Transformation of Rodent Cells by a Cloned DNA Fragment of Herpes Simplex Virus Type 2

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Transformation of rodent cells with isolated restriction endonuclease fragments of herpes simplex virus type 2 DNA identified a region of the genome located between map positions 0.58 and 0.62. These sequences were cloned into pBR322, and the recombinant plasmid was used to transform primary rat embryo cells and NIH 3T3 cells. The transformants were selected for their ability to form dense foci on a monolayer or to form colonies in semisolid medium. In contrast to the parental rat or mouse cells, cell lines transformed with the cloned herpes simplex virus type 2 fragment grow to high saturation densities, replicate in medium containing 1% serum, form colonies in dilute methylcellulose, show reduced levels of fibronectin, and are tumorigenic in nude mice and in their syngeneic hosts. Southern blot hybridizations have detected sequences homologous to the viral fragment in high-molecular-weight DNA from the transformed cell lines that are not present in DNA from normal rodents. In all cases, the plasmid DNA was present in less than one copy per cell, and the patterns of viral sequences changed with passage of the cell line *in vivo*.

In the decade that has elapsed since Duff and Rapp (11, 12) first reported that herpes simplex virus (HSV) can induce the morphological transformation of rodent cells, these observations have been repeated and extended with a variety of debilitated forms of HSV, including UV-inactivated virus (4, 11, 12), photodynamically inactivated virus (36), temperature-sensitive mutants (28, 33, 45), virus at supraoptimal temperatures (9, 29), sheared viral DNA (49), and defined fragments of the viral genome (5, 26, 38). Numerous studies have described the phenotypic properties, tumorigenicity, cytogenetics, and expression of viral antigens in cells transformed by HSV (for a review, see reference 37). Nevertheless, the molecular aspects of transformation by HSV have remained elusive.

Central to the dogma of transformation by DNA viruses is the idea that at least a portion of the viral genome integrates into the DNA of the host cell, where it is stably transmitted to the descendants of the cell and transcribed into mRNA which is in turn translated into a protein(s), and that the expression of these sequences is interconnected with the characteristic phenotypic changes associated with the transformed state. These products have been difficult to identify in cells transformed by HSV. Virus-specific antigens have been associated with transformed cells (5, 11, 12, 14, 26, 44); however, no protein common to all of the lines has been identified. The presence of viral RNA (7, 8) and

DNA (15, 17, 27, 34) has also been reported, but the sequences which are necessary for morphological transformation were not fully defined.

Two laboratories have reported that a fragment of HSV-1 DNA located between map positions 0.31 and 0.42 is able to transform hamster embryo cells (5, 38). The initial report (5) indicated that a membrane glycoprotein which is encoded by these sequences was present in the transformed cells, but no evidence concerning the content of viral sequences was available. In subsequent studies (38), viral DNA has not been detected in cells transformed by fragments of HSV-1 DNA.

Two different regions of the HSV-2 genome have been reported to have transforming potential, neither of which is homologous to the HSV-1 sequences. Reyes et al. (38) used the *Bgl*III N fragment of HSV-2 (333) which maps between positions 0.58 and 0.63 to alter the growth properties of hamster embryo cells and BALB 3T3 cells. After fractionation on RPC-5 columns, DNA homologous to the viral fragment could be detected in the transformed cells. However, sequences homologous to the *Bgl*III I fragment (positions 0.63 to 0.72) were also found in cells transformed with an *Hpa*I/*Xba*I fragment (0.575 to 0.702 map units). Unlike others, who used the focus formation assay (5, 38), Jariwalla et al. (26) selected transformed cells which grew out of continuous passage after exposure to fragments of HSV-2 (333 or S₁) DNA. These experiments

identified a fragment of DNA with transforming potential located between positions 0.43 and 0.58 on the HSV-2 genome. The transformed cells were tumorigenic and expressed a viral antigen, ICP-10. Data to demonstrate the presence of viral DNA in these cells were not available.

This study was undertaken to identify sequences of HSV-2 DNA that have transforming activity and to use cloned fragments of the viral genome to establish transformed cell lines. Our results indicate that a single region of the HSV-2 genome located between map positions 0.58 and 0.63 has the ability to alter the morphological phenotype of rodent cells. The transformed cells were investigated with regard to their growth properties, tumorigenicity, and arrangement of viral DNA sequences.

MATERIALS AND METHODS

Cells and viruses. HSV-2 strain 333 was propagated on monolayers of BSC-1 cells infected at a low multiplicity (0.01 PFU per cell). Viral DNA was isolated by equilibrium gradient centrifugation through sodium iodide gradients containing ethidium bromide (47). Cultures of primary rat cells were obtained by trypsinizing 14-day-old Wistar rat embryos. NIH 3T3 cells were obtained from F. Yoshimura. All cells were cultured in an atmosphere of 10% CO₂, using the Dulbecco modification of Eagle medium (DME; GIBCO Laboratories) with 10% fetal calf serum (FCS; Microbiological Associates) on plastic tissue culture plates (Falcon Plastics).

Plasmids. The construction and characterization of plasmids derived from pBR322 which contain fragments of the HSV-2 genome have been described previously (19). The plasmids were propagated in *Escherichia coli* HB101. Plasmid DNA isolated by the cleared lysate technique (21) was purified by gradient centrifugation through CsCl.

Isolation of transformed cell lines. Viral DNA fragments which were isolated from agarose gels or plasmid DNA which had been cleaved with a single-cut restriction enzyme (0.1 to 20 µg per 60-mm plate) was precipitated by the calcium phosphate technique (20) onto monolayers with 5×10^5 cells per 60-mm dish as described previously (48). Transformants were selected in two different ways. In the first assay, the medium (DME containing 10% FCS) was changed at 4 h after the DNA precipitate had been added, and at 24 h after the change of medium, the cells were subcultured from 1:3. The cells were fed every 2 days for 1 week, and thereafter the serum was lowered to 2%. The cells were fed twice weekly without further subculturing, and within 4 to 6 weeks transformants appeared as dense foci on the monolayers. These were teased from the plates with a Pasteur pipette, trypsinized, and replated. In the other assay, the medium was replaced at 4 h after the DNA had been added, and at 24 h after the change of medium, the cells were trypsinized and seeded into plates containing 1.2% methylcellulose (10⁵ cells per 60-mm plate). Cells were fed weekly with DME containing 10% FCS plus methylcellulose. After 3 to 5 weeks, colonies appeared in the

semisolid medium. Colonies of NIH 3T3-derived cells could be picked at that time; however, colonies from the primary rat cells were not large enough to be removed until 6 to 8 weeks. The colonies were trypsinized, replated, and maintained in DME containing 10% FCS. Isolation of high-molecular-weight DNA from the transformed cell lines was as described previously (2).

Assays for transformed cell properties. The transformed cell lines were assayed as described previously (18) for growth in medium containing 1% serum, saturation densities, and efficiency of plating in 1.2% methylcellulose. The level of fibronectin was determined by an immunofluorescence assay as described previously (6), using antisera generously provided by L. B. Chen.

Tumorigenicity tests. Cells were removed from the dish by scraping, washed in phosphate-buffered saline, and suspended in a small volume of serum-free DME. Cells (5×10^5 to 5×10^6) were inoculated subcutaneously or intraperitoneally (0.1 ml per inoculum) into 3-week-old congenitally athymic nude mice or newborn NIH/Swiss mice. Animals were monitored daily for palpable tumors. Once a substantial mass developed, the animal was sacrificed, the tumor was excised, and DNA was extracted from half of the tissue; the remaining piece was minced and trypsinized, and a cell culture was established from the tumor cells.

Assay for type C particles. Transformed cell lines derived from NIH 3T3 cells were tested for type C particles at passage 10 by assaying for reverse transcriptase activity. Cell debris was removed from the tissue culture fluid by low-speed centrifugation, and the supernatant was centrifuged in a Beckman type 30 rotor for 1 h at 25,000 rpm. The pellet was assayed for incorporation of [³H]dGTP (New England Nuclear Corp.) into acid-precipitable material, using 1 µg of (C)_n (dG)₁₂₋₁₈. The lines were also tested by a focus formation assay, using the rat cell line NRK-2 as an indicator. The NIH 3T3-derived cells were cocultivated with NRK (MSV-C11a) cells, and the two were overlaid with NRK-2 cells. As a positive control, NRK (MSV-C11a) cells infected with BALB virus 2 were used. Advice for this assay and the cell lines used in it were provided by R. Nowinski.

Restriction enzymes, gel electrophoresis, blotting, nick translation, and hybridization. All of the restriction enzymes were purchased from Bethesda Research Laboratories, Rockville, Md., and used under the specified conditions. Agarose gel electrophoresis was carried out in horizontal slab gels, using 0.4% agarose to separate fragments of HSV-2 DNA and 0.7% agarose to fractionate DNA from the recombinant plasmids and transformed cells. The gels were stained with ethidium bromide and photographed. DNA was denatured in situ and transferred to nitrocellulose sheets (42) as described previously (3). The introduction of ³²P-labeled nucleotides into viral DNA for use as hybridization probes was carried out as described (31), using the nick translation method (39). DNA polymerase I was purchased from Boehringer Mannheim Corp. Specific activities of 5×10^7 to 1×10^8 cpm/µg were routinely obtained, using two radio-labeled triphosphates (New England Nuclear Corp.;

specific activity, approximately 500 Ci/mmol). The hybridization procedure, pretreatment of the nitrocellulose filters, and their subsequent washing was performed as described previously (3). The filters were placed in contact with Kodak XR-1 film and Du Pont Lightning Plus intensifier screens at -70°C for periods of 5 to 14 days.

RESULTS

Identification of the *Bgl*II N fragment with transforming activity. HSV-2 DNA was digested with several restriction endonucleases, and the entire collection of fragments was used in transformation experiments. Transformants were produced with DNA digested with *Hind*III, *Bgl*II, and *Xba*I (data not shown). Fragments of *Bgl*II-cleaved DNA were chosen for further study because at least one produced transformants and because a reasonable number of fragments were generated. *Bgl*II recognizes 12 sites in HSV-2 DNA (40), which produces 18 fragments because of the heterogeneity resulting from isomerization of the viral genome (25) (Fig. 1). A 100- μg amount of viral DNA was fractionated through 0.4% agarose, and the individual fragments or group of fragments (Fig. 1) was extracted from the agarose by electroelution and extraction with phenol. No attempt was made to add the equivalent amount of DNA to each plate, so the quantity of a given fragment ranged from approximately 0.5 μg of *Bgl*II-OP to 5 μg of *Bgl*II-CDEF. The only fragment which altered cells in a way that allowed them to form colonies in methylcellulose or to form dense foci was the *Bgl*II N fragment. These experiments are not presented fully because they merely confirm the observations of Reyes et al. (38).

To study the transformation of rodent cells by fragments of the HSV-2 genome in greater detail, the *Bgl*II N fragment was cloned into pBR322 (1). The description of its construction, selection, and characterization was reported previously (19). The sites of cleavage with 16 restriction endonucleases were determined (Fig. 2). The size of the entire plasmid, designated pDG401, is approximately 11.46 kilobases (kb), suggesting that the size of the inserted *Bgl*II N fragment is 7.1 kb. Restriction and hybridization analyses of pDG401 have revealed the following features: (i) the sites at which the *Bgl*II N fragment is ligated to the *Bam*I site of pBR322 are resistant to cleavage by both *Bam*I and *Bgl*II; (ii) the orientation of the *Bgl*II N fragment is such that the left end (position 0.582) is near the *Hind*III site of pBR322 (1); (iii) the distance between the *Bgl*II site at position 0.582 and the *Bam*I site at position 0.584 (38) is shorter by 300 nucleotides than the size predicted by digestion of the authentic viral fragment; (iv)

FIG. 1. Restriction endonuclease cleavage pattern of HSV-2 DNA after hydrolysis with *Bgl*II. Purified (47) HSV-2 DNA (100 μg) was digested with *Bgl*II, and the fragments were separated by electrophoresis through a horizontal slab of 0.4% agarose. Seven fragments or groups of fragments, as indicated by the roman numerals, were sliced from the gel. DNA was removed from the agarose by electrophoresis into dialysis tubing, extracted with phenol, and precipitated with ethanol. The position of the fragments (40) along the HSV-2 genome is shown in the bottom line. *Bgl*II-N is located between map positions 0.58 and 0.62.

the cleavage site for *Xho*I near position 0.582 (38) is not present; (v) there are no cleavage sites for *Bgl*II, *Xba*I, and *Kpn*I in pDG401; (vi) with the exception of the 300-base-pair deletion, the recombinant plasmid appears to be identical to the authentic viral fragment and to pBR322; and (vii) sequences from the HSV-2 genome, other than those from the *Bgl*II N fragment, could not be detected in pDG401 (19).

Previous experience with the transfer of the thymidine kinase phenotype to mouse cells, using a cloned fragment of viral DNA, indicated that the efficiency of transformation was improved by cleaving DNA from a circular plasmid to render it linear (32). Therefore, before a DNA-calcium precipitate was formed, pDG401 was cleaved with *Hind*III, which recognizes a single site in the plasmid located within the sequences of pBR322. Other plasmid DNAs which were used as controls were similarly cleaved whenever a single-cut restriction enzyme could be identified. In addition to pDG401, NIH 3T3 cells and embryonic rat cells were exposed to DNA from the following plasmids: pBR322, the parental

FIG. 2. Restriction map of pDG401 DNA. The thick line indicates the sequences homologous to the HSV-2 *BglIII* N fragment, and the thin line indicates the pBR322 moiety of the molecule. The junction between the viral and plasmid sequences contains a hybrid site which is resistant to cleavage by both *BamI* and *BglII*. The numbers inside the circle refer to the size of the plasmid (kb).

vector, and pDG305, pDG304, pDG301, pDG206, pDG201, and pDG102. The location of sequences of HSV-2 DNA contained in these plasmids is (Fig. 1) *BglIII*-G, *BglIII*-J, *BglIII*-O, a *BamI* fragment located within *BglIII*-I, a *BamI* fragment which spans the L-S junction, and a *HindIII* fragment located within the unique S region, respectively (19).

We used four concentrations of plasmid DNA in the transformation experiments: 0.5, 1, 5, and 20 μ g of DNA per 60-mm plate. For the first three samples, calf thymus DNA, NIH 3T3 DNA, or rat embryo DNA was used as a carrier, so that the final concentration of DNA was 20 μ g per 60-mm plate. After exposure to DNA, transformed cells were selected by either the focus or colony formation assay as described above. In general, the colony formation assay

proved to be more satisfactory because colonies could be recognized unambiguously, whereas dead clumps of cells occasionally appeared as dense foci. A summary of the frequency of transformation of DNA from pDG401 is given in Table 1. Transformation was not obtained with DNA from any of the other plasmids. In one experiment, colonies were observed 2 weeks after exposure to DNA from pDG305 and pDG304, which contain the *BglIII* G and J fragments. By 4 to 5 weeks, the colonies began to disintegrate, and attempts to remove the cells from methylcellulose and establish tissue culture lines were negative. This phenomenon was only observed once, and whether it represents an abortive transformation has not been determined. The only cells which regularly formed colonies or foci and from which permanent cell lines could be

established were those that had been exposed to pDG401. The transformation efficiency per microgram of the *Bgl*II N sequences in pDG401 was four foci or colonies per 10^6 cells, which is similar to that reported for the purified *Bgl*II N fragment (38) of HSV-2 and for the *Hsu*I G fragments of adenovirus types 2 and 5 (46).

Properties of the transformed cells. DNA virus transformants are commonly defined by an alteration in growth properties. Typically, transformed cells are able to grow to high densities, proliferate in medium which is low in serum, and form colonies in methylcellulose at an appreciable frequency. When the HSV-2 DNA transformants were tested by these criteria, the results shown in Table 2 were observed. All of the cell lines derived from NIH 3T3 cells are able to grow to high saturation densities and to grow well in low serum, as compared with the parental cells. The cell lines derived from rat embryo cells did not exhibit such marked changes. The efficiency of plating in methylcellulose was significantly high among the transformants and increased with passage of the cells in vitro and, particularly, in vivo. The initial values for cloning

in methylcellulose were in the same range as those obtained with cells transformed by the left-hand 7.5% of the adenovirus genome (0.03 to 0.85%) (46), but were considerably lower than the values reported for hamster cells transformed with the HSV-2 *Bgl*II C fragment (26).

The in vitro morphology of the cell lines was variable. The lines which were derived from NIH 3T3 cells showed greater variation, ranging from purely fibroblastic to purely epithelial. The characteristic morphology was maintained over many passages. The lines which were derived from rat embryo cells were generally more fibroblastic in appearance. The rat cell lines showed some variation in their growth patterns in tissue culture dishes in that some lines always formed multilayered arrangements, whereas other lines grew as densely packed foci. Figure 3 shows two of the more common morphologies, with a colony growing in methylcellulose and the establishment and growth of the line in tissue culture.

The cytogenetics of hamster cell lines transformed by UV-inactivated HSV-2 suggested that the lines were generally hyperdiploid and contained a spectrum of marker chromosomes (8, 35). Our findings with the rat cell lines are consistent with these observations (data not shown).

The tumorigenicity of cells transformed by fragments of HSV-2 was investigated by inoculating 10^6 cells either subcutaneously or intraperitoneally into 3-week-old athymic nude mice (Table 3). Even at early passages, most of the transformed cell lines examined (three of four) were tumorigenic in 100% of the animals by 4 weeks post-inoculation. The tumors appeared to be fibrosarcomas, and in some cases, invasion into the muscular walls could be detected. Histological examination confirmed these observations (C. Fenoglio, unpublished data). The tumors showed no signs of regression during the

TABLE 1. Frequency of transformation by pDG401

DNA (μ g) ^a	No. of foci per 60-mm dish		No. of colonies per 60-mm dish	
	Rat embryo	NIH 3T3 cells	Rat embryo	NIH 3T3
0.5	0, 0	2, 3	0, 0	1, 3
1.0	3, 2	2, 4	1, 0	2, 5
5.0	6, 6	5, 8	3, 4	4, 7
20.0	7, 9	11, 15	11, 9	14, 20

^a Amount of pDG401 DNA cleaved with *Hind*III and mixed with carrier DNA to a total of 20 μ g, which was precipitated on each 60-mm dish. The results of two experiments are given.

TABLE 2. Growth properties of transformed cells

Cell line ^a	Passage	Doubling time ratio (10% FCS/1% FCS)	Saturation density (cells per $\text{cm}^2 \times 10^5$)	Saturation density ratio (10% FCS/1% FCS)	Efficiency of plating (%) in methylcellulose (10% FCS)
XRH	10	0.81	3.2	2.5	0.80
XRH-mc	20	0.84	4.3	2.8	80.0
401 RH4	10	0.63	1.4	6.0	0.06
Rat embryo	10	∞^b	1.3	$>10.0^b$	<0.002
401 NIH-1	10	0.77	5.2	5.9	72.0
401-T1	3	NT ^c	NT	NT	74.0
401 NIH-0-1	10	0.75	10.4	4.5	0.64
401-0-T1	3	NT	NT	NT	58.0
NIH 3T3	10	0.33	1.2	7.9	<0.002

^a The first three cell lines are derived from rat embryo cells. XRH-mc is derived from a methylcellulose colony of XRH. The second set of cell lines are derived from NIH 3T3 cells or tumors (T1 and 0-T1) induced in nude mice.

^b Rat embryo cells did not grow in 1% serum.

^c NT, Not tested.

FIG. 3. *Morphology of transformed cell lines. The cell lines shown are NIH 3T3 (A), 401 NIH-0-1 (B), a multicellular colony growing in methylcellulose from which 401 RH4 was established (C), and 401 RH4 (D).*

entire observation period. Tissue culture lines were established from the tumors which were excised from the nude mice, and the tumorigenicity of these cells was assayed in newborn animals (Table 3). The tumor lines were highly oncogenic in newborn NIH/Swiss mice, often producing palpable tumors in 7 days which grew to such a large size by 14 days that the animals had to be sacrificed.

In numerous studies, the relationship between tumor induction, the loss of fibronectin, and the loss of anchorage independence has been investigated. The number of fibronectin-positive cells among the initial transformants and the tumor lines was examined by immunofluorescence (Table 3). Our results do not show any direct correlation between the ability to form tumors and the absence of fibronectin or the efficiency of cloning in methylcellulose. However, the cell lines derived from tumors had no fibronectin on the surface of the cells and exhibited a higher

efficiency of growth in methylcellulose. These results argue that growth *in vivo* selects for cells with these phenotypes, in agreement with Galimore et al. (16).

The appearance of type C virus is not uncommon in cultures transformed by HSV (13, 22) or other agents (23). In cultures of Swiss 3T3 cells transformed by UV-inactivated HSV-2, the medium contained particles with a density characteristic of type C particles by passage 10, and oncornavirus antigens could be detected in the cells by immunofluorescence (13). NIH 3T3 cells do not produce ecotropic or xenotropic murine leukemia virus either spontaneously or after exposure to halogenated pyrimidines (43); however, they contain a xenotropic murine leukemia virus (30). We wished to determine whether transformation of NIH 3T3 cells induced the endogenous type C virus. Attempts to detect reverse transcriptase activity in medium from transformed cells gave negative results. We

TABLE 3. *Tumorigenicity and levels of fibronectin in transformed cells*

Cell line	Tumors in ^a :		Fibro- nectin- positive Cells (%)
	Nude mice	NIH/ Swiss mice	
XRH	4/4 (43) ^b		70
XRH Tu1			0
401 RH4	0/4 ^c		50
RE	0.4		95
401 NIH-1	4/4 (29)		50
401 T1		4/4	0
401 NIH-O-1	4/4 (26)		20
401 OT1		4/4	0
NIH 3T3	0/4		95

^a Animals were inoculated with 10^6 cells intraperitoneally and subcutaneously.

^b Number within parentheses indicates the number of days at which 100% of the mice had tumors.

^c Animals died at 41 days.

turned to a more sensitive assay in which the cells could be assayed directly. The transformed cells were cocultivated with permissive nonproducer rat cells and tested for the synthesis of focus-forming virus on rat cells. The results of these experiments were also negative, suggesting that transformation of NIH 3T3 cells with pDG401 does not activate a type C virus except, perhaps, transiently or at levels which cannot be detected by these assays.

Analysis of viral DNA in the transformed cells. DNA from the transformed cells was digested with restriction endonucleases, transferred to nitrocellulose filters, and hybridized to 32 P-labeled pDG401. Initial attempts to detect viral sequences in 5 μ g of DNA from the transformed cells were encouraging but not convincing. When 20 μ g of transformed cell DNA was analyzed, radioactive bands could be consistently detected. An example of the pattern of viral DNA sequences observed in two cell lines, one derived from NIH 3T3 cells and one derived from rat embryo cells, is shown in Fig. 4. The arrangement of viral sequences was examined in four lines of cells which had been transformed by pDG401 and in two tumors from nude mice which had been inoculated with cells from one of the transformants: 401 RH2, 401 RH4, 401 NIH-1, 401 NIH-O-1, 401 NIH-O-1 Tu1, and 401 NIH-O-1 Tu2. All of the DNAs shared common features with regard to the pattern of viral sequences: (i) sequences homologous to pDG401 were detected in DNA from all six lines, and those sequences were not present in DNA from normal mice or rats (Fig. 4 and 5; data not shown); (ii) the virus-specific sequences were associated with high-molecular-weight DNA

(Fig. 4, 5, and 6); (iii) the position (i.e., electrophoretic mobility) of the radioactive bands was not uniform among the various cell lines (Fig. 4, 5, and 6); (iv) the intensity of the radioactive bands as compared with that from a reconstruction suggested that the content of pDG401 sequences in the four transformed cell DNAs was approximately 0.1 copy of plasmid DNA per diploid genome of cell DNA, whereas the intensity of the bands from the tumor tissue was slightly greater, suggesting a content of about 0.5 copy (Fig. 5 and 6); (v) cleavage of pDG401 with *Bam*I produced an authentic 3.5-kb viral fragment, which was not detectable when the transformed cell DNA was cleaved with *Bam*I (Fig. 4 and 6), and similarly, no bands corresponding to authentic *Sac*I fragments could be detected (data not shown); (vi) cleavage of pDG401 DNA with *Sa*II produced three fragments of 8.4, 1.6, and 1.4 kb, and for at least one

FIG. 4. Detection of fragments of DNA that contain pDG401 sequences after hydrolysis of transformed cell DNA with endonuclease *Bam*I. High-molecular-weight cell DNA was extracted from the transformed cells, and 20 μ g of DNA was digested with *Bam*I. The products of digestion were fractionated by electrophoresis in slab gels through 0.7% agarose, transferred to a nitrocellulose filter, hybridized to 32 P-labeled pDG401 DNA, and exposed for autoradiography. The slots labeled C and R each contained 20 μ g of DNA extracted from NIH 3T3 cells, and R also contained 10^{-5} μ g of pDG401 DNA which represents 0.5 copy of plasmid DNA per diploid genome of rodent DNA. The sites at which pDG401 DNA are cleaved by *Hind*III (H) and *Bam*I (B) are shown at bottom of figure.

FIG. 5. Detection of fragments of DNA that contain pDG401 sequences after hydrolysis of transformed mouse cell DNA and tumor DNA with endonuclease *Hind*III. High-molecular-weight cell DNA was extracted from the transformed cells and from two tumors excised from a nude mouse which had been inoculated with 10^6 401 NIH-O-1 cells, and 20 μ g was digested with *Hind*III. The products of digestion were treated as described in the legend to Fig. 4. The nitrocellulose filter was hybridized to ³²P-labeled pDG401 DNA (A) and to ³²P-labeled pBR322 DNA (B).

line, a fragment corresponding to a 1.6-kb fragment of authentic viral DNA could be detected (Fig. 7B); (vii) hybridization with specific subfragments of pDG401 revealed that the transformed cells retained sequences homologous to pBR322 (Fig. 5B) and to HSV-2 when probed with a *Bst*EII fragment (Fig. 7). Sequences homologous to the 3.9-kb *Bam*I fragment could not be detected (data not shown). These results locate the transforming sequences within the right half of the *Bgl*III N fragment, and they suggest that the plasmid DNA may be integrated at random locations into the rodent genome.

An interesting observation was made in examining the pattern of viral sequences present in tumor tissue. Cleavage of DNA from 401 NIH-O-1 with *Hind*III or *Bam*I produced a single virus-specific band of approximately 12 and 8 kb, respectively (Fig. 5 and 6). The cells were tumorigenic in nude mice, and in one animal two tumors were excised, and the DNA from each tumor was analyzed. The pattern of viral sequences after digestion with either *Hind*III (Fig. 5) or *Bam*I (Fig. 6) was not identical in the two tumors, nor did the viral sequences in either

tumor resemble the band seen in the transformed cell DNA. The DNA from Tu1 gave two bands upon cleavage with *Hind*III, a prominent high-molecular-weight band (<15 kb) and a faint band of approximately 8 kb. Cleavage of DNA from Tu2 resulted in a single virus-specific band with a lower molecular weight of around 7 kb. When pBR322 was used as a probe (Fig. 5B), it was apparent that the larger band in Tu1 DNA did not contain any sequences homologous to the pBR322 moiety of the plasmid; however, these sequences were present in the smaller fragment of Tu1 DNA as well as in the DNA of Tu2 and 401 NIH-O-1. The same phenomenon was observed after cleavage of the transformed cell and tumor DNAs with *Bam*I, in that neither of the three patterns was identical. Tu1 and Tu2 each showed a single virus-specific fragment of *Bam*I-cleaved DNA of about 5.5 and 5 kb, respectively (the spot above the band in Fig. 6A is an artifact), which can be detected by hybridization with DNA from pDG401 (Fig. 6A), pBR322 (Fig. 6B), or the *Bst*EII fragment of pDG401 (Fig. 7A). Taken together, these results indicate that at least a fraction of the cells in each cell line which was tested retain sequences

homologous to a portion of pDG401. The implication of these findings will be discussed in the next section.

DISCUSSION

The use of a cloned fragment of HSV-2 DNA in transformation experiments has demonstrated unambiguously that sequences of viral DNA located between map positions 0.58 and 0.62 on the HSV-2 genome can alter the phenotype of primary rat cells and NIH 3T3 cells in a fashion which allows them to grow in medium containing low serum, reach high saturation densities, form colonies in semisolid medium, and produce tumors in nude mice and newborn animals. The cell lines which have been obtained after exposure to pDG401 have several features which are characteristic of DNA virus transformants generally and of herpesvirus transformants specifically. Although the HSV-transformed cells reported on here and elsewhere (5; C. D. Copple, unpublished data) replicate better in low serum than do nontransformed cells, they do not show the extreme differences observed with simian virus 40 transformants. Similarly, the saturation densities of the transformants are higher than normal but not extraordinarily so, in agreement with others (24). The most pro-

nounced difference between cells transformed by pDG401 and their parental cells is the loss of anchorage dependence, as measured by colony formation in methylcellulose and tumor formation in animals. The transformants show varying efficiencies of cloning in methylcellulose, whereas rat embryo cells and the NIH 3T3 cells used in this study have virtually no capacity for growth in semisolid medium and are not tumorigenic.

A preliminary assessment of the arrangement of viral sequences in cells transformed by pDG401 suggests some unusual possibilities not generally associated with DNA virus transformants, but which are not inconsistent with previous observations about the content of HSV DNA in transformed cells. Measuring the content of viral sequences in transformed cells by estimating the intensity of bands on an autoradiograph is not the most accurate method, but despite the pitfalls (e.g., efficiency of transfer), there generally has been a good correlation between intensity and copy number when measured by reassociation kinetics (18). It seems a reasonable estimate, then, that cells transformed by pDG401 retain an equivalent of 0.1 copy per diploid genome. The persistence of a small quantity of viral DNA may be a general feature of

FIG. 6. Detection of fragments of DNA that contain pDG401 sequences after hydrolysis of transformed mouse cell DNA and tumor DNA with endonuclease *Bam*I. High-molecular-weight DNA extracted from the transformed cells and tumors described in the legend to Fig. 5 was digested with *Bam*I and treated as described. (A) and (B) show the results of hybridization with ³²P-labeled pDG401 DNA and ³²P-labeled pBR322 DNA, respectively.

FIG. 7. Detection of fragments of DNA that contain herpesvirus sequences after hydrolysis of tumor DNA with endonuclease *Bam*I and *Sal*I. High-molecular-weight DNA was extracted from the tumors described in the legend to Fig. 5, and 20 μ g of DNA was digested with *Bam*I (A) or *Sal*I (B). The nitrocellulose filters were hybridized with a 32 P-labeled authentic herpesvirus fragment obtained by cleavage of pDG401 DNA with *Bst*EII. In data not shown, the *Bst*EII fragment was hybridized to pDG401 DNA which had been cleaved with *Bam*I or *Sal*I. Both *Bam*I fragments were detected, and the larger two of three *Sal*I fragments were detected. The smallest *Sal*I fragment spanning the pBR322-HSV junction was not detected; therefore, the presence of these sequences in the tumor DNA could be determined. (A) and (B) are not from the same gel; therefore, the mobility of DNA fragments cannot be compared.

cells transformed by fragments of HSV DNA and may explain the difficulty in detecting viral sequences in these cells (38) and perhaps even in cells transformed by UV-inactivated HSV (10, 34).

There are three possible explanations for the small amount of pDG401 DNA in the transformed cells: (i) each cell retains approximately 0.1 copy of plasmid DNA (1.14 kb); (ii) the cell population is extremely heterogeneous with respect to the sites in the genome at which viral sequences are retained, so that cleavage with a restriction enzyme does not produce a distinct set of bands; or (iii) not all cells retain viral sequences, and those that do contain more than 0.1 copy. The first possibility does not seem likely because the cells retain sequences homologous to pBR322 as well as the HSV-2 moiety, so that the viral information could only specify

a protein of less than 30,00 daltons. In addition, an authentic 1.6-kb *Sal*I fragment has been identified in a cell line which also contains sequences homologous to pBR322. The most likely arrangement would be that these cells retain at least 3 kb of pDG401 DNA. The second possibility is harder to rule out because although the transformed cell lines have a clonal origin, rapid heterogeneity could occur during passage of the cells in tissue culture. This situation is not observed with simian virus 40- or adenovirus-transformed cells in which no alteration is observed in the arrangement of integrated viral DNA or its flanking cellular sequences when transformed cells are grown in culture for hundreds of generations (3, 41). One fact which argues against this possibility is that the internal fragment produced by cleavage with *Sal*I is not more intense than the joint fragments, which would be predicted if every cell retained the same set of viral sequences located at different sites within the host cell genome.

At this time, we favor the possibility that not every cell retains plasmid sequences. Whether those cells that do not contain viral sequences remain fully transformed by a hit-and-run mechanism or whether they die with passage of the cells is not known. Based on the observation that two tumors seemingly retained more viral sequences per cell than did the original transformed cell line, it is tempting to speculate that growth in vivo selects for cells which retain viral DNA. Experiments to test this hypothesis are in progress. These data shed little information as to the state of the viral sequences within the transformed cell. That the viral bands are associated with high-molecular-weight DNA is suggestive but not conclusive evidence that the sequences are integrated. On the other hand, if the plasmid DNA is episomal it could be lost with passage of the cells, and that would explain why not every cell retains viral sequences. The observed changes in the pattern of viral DNA in two tumors from the same animal and the transformed cell line used to induce the tumors are intriguing. It indicates that rearrangements of the transforming sequences occur very rapidly during growth of the cells in vivo; yet a mechanism to generate the rearrangement is unclear. Recombination, excision and reintegration, integration of previously unintegrated DNA, or selection of different cells from a heterogeneous population are all possible explanations for the genesis of rearrangements.

It may be appropriate to ask whether we have moved any closer toward understanding the factors involved in transformation by HSV. Our results indicate that a single region of the HSV-2 genome, defined by the *Bgl*II N fragment,

alters the phenotype of normal rodent cells to cause them to have abnormal growth properties and to become tumorigenic. The method of selecting transformed cells (focus or colony formation versus continuous passage) may explain why one region of the HSV-2 genome confers this phenotype in one assay (38; these results) and a different region of the genome confers the same phenotype when a different selection procedure is used (26). The results of experiments to locate transforming sequences of HSV-2 DNA in a position homologous to the HSV-1 transforming gene have been negative, but the experiments may have failed due to an unfortunate set of restriction sites. The proof that at least a portion of pDG401 causes transformation awaits the creation of transformation-deficient mutants. The identification of a protein(s) encoded by pDG401 which may mediate transformation will be an essential step toward understanding the mechanism by which HSV-2-causes-oncogenic-transformation.

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