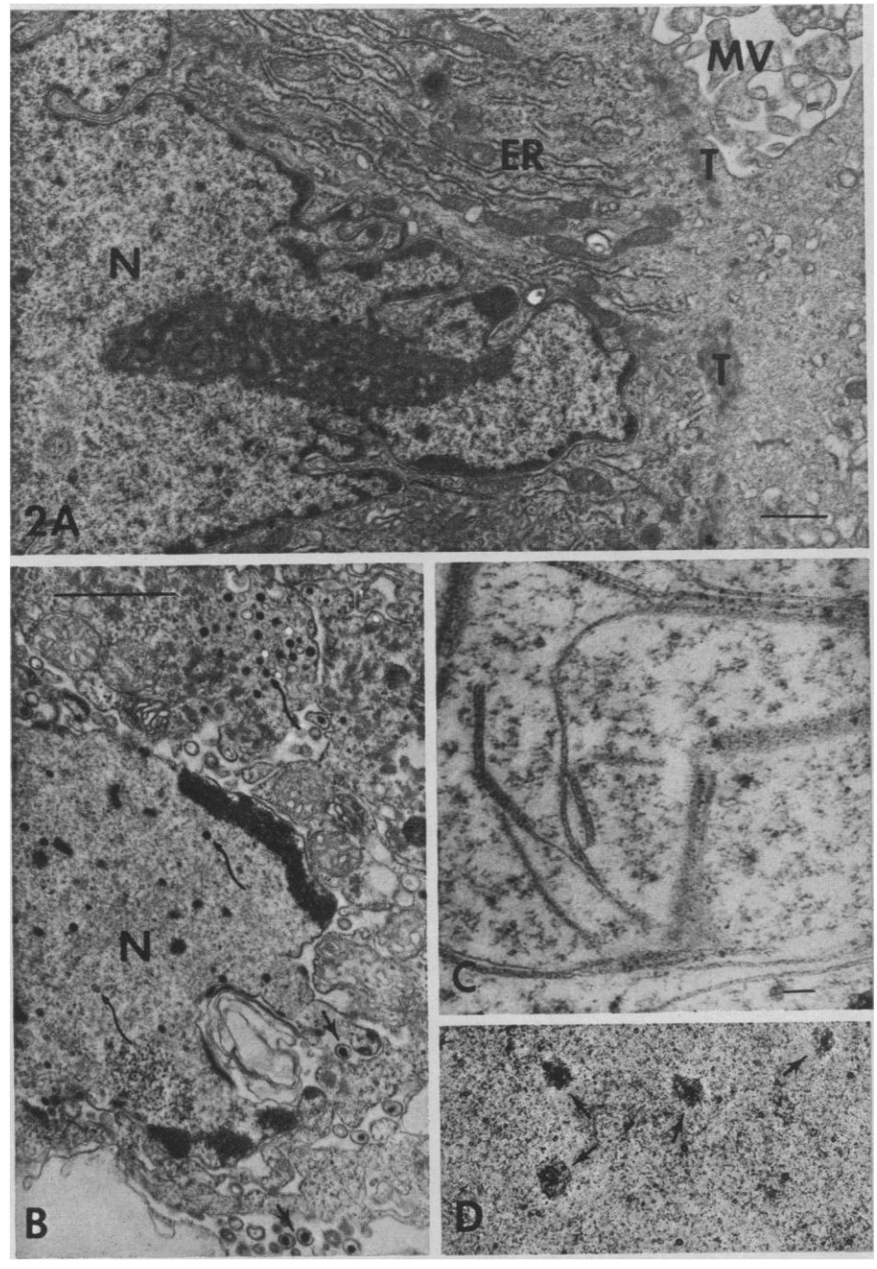

Fig. 2. (A) Electron micrograph of a viable S332G cell. Note lobulated nucleus (*N*) with marginated chromatin, extensive endoplasmic reticulum (*ER*), numerous microvilli (*MV*), and tonofibrils (*T*) (scale represents 1 μm). (B) HEp-2 cell infected with S-1 isolate, containing nucleocapsids (long arrows) in nucleus (*N*) and cytoplasm and enveloped virions (short arrows) (scale represents 1 μm). (C) Intranuclear microtubules in S-1 infected HEp-2 cells (scale represents 100 nm). (D) Plaques (arrows) produced by S-1 in infected HEp-2 cells, stained with Giemsa (× 25).

serum neutralizes HSV-MP preferentially in a mixture of HSV-2 and HSV-MP. On the basis of these results, an unknown strain can be classified as type 1 or type 2 by exposure in an artificial mixture with HSV-MP to either Ra-1 or Ra-2 serum. The test, a plaque reduction procedure (*2*, *6*), is made possible by the observation that isolates from S332G cells give rise to plaques consisting of small loose clumps of rounded cells (Fig. 2D) similar to those produced by a prototype of HSV-2 (*2*) and easily differentiated from the polykaryocytes induced by HSV-MP (*13*). Briefly, artificial mixtures of equal titers of HSV-MP and either one of the four isolates from degenerated S332G cells are exposed to varying amounts of Ra-1 or Ra-2 serum. After incubation at 37°C for 48 hours, the cells are fixed and stained with Giemsa, and the plaques formed by the two viruses are counted. As shown in Fig. 3, Ra-2 serum is more effective than Ra-1 in neutralizing isolate S-1; isolate S-1 is therefore immunologically a type 2 herpesvirus. The three other isolates are identical to isolate S-1 with respect to plaque morphology in HEp-2 cells and immunologic specificity as determined by neutralization.

Since degeneration of the S332G cells and virus isolation were consistently associated with an increase in the *p*H of the medium, an experiment was done to study the effect of *p*H on virus induction in S332G cells. After two cultures originating from the same seed of cells were subdivided to yield four cultures, duplicate cultures were grown for 3 weeks in MEM with 10 percent fetal calf serum, with and without a 5 percent CO_2 atmosphere. The *p*H of the medium was determined at 2-hour intervals for the first 10 hours after transfer and once a day during the next 5 days. Under the CO_2 atmosphere, normal growth and an even *p*H (7.2 to 7.3) were characteristic to the cultures, whereas extreme *p*H ranges (7.7 to 8.7) and poor growth were observed in the cultures grown without CO_2. At the end of 3 weeks typical cytopathogenic changes were observed in the latter, and the cells stained preferentially with Ra-2 serum.

The focal point of our study is the isolation from a line of cervical tumor cells of a virus identical to type 2 herpesvirus with respect to two biologic properties (plaque morphology and microtubule formation in infected HEp-2 cells) and immunologic specificity, as determined by immunofluo-

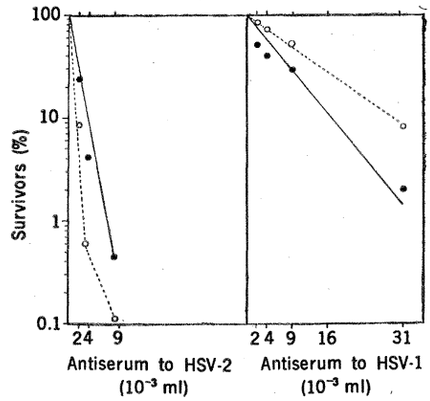

Fig. 3. Neutralization of artificial mixtures of S-1 and HSV-MP with rabbit antiserum to HSV-2 (Ra-2) and rabbit antiserum to HSV-1 (Ra-1). Open circles, S-1; solid circles, HSV-MP.

resence and neutralization tests. The data are susceptible to three interpretations. First, it is possible that the observations are due to the external contamination of the cultures with a type 2 herpesvirus. The failure to isolate infectious virus and detect viral antigens and particles in cultures of nondegenerating S332G cells studied at the 10th, 12th, and 15th transfers and the isolation of a type 2 herspesvirus from duplicate degenerating cultures at these transfers argue against this possibility. A second interpretation of the data is that S332G cells are chronically infected as a low grade infection (*14*). Arguing against this interpretation are three observations. (i) Normally replicating S332G cells studied prior to the first observed degeneration (3rd, 5th, and 8th transfers) did not contain virus antigens and particles; (ii) viable cultures studied simultaneously with their degenerating duplicates (10th, 12th, and 15th transfers) did not show evidence of virus presence, and viral antigens were not observed in viable cells studied after the last observed degeneration (20th transfer); and (iii) viable S332G cells are capable of supporting the replication of a prototype of HSV-2 (*2*) as determined by immunofluorescence and plaque formation on HEp-2 cells with an extract of the infected S332G cells (unpublished data).

The third interpretation is that the cells harbor a viral genome that under specific conditions, such as high *p*H, may express its genetic potentialities resulting in the formation of infectious virus and concomitant cell death. Indeed, the suggestion has been made that virus persistence in latently infected cells depends on the arrest of virus multiplication in the infected cells after the DNA is uncoated, but before the early functions leading to host inhibition and modification are expressed (*15*). The failure to detect herpesvirus antigens and complete or incomplete particles in the viable S332G cells, prior to, at the time of, and after the isolation of HSV-2 from the degenerating duplicate cultures, gives support to this hypothesis and suggests that all or possibly some of these cells harbor the viral genome. In that event they should contain herpesvirus DNA.

The isolation of herpesvirus from tumor cells grown in vitro under suboptimum conditions, such as partial malnutrition or low temperature, is not without precedent (*16*). Our data indicate that virus induction in S332G cells is dependent on growth of the cells at a relatively high *p*H. However, prior to virus isolation the cells were grown for 6 months under conditions of partial malnutrition consisting of MEM supplemented with 10 rather than 20 percent fetal calf serum. The precise physiologic manipulations associated with virus replication need to be established.

More broadly, our findings strengthen the evidence for the association of HSV-2 with carcinoma of the cervix; however, the role of the virus in the causation of the tumors is still unknown. Its elucidation must await further experimentation.

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Mendelian and Uniparental Alterations in Erythromycin Binding by Plastid Ribosomes

Abstract. *Erythromycin binds specifically to the 52S subunit of the chloroplast ribosome of* Chlamydomonas reinhardi. *A number of erythromycin-resistant mutants whose ribosomes have lost their affinity for the antibiotic have been isolated, but the sedimentation properties of their ribosomes are indistinguishable from those of the wild-type strain. These mutants represent at least three genetic loci. Two of them show Mendelian inheritance, and one of them is inherited in a uniparental manner.*

Erythromycin resistance is a valuable genetic marker for structural components of bacterial ribosomes. In *Escherichia coli*, the alteration leading to resistance has been traced, for a number of mutants, to a single protein of the 50*S* ribosomal subunit (*1*). In a resistant mutant of *Staphylococcus aureus*, where no altered proteins could be detected, changes in the 23*S* RNA of the 50*S* subunit were found (*2*).

Sensitivity to erythromycin and loss of sensitivity by mutation seem to be common characteristics of microbial eukaryotic organisms as well. *Saccharomyces* (*3*, *4*), *Paramecium* (*5*), and *Chlamydomonas* (*6*) are among the genera in which genetic studies of these characteristics have been carried out. Linnane *et al.* (*3*) have obtained indirect evidence that some of the resistance mutations in yeast lead to functionally altered mitochondrial ribosomes. We have made a search of erythromycin-resistant mutants in *Chlamydomonas reinhardi* in order to identify and map genes coding for structural features of the sensitive ribosomes.

Wild-type, mating-type plus cells (from strain 137c obtained from R. P. Levine) were treated with ethyl methanesulfonate (*7*), distributed in portions to a large number of tubes, and kept in liquid minimal medium (*8*) for 24 hours. The cells from each tube were then transferred to individual plates of minimal medium containing $5 \times 10^{-4} M$ erythromycin. After the plates were incubated for 1 week, approximately 75 isolates were picked, one from each plate, and an attempt was made to take a representative sample of various colony sizes and morphologies. All the stable resistant isolates were resistant when grown either in the light on minimal medium or in the dark on medium supplemented with acetate. Nine of these strains, again a representative sample, were selected for further study and have been maintained in the complete absence of erythromycin for 12 months.

Ribosomes were isolated from cells in the late log phase of growth and were broken by forcing them under pressure through a phase transition at $-20°C$ in a modified Hughes press (*9*). The frozen cell lysate was thawed in three volumes of buffer (25 m*M* KCl, 25 m*M* $MgCl_2$, 25 m*M* tris-Cl, *p*H 7.5), and the cell debris and membrane material were removed by centrifugation at 27,000*g* for 20 minutes. The supernatant was layered over buffer made 1*M* in sucrose, and centrifuged at 350,000*g* for 3 hours. The clear pellet, taken up in buffer, was the standard crude preparation of ribosomes.

Table 1. Genetic characterization of the six erythromycin-resistant mutants known to have altered chloroplast ribosomes.

Mutant	Segregation	Linked to 15S4?
2L1	4 : 0	
12S3	2 : 2	No
15S4	2 : 2	(Yes)
1L5	2 : 2	Yes
11S7	2 : 2	Yes
13L1	2 : 2	Yes

In order to distinguish mutants with altered ribosomes from possible mutants which gain resistance by either reduced permeability to erythromycin or the ability to detoxify the drug, the binding of [^{14}C]erythromycin to mutant and wild-type ribosomes was studied. Erythromycin binding was measured by the retention of erythromycin-ribosome complexes by Millipore filters. The procedure was essentially that of Teraoka (*10*), with appropriate modifications of the buffer. The closed circles in Fig. 1a show the binding of erythromycin to wild-type ribosomes as a function of erythromycin concentration. This binding requires K^+ at 25 m*M* or higher concentrations. In contrast to the requirements for binding to bacterial ribosomes (*10*), NH_4^+ will not fully substitute for K^+, and at 30 m*M* or higher, NH_4^+ inhibits the binding; Na^+ does not substitute at all. The binding requires Mg^{2+} at concentrations of 10 m*M* or higher and is insensitive to change in *p*H from 6 to 9. The binding is abolished if the ribosomes are treated with Triton X-100 at any point during their preparation [a treatment which is part of the isolation procedure of Hoober and Blobel (*11*)].

The binding constant for the reaction

$$\text{Ribosome} + \text{erythromycin} \rightleftharpoons \text{erythromycin-ribosome complex}$$

and the number of binding sites in the reaction mixture can be directly determined from the double reciprocal plot shown in Fig. 1b. The equilibrium constant, as determined from the average of several experiments, is about 8×10^4 mole^{-1}, and the typical preparation of crude ribosomes contains one binding site for every 8×10^6 to 9×10^6 daltons of nucleic acid. For ribosomes from wild-type cells, this type of plot always yields a straight line, indicating the homogeneity of the binding sites in their affinity for the antibiotic.

For the resistant mutants listed in