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Analysis of viral DNA sequences in hamster cells transformed by herpes simplex virus type 2

(renaturation kinetics/HSV-2 strain 333/ultraviolet light-inactivated virus transformants)

DENISE A. GALLOWAY*†‡, CHRISTINE D. COPPLE*, AND JAMES K. MCDOUGALL*†‡

*Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724; and †Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104

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ABSTRACT **Herpes simplex virus type 2 (HSV-2) DNA was treated with four restriction endonucleases (*Eco*RI, *Hin*cIII, *Bgl* II, and *Xba* I) and eight fragments were purified and labeled with ^{32}P *in vitro*. The kinetics of renaturation of each of the fragments was measured in the presence of DNA extracted from 333-8-9, a hamster cell line transformed by UV light-inactivated HSV-2 strain 333, and from a series of cloned derivatives and their tumor lines. All of the lines examined contained a partial set of viral sequences present at only a few copies per cell. Passage of the cell lines in tissue culture or in animals resulted in partial loss of viral DNA. Two blocks of sequences were present in most of the lines examined; those mapping at positions 21–33 of the HSV-2 genome were detected in seven of seven cell lines tested and those at positions 60–65 were detected in six of eight. Other sequences from the L component can also be present in the DNA of HSV-2-transformed hamster cells.**

Primary rodent cells show altered growth properties after exposure to various debilitated forms of herpes simplex virus types 1 and 2 (HSV-1, HSV-2) (1–5). Of these transformants, the best characterized are hamster cells transformed by HSV-2 strain 333 which had been inactivated by ultraviolet light (1). A focus of dense cells was picked and became the cell line known as 333-8-9, which was subsequently discovered to be highly pleomorphic (3, 6). Cytogenetic observations showed a wide spectrum of marker chromosomes (6, 7). In order to produce more homogeneous cell lines, and with the hope that various properties of the transformants would segregate, the original cell line was cloned (6). Twenty clones were tested and showed segregation of marker chromosomes, of *in vitro* growth properties, and of oncogenicity (6). The viral DNA sequences in the cloned derivatives were analyzed in order to determine whether a common set of viral DNA sequences is retained by HSV-2 transformed cells. The results are presented in this paper.

The first evidence that 333-8-9 cells contained viral information came from the detection of viral RNA by molecular hybridization (8). The sequences transcribed represented 11% of the HSV genome and were common to both HSV-1 and HSV-2. The literature concerning attempts to detect viral DNA sequences is controversial, in part because of previous difficulties in the preparation of sufficiently sensitive radioactive probes (for a discussion, see ref. 9). An early reported attempt to detect viral DNA sequences in 333-8-9 was negative (10). Subsequently, viral DNA was detected in 333-8-9, in a series of other hamster cells transformed by HSV-2, and in a tumor induced by the inoculation of 333-8-9 cells (11). Using HSV-2 DNA labeled *in vitro* with ^{32}P (5×10^8 cpm/μg) as probe, Frenkel *et al.* (11) showed that various lines contained sequences comprising 8–32% of the HSV genome present at one to three copies per cell. Another study (12) detected 40% of the HSV-2 genome in an early passage, 45, of 333-8-9 cells but no viral sequences in cells at a later passage, 80. By *in situ* hybridization using an iodinated HSV DNA probe, viral RNA was visualized in most of the clonal derivatives of 333-8-9 (6) although a search for viral DNA gave negative results (12).

It has been clearly demonstrated that the most accurate method of measuring the amount of viral DNA in transformed cells that contain only a partial copy of the viral genome or that contain some parts of the viral genome in a much higher frequency than others is to use defined segments of viral DNA as probes in the hybridization reaction (13, 14). To this end, restriction endonuclease fragments of HSV-2 DNA were produced. Cleavage of the HSV genome with restriction endonucleases yields fragments in various molarities as a result of the four configurations of the viral genome (15, 16). Some of these fragments were isolated and made highly radioactive *in vitro* (17), and a small amount was hybridized with large quantities of unlabeled DNA isolated from transformed and control cells.

MATERIALS AND METHODS

Cells and Viruses. The properties of the transformed cells used in this study have been described (6). Cells were cultured in an atmosphere of 10% CO_2/90% air in Dulbecco's modification of Eagle's medium (Bio-Rad) with 10% fetal calf serum (Irvine) on plastic tissue culture plates (Falcon). HSV-2 strain 333 was propagated on monolayers of BSC-1 cells infected at low multiplicity (0.01 plaque-forming units per cell). Viral DNA was isolated by equilibrium gradient centrifugation through sodium iodide gradients containing ethidium bromide (18).

Restriction Enzymes, Gel Electrophoresis, Blotting, Nick-Translation, and Hybridization. All of the restriction enzymes used were either purchased from Bethesda Research Labs., Rockville, MD or were the generous gift of Ronni Greene. The reaction conditions have been described (19). HSV-2 DNA was fractionated by gel electrophoresis in horizontal slab gels cast with 0.4% agarose. The gels were stained with ethidium bromide and photographed (20). DNA was denatured *in situ* and transferred to nitrocellulose sheets (21) as described (22). Introduction of ^{32}P-labeled nucleotides into viral DNA for use as hybridization probes was carried out as described (17). DNA polymerase I was purchased from Boehringer Mannheim. The purification of the probe and tests of its integrity are described in *Results*. The hybridization procedure, pretreatment of the nitrocellulose filters, and their subsequent washing were performed as described (22).

DNA Reassociation. The preparation of DNA from transformed cells, hybridization conditions, detection of hybrids by

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Abbreviations: HSV-2, herpes simplex virus type 2; mtr, morphological transforming region.

‡ Present address: Fred Hutchinson Cancer Research Center.

hydroxyapatite chromatography, and calculations were carried out as described (13). Instead of using viral DNA labeled *in vivo*, we prepared fragments of unlabeled viral DNA and then made them radioactive *in vitro*.

RESULTS

Preparation and Characterization of Fragments of HSV-2 DNA Cleaved by Restriction Endonucleases. Restriction endonuclease maps for HSV-1 and HSV-2 DNAs have been published in several reports and were recently summarized (23). As a consequence of the inversions of the L and S components, fragments that come from the terminal or junction portions of the genome are less frequently represented among the digestion products and are therefore difficult to isolate. Eight fragments from four different restriction enzyme digests were purified. Although not providing complete coverage of the viral genome, 80% of the unique sequences as well as the repetitive DNA of the short component are represented. A further advantage is that these fragments can be isolated with minimal contamination. The fragments used as probes are shown in Fig. 1.

After digestion with an endonuclease, HSV-2 DNA (100 μg) was fractionated by electrophoresis through 0.4% agarose, visualized with ethidium bromide, removed from the gel by electro-elution, extracted with phenol, and precipitated with ethanol. The fragments (0.5 μg) were made radioactive by nick-translation with deoxynucleoside [^{32}P]triphosphates to specific activities of 2.5×10^7 to 8×10^7 cpm/μg. The purity of the ^{32}P-labeled fragments was determined by hybridization to nitrocellulose strips containing fragments of HSV-2 DNA produced by digestion with *Eco*RI and *Bgl* II. Two examples are shown in Fig. 2: the *Hin* III B probe hybridized to the terminal and junction fragments *Eco*RI A, B, and D and to the internal fragments *Eco*RI I and *Bgl* II G and P. Slight contamination was detected by faint hybridization to *Eco*RI K and to *Bgl* II I and N. With the *Eco*RI K fragments as probe, hybridization was only detected with *Eco*RI K and *Bgl* II I. These two examples represent the extremes; in one case no contamination was detected whereas, with the larger fragment, cross-hybridization was easily seen. All of the fragments used were estimated to be greater than 90% pure.

Sequences of HSV-2 Present in Transformed Cells. Before using the fragments of HSV-2 DNA to analyze the viral sequences in the genomes of transformed hamster cells, it was necessary to show that the fragments were capable of hybridizing to unlabeled viral DNA. Each panel in Fig. 3 shows that the rates of hybridization of the ^{32}P-labeled DNAs were accelerated in the presence of unlabeled viral DNA by roughly the amounts expected for the concentration of HSV-2 DNA

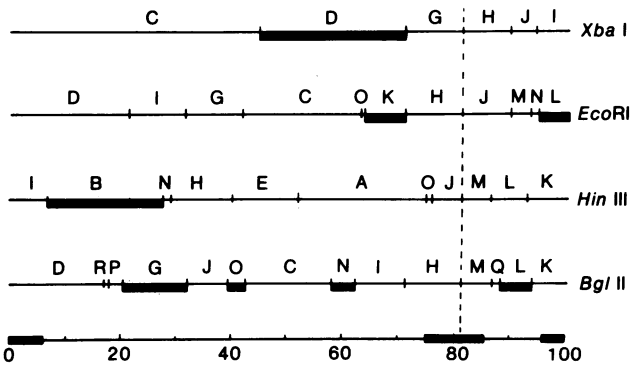

FIG. 1. Location of hybridization probes along the restriction endonuclease cleavage maps of HSV-2 DNA. The maps are taken from published data (15, 16, 23). The solid blocks indicate the fragments that were used as probes.

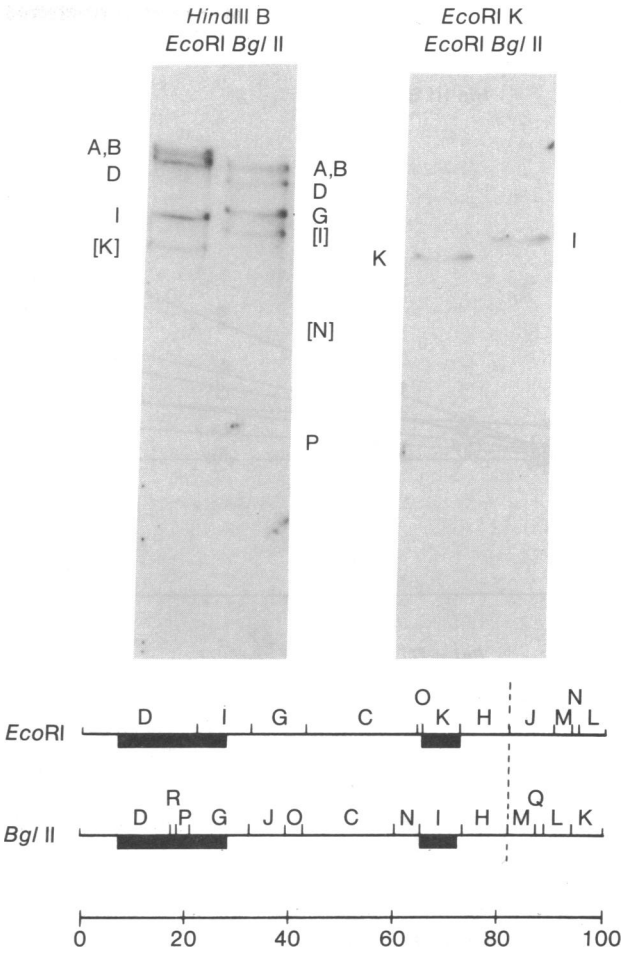

FIG. 2. Hybridization of ^{32}P-labeled fragment probes to restriction digests of HSV-2 DNA. HSV-2 DNA (1 μg per lane) was digested with *Eco*RI or *Bgl* II at 37°C for 2 hr in a total volume of 0.1 ml containing 0.1 M Tris (pH 7.2), 0.05 M NaCl, 5 mM $MgCl_2$, and 2 mM 2-mercaptoethanol or 0.02 M Tris (pH 7.4), 7 mM $MgCl_2$, and 7 mM 2-mercaptoethanol, respectively. The DNA was fractionated by gel electrophoresis and transferred to nitrocellulose sheets. Purified fragments were nick-translated to specific activities of about 6×10^7 cpm/μg. The fragments (10^7 cpm) were hybridized to strips for 16 hr at 68°C, washed, and exposed for autoradiography as described (19, 22).

added. The rate of renaturation was nearly as predicted when 1 copy of HSV-2 was added, but the acceleration was consistently slower than expected in the presence of 5 or 10 copies of HSV-2—e.g., the acceleration of *Bgl* II G was 6-fold when 10 copies were added, and for *Bgl* II N, 2.4-fold faster when 5 copies were added.

In order to assay DNA extracted from transformed cells for sequences homologous to each of the fragments of HSV-2 DNA, the rate of hybridization of the ^{32}P-labeled fragments was measured in the presence of unlabeled DNA extracted from the HSV-2-transformed hamster cell line 333-8-9, several of the clonal derivatives (e.g., C 1, C 8, C 18), subclones of one of those lines (e.g., C 18.1, C 18.4), lines established from tumors induced by the inoculation of C 18.4 cells into hamsters (e.g., Tu 35, 2 Tu 1), and control cells that had never been exposed to the virus (BHK, and a hamster line transformed by simian virus 40). Each hybridization mixture contained the same concentration of ^{32}P-labeled DNA and identical concentrations of cell DNA; this has been judged to be the most sensitive method for determining the absence, from transformed cells, of parts of the probe (13). Thus, the ratio of the number of cellular genomes

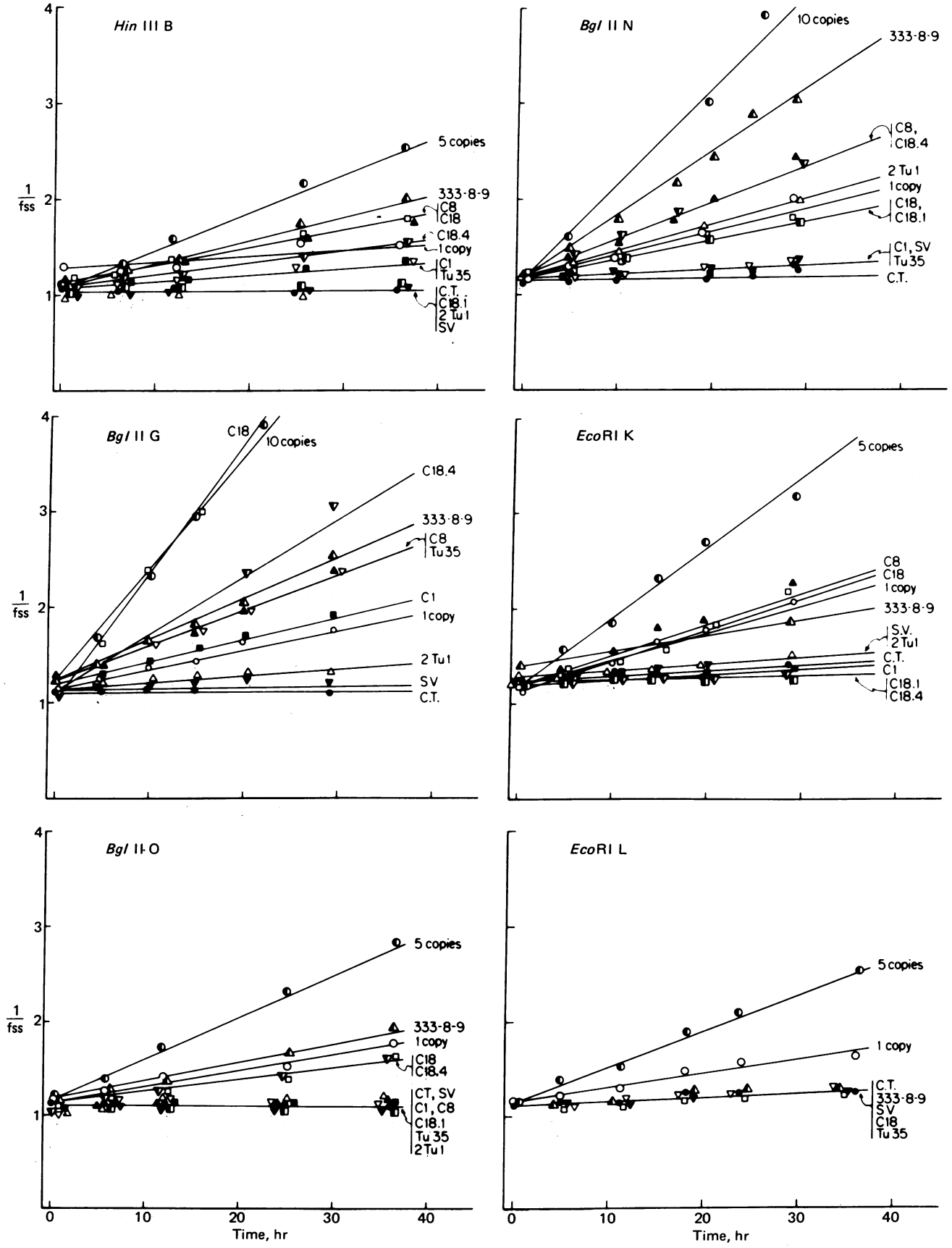

FIG. 3. (*Legend appears at bottom of the next page.*)

Table 1. Number of copies of various viral DNA sequences from hamster cells transformed by HSV-2

Cell line	No. copies present in diploid quantities							
	Hin III B	*Bgl* II G	*Bgl* II O	*Xba* I D	*Bgl* II N	*Eco*RI K	*Bgl* II L	*Eco*RI L
333-8-9	2.0	2.5	1.0	1.0	5.0	1.0	0.0	0.0
C 1	0.5	1.5	0.0	0.5	0.0	0.0	0.0	0.0
C 8	1.5	3.0	0.0	ND	2.0	1.0	0.0	0.0
C 18	1.5	10.0	0.5	0.5	1.0	1.0	0.0	0.0
C 18.1	0.0	ND	0.0	<0.5	1.0	0.0	0.0	0.0
C 18.4	1.0	3.0	0.5	1.0	2.0	0.0	0.0	0.0
Tu 35	0.5	1.0	0.0	0.5	0.0	ND	0.0	0.0
2 Tu 1	0.0	<0.5	0.0	1.0	3.0	0.0	0.0	0.0

The number of equivalents was obtained by comparing the acceleration of renaturation for the transformed cell DNA with the renaturation of calf thymus DNA containing one copy of a fragment of viral DNA. The calculations assumed that the transformed cells contained the entire viral probe, that the molecular weight of a diploid quantity of hamster DNA is 3×10^{12}, and that the transformed cell contained a diploid quantity of DNA. ND, not determined.

to the number of copies of ^{32}P-labeled DNAs varied with the size of the fragment.

The extent to which the radioactive probe hybridized in the presence of transformed cell DNA, calf thymus DNA, and 1, 5, or 10 copies of HSV-2 DNA was measured in a reaction mixture containing 1.8 mg of cellular DNA and 0.48 ng of ^{32}P-labeled fragment per ml. Results with six different fragments are shown in Fig. 3. Viral sequences homologous to the *Bgl* II G fragment were present in seven of seven cell lines tested and at a level of at least 1 copy per diploid genome of cell DNA in six of seven lines. Additionally, sequences homologous to the *Bgl* II N fragment were present in the DNA of six of eight lines. When other fragments located along the unique sequences of the L component were used—e.g., *Bgl* II O or *Eco*RI K—only a few cell lines retained these sequences. The *Hin* III B fragment which overlaps the right-hand end of the *Bgl* II G fragment (see Fig. 1) hybridized to DNA from six of eight lines; in the two cases in which no sequences were detected, 2 Tu 1 showed little hybridization to *Bgl* II G and C 18.1, which was not tested with *Bgl* II G, generally contained small amounts of all HSV-2 sequences. The rate of renaturation of *Hin* III B was slower than that of *Bgl* II G in all lines, suggesting that perhaps only a portion of the fragment was present in the DNA of the transformed cells although no deviation from second-order kinetics was observed. None of the lines tested hybridized to fragments representing sequences from the short component of the HSV-2 genome—e.g., *Eco*RI L (Fig. 3) or *Bgl* II L (data not shown).

A summary of the data indicating the number of copies of the various segments of HSV-2 DNA are present in diploid quantities of DNA extracted from each of the transformed cell lines is shown in Table 1. The confidence with which we can state that all of lines examined contain viral DNA hinges on the relative concentrations of probe and transformed cell DNA. *N* has been defined as the ratio of genome equivalents of cell DNA to the genome equivalents of viral probe DNA in the hybridization (11). For example, for *Bgl* II N $N = 6.25$ and for *Xba* I D $N = 33$. Given the specific activities of the probes (N, 5×10^7 cpm/μg) and the size of the fragments, we can detect one copy of the *Bgl* II N fragment. Therefore, we cannot exclude the possibility that some cell lines (e.g., C 1, Tu 35) contain sequences homologous to at least a portion of the *Bgl* II N fragment at a level below detection.

DISCUSSION

Our results demonstrate that hamster cells transformed by ultraviolet light-inactivated HSV-2 retain viral DNA sequences after extensive passage in tissue culture, cloning, and passage in animals. In all of the lines examined, only a portion of the complete viral genome was retained. Some lines (333-8-9, C 8) contained an extensive set of sequences whereas other lines (C 18.1, 2 Tu 1) retained a greatly decreased quantity of HSV sequences. In all cases the viral information was present at only a few copies per cell. That the cells contain few copies of a limited region of the viral genome could explain previous failures to detect viral sequences by using less sensitive probes (10, 12). In a host cell permissive for viral replication, transformants cannot arise without limiting the extent of the viral genome retained by the cells; therefore, it is not surprising that only a portion of the HSV genome is retained by transformed hamster cells. The picture is very much like that seen in the transformation of rodent cells by adenovirus 2 or 5, in which only a limited region of the adenoviral genome is retained at a few copies (24).

This and other studies (10, 11) have shown that viral DNA sequences are lost during passage of the transformed cells *in vivo* or *in vitro*. The interval in which viral sequences reach a stable and permanent relationship with the cellular genome is much longer than the time required by simian virus 40, adenovirus 2, or adenovirus–simian virus 40 hybrids (19, 22). This raises the possibility that some or all of the HSV sequences present in transformed cells are not integrated into the host genome and can be lost by dilution during passage. It is essential to determine whether, ultimately, a subset of viral sequences must be retained and express a viral gene product that is necessary for the maintenance of the transformed state or whether

FIG. 3 (*on preceding page*). Kinetics of reassociation of ^{32}P-labeled fragments of HSV-2 DNA in the presence of control and transformed cell DNA. The fragments were labeled *in vitro* with ^{32}P to the following specific activities (cpm/μg): *Hin* III B, 4.4×10^7; *Bgl* II G, 3.8×10^7; *Bgl* II O, 5.2×10^7; *Bgl* II N, 2.5×10^7; *Eco*RI K, 6.5×10^7; *Eco*RI L, 2.8×10^7. Each hybridization reaction mixture contained either calf thymus DNA or DNA (1.8 mg/ml) extracted from one of the transformed cell lines and 0.48 ng of ^{32}P-labeled DNA. To the reconstruction reactions, 1, 5, or 10 copies of unlabeled HSV-2 DNA was added to concentrations of 0.06, 0.31, and 0.62 μg/ml, respectively. Before hybridization, both cellular and viral DNAs were degraded to lengths of about 300 nucleotides by boiling in 0.3 M NaOH for 15 min. The hybridizations were carried out at 68°C in 1 M NaCl/0.14 M sodium phosphate, pH 6.8/0.1% $NaDodSO_4$. Samples were removed at intervals over the next 60 hr and passed over hydroxyapatite columns to determine the amount of ^{32}P-labeled DNA that remained single-stranded (13). The fragments were renatured in the presence of DNA from: calf thymus (●), calf thymus plus 1 copy of HSV-2 DNA (○), calf thymus plus 5 or 10 copies of HSV-2 DNA (◐), simian virus 40-transformed hamster cell line (▼), 333-8-9 (▲), C 1 (■), C 8 (▲), C 18 (□), C 18.1 (◧), C 18.4 (▼), Tu 35 (▽), and 2 Tu 1 (△).

all viral sequences can eventually be lost and the cell remain transformed by mechanisms consistent with a hit-and-run hypothesis.

We have found two blocks of viral sequences that are common to most of the transformed cell genomes. These sequences fall between positions 21 and 33 and between positions 60 and 65 on the HSV-2 genome. Because of the limited selection of hybridization probes we cannot delineate the boundaries more precisely. Isolated restriction endonuclease fragments of HSV-1 and HSV-2 DNA have been used to produce transformed cells and have identified two regions with transforming activity: positions 30–45 on the HSV-1 genome (25) and 58–62 on the HSV-2 genome (26), designated morphological transforming region I (mtr I) and mtr II. The two regions show no detectable cross-hybridization, and fragments from the homologous regions of the HSV-1 or HSV-2 genome do not yield significant numbers of foci. The fact that we detect sequences around both these regions in cells transformed by ultraviolet light-inactivated HSV-2 may indicate that the expression of more than one gene is involved in the transformed or tumorigenic phenotype of these cells. The mtr II region is sufficient to enable cells to form foci in low-serum medium and to grow in agarose; if sequences of HSV-2 DNA from the mtr I region are involved in transformation, they may be inactivated by cleavage with *Bgl* II and have thus gone undetected or function at a much lower efficiency than mtr II.

Induction and maintenance of the transformed phenotype by smaller DNA viruses require genes that are expressed before the onset of viral replication. In HSV-1 infected cells, an immediate early RNA accumulates in the presence of cycloheximide or emetine which maps in the region mtr II, but no early RNAs could be detected between positions 18 and 53 (27). Whether this RNA(s) is involved in transformation remains to be seen, as well as answers to the question of how mtr I genes can transform cells without the expression of an early function.

Useful as these experiments are in defining the genes of HSV-2 that are involved in transformation, they merely present a catalogue of viral sequences. The next step is to determine how the sequences are arranged and, in particular, to determine when and if integration of HSV-2 sequences occurs. It is difficult at present to understand the initial interaction between the HSV and hamster genomes that results in the molecular pattern we detect generations later.

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