JOURNAL OF VIROLOGY, Apr. 1981, p. 125-135
0022-538X/81/040125-11$02.00/0

Vol. 38, No. 1

Structure of the Provirus Within NIH 3T3 Cells Transfected with Harvey Sarcoma Virus DNA

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Received 2 September 1980/Accepted 16 December 1980

NIH 3T3 cells transformed with unintegrated Harvey sarcoma virus (HSV) linear DNA generally acquired a complete HSV provirus. Infection of these transformed cells with Moloney murine leukemia helper virus was followed by release of infectious particles. The HSV provirus within these transfected cells was convalently joined to nonviral DNA sequences and was termed "cell-linked" HSV DNA. The association of this cell-virus DNA sequence with the chromosomal DNA of a transfected cell was unclear. NIH 3T3 cells could also become transformed by transfection with this cell-linked HSV DNA. In this case, the recipient cells generally acquired a donor DNA fragment containing both the HSV provirus and its flanking nonviral sequences. After cells acquired either unintegrated or cell-linked HSV DNA, the newly established provirus and flanking cellular sequences underwent amplification to between 5 and 100 copies per diploid cell. NIH 3T3 cells transfected with HSV DNA may acquire deleted proviral DNA lacking at least 1.3 kilobase pairs from the right end of full-length HSV 6-kilobase-pair DNA (corresponding to the 3'-proximal portion of wild-type HSV RNA). Cells bearing such deleted HSV genomes were transformed, indicating that the viral transformation gene lies in the middle or 5'-proximal portion of the HSV RNA genome. However, when these cells were infected with Moloney murine leukemia helper virus, only low levels of biologically active sarcoma virus particles were released. Therefore, the 3' end of full-length HSV RNA was required for efficient transmission of the viral genome.

Harvey sarcoma virus (HSV) is a retrovirus that induces fibrosarcomas in infected rats and mice and causes morphological transformation of fibroblasts cultured in vitro. HSV originated through genetic recombination between a nonsarcomagenic retrovirus, Moloney murine leukemia virus (M-MLV), and rat cellular sequences (16, 24). As Fig. 1 shows, HSV genomic RNA (size, 5.4 kilobases) contains a large internal rat cellular sequence, whereas the RNA 5' and 3' ends are derived from the 5' and 3' ends of genomic M-MLV RNA (5).

The HSV genome replicates within an infected cell through DNA intermediates analogous to the DNA intermediates of other retroviruses. Shortly after infection, HSV RNA is reverse transcribed to generate a linear double-stranded DNA which is slightly longer (6.0 kilobase pairs [kbp]) than genomic RNA (11, 20). As Fig. 1 shows, the 6-kbp DNA bears a terminal sequence repetition; nucleotide sequences derived from the 5' and 3' ends of the genomic RNA are present at both ends of the viral DNA (9). Newly synthesized viral DNA integrates into chromosomal DNA, thereby generating a stable DNA provirus whose sequence is colinear and nonpermuted with respect to the unintegrated linear viral DNA (18).

In the replication cycles of many retroviruses, the establishment of an integrated provirus is followed by synthesis of viral RNA and proteins, which are assembled to form new virus particles. However, the HSV genome does not code for viral structural proteins. As a consequence, a fibroblast infected with HSV acquires a DNA provirus and becomes transformed, but no virus particles are released. Superinfection of the nonproducer transformed cell with a replication-competent helper virus (such as M-MLV) results in rescue of infectious sarcoma virus, the HSV genomic RNA being pseudotyped into virions derived from helper virus proteins (1).

We sought to understand how newly synthesized HSV DNA integrates into the chromosome of an infected cell. Toward this end, we applied

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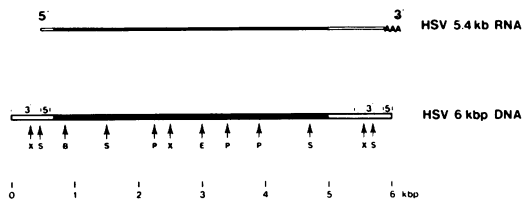

FIG. 1. *Structures of HSV RNA and DNA. The M-MLV and rat-specific sequence components (white and black regions, respectively) of polyadenylated HSV virion 5.4-kilobase (kb) RNA were mapped by Chien et al. (5). The HSV RNA is reverse-transcribed in vivo to generate HSV 6.0-kbp DNA, whose sequence is colinear with HSV RNA; this DNA contains terminally redundant ends (9) derived from 5'- and 3'-terminal RNA sequences, as indicated on the figure. The restriction endonuclease cleavage sites along HSV 6.0-kbp DNA were mapped previously (11). B, BamHI; E, EcoRI; P, PstI; S, SmaI; X, XbaI.*

viral DNA directly to uninfected mouse cells, a process termed transfection. Transfection of mouse NIH 3T3 fibroblasts with HSV 6-kbp DNA was shown to induce transformation of a small fraction of the treated cells (11, 20). When nonproducer transformed cells derived by transfection were infected with M-MLV helper, infectious sarcoma virus was rescued in high titers from these cells, demonstrating that the transformants had acquired a functional HSV provirus.

Transfection of cells with retrovirus DNA can lead to successful establishment of viral genomes via two routes. Either the transfected donor DNA is integrated directly into DNA within the recipient cells, or the donor DNA can serve, even while not integrated, as a template for the synthesis of progeny virus particles which are then able to spread horizontally through the transfected culture (6). In the case of HSV, the replication defectiveness of HSV precludes this infectious spread. Thus, we (11) and others (20) concluded that the donor DNA became established directly within the successfully transfected cells.

These earlier experiments did not address the nature of the HSV DNA acquired within these transfected cells. In the present work, we analyzed the structure of transfected HSV genomes. This analysis yielded unexpected insights into the process of transfection of retrovirus DNA and led to an understanding of some of the functions encoded by different segments of the HSV genome.

MATERIALS AND METHODS

Preparation of unintegrated and cell-linked HSV DNA. A DNA fraction enriched for unintegrated linear HSV DNA was prepared from JLS V9 BALB/c cells infected with HSV 24 h before extraction. The use of the Hirt extract procedure (17) to enrich for low-molecular-weight DNA and the use of cesium chloride-ethidium bromide gradients to separate linear DNA from closed circular molecules have been described previously (11). Where necessary, HSV linear 6-kbp DNA was freed of contaminating leukemia viral DNA by agarose gel electrophoresis.

Cellular DNA from HSV DNA-transfected cells bearing "cell-linked" HSV proviral DNA (see below) was prepared by sodium dodecyl sulfate-proteinase K lysis of cells and phenol-chloroform extraction of the lysate (27).

DNA transfection. Transfection of NIH 3T3 fibroblasts with HSV DNA by the calcium phosphate-DNA precipitation method (13) has been described previously (2). Cellular DNA (containing cell-linked HSV DNA) was sheared by two passages through a 20-gauge needle before use in transfection.

Southern filter hybridization. Cellular DNA was cleaved with restriction endonucleases (purchased from New England Biolabs) according to the instructions of the supplier. Cleaved DNA was subjected to agarose slab gel electrophoresis, and the DNA contents of the gel were transferred to a nitrocellulose filter by using the method first described by Southern (26). The filter was hybridized to [^{32}P]DNA as described elsewhere (27).

Radiolabeled compounds employed. α-^{32}P-labeled nucleoside triphosphates were purchased from Amersham. [γ-^{32}P]ATP was purchased from New England Nuclear Corp.

Preparation of HSV ^{32}P-labeled cDNA. Representative ^{32}P-labeled complementary DNA (cDNA) that was complementary to HSV 30S RNA was synthesized in vitro by using avian myeloblastosis virus reverse transcriptase, as described previously (11). To purify the rat-specific cDNA sequences, the ^{32}P-labeled cDNA was hybridized to a 50-fold excess of M-MLV 38S RNA at 67°C in a reaction mixture containing 0.6 M NaCl, 10 M Tris (pH 7.4), 1 mM EDTA, and 0.1% sodium dodecyl sulfate. After hybridization, unhybridized rat-specific ^{32}P-labeled cDNA was separated from M-MLV RNA-homologous cDNA hybrids by isopycnic banding in guanidine hydrochloride-cesium chloride gradients (7).

Host-vector systems for recombinant DNA experiments. Recombinant DNA experiments were conducted under P2 containment and employed National Institutes of Health-certified host-vector systems.

Recombinant λ bacteriophage was grown on *Escherichia coli* strain DP50 *supF* (F$^-$ *dapD8 lacY*$^-$ Δ[*gal-uvrB*]Δ*thyA nal-A*$^-$ hsds suII suIII).

Wild-type and recombinant pBR322 plasmids were propagated in *E. coli* strain χ1776.

Molecular cloning of the 3.2-kbp *Sma*I fragment of HSV DNA into pBR322. *Eco*RI-permuted HSV 6.0-kbp DNA cloned into the λgtWES:λB vector (15) was a kind gift from D. Lowy and E. M. Scolnick. The HSV-λ hybrid phage was grown on *E. coli* strain DP50 *supF*, and phage DNA was extracted from CsCl gradient-purified phage particles (21). A 100-μg amount of phage DNA was digested with *Eco*RI, the

DNA was incubated at 42°C in 1 ml of 1 M NaCl-10 mM Tris (pH 7.5)-1 mM EDTA to allow the λ DNA cohesive ends to anneal, and the HSV DNA insertion was purified away from the λ DNA by sucrose gradient sedimentation (10 to 40% [wt/wt] sucrose in the above-described incubation buffer; 24,000 rpm for 27 h in a Beckman SW27 rotor) (21). Purified HSV DNA was circularized with T4 ligase (New England Biolabs) in a reaction mixture containing 66 mM Tris (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 40 mM β-mercaptoethanol, and 1 μg of HSV DNA per ml, and the circularized DNA was cleaved with *Sma*I to generate fragments of 3.2, 1.2, 1.0, and 0.6 kbp (Fig. 1). One thousand picomoles of *Bam*HI decanucleotide linkers (Collaborative Research, Inc.) which had been ^{32}P end labeled with [γ-^{32}P]ATP and polynucleotide kinase (Boehringer Mannheim Corp.) was blunt end ligated to the *Sma*I HSV DNA fragments (2-pmol ends) in a 30-μl reaction mixture incubated at 25°C for 16 h, and the DNA was subsequently digested with *Bam*HI endonuclease.

The *Sma*I HSV DNA fragments bearing *Bam*HI linkers (120 ng) were ligated to 10 μg of pBR322 DNA which had been cleaved with *Bam*HI and treated with calf intestinal phosphatase. The ligated DNA was used to transform *E. coli* χ1776 cells, and plasmid-bearing colonies were selected by resistance to ampicillin (29). Plasmid-bearing clones containing HSV DNA insertions were identified by the colony filter hybridization method (14), using representative HSV ^{32}P-labeled cDNA as a hybridization probe, and the sizes of the HSV DNA insertions were measured by *Bam*HI cleavage of HSV-pBR hybrid DNAs and subsequent gel electrophoresis.

^{32}P nick translation of plasmid DNA. HSV-pBR322 recombinant plasmid DNA was labeled with ^{32}P by DNase I-DNA polymerase I nick translation, using [α-^{32}P]dCTP and [α-^{32}P]dGTP (22). When the HSV DNA required purification from pBR322 sequences, the ^{32}P-labeled DNA was cleaved with *Bam*HI and subjected to neutral agarose gel electrophoresis.

Hybridization in solution. Reannealing of nick-translated HSV [^{32}P]DNA in the presence of cellular DNA was conducted in 10-μl reaction mixtures containing 0.7 M NaCl, 70 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] (pH 6.1), 2 mM EDTA 0.4% sodium dodecyl sulfate, 2 ng of [^{32}P]DNA per ml, and 4 mg of cellular DNA per ml. Cellular DNA was sheared by two passages through a 25-gauge needle before use. Samples sealed in capillary pipettes were boiled for 10 min and then incubated at 67°C. Given the Na^+ concentration in these reactions, 1 h of reannealing was equivalent to ^{32}P-labeled viral DNA $C_0t \times 1.1 \times 10^{-4}$ (3). Reactions were stopped by rapidly chilling to 0°C and then diluting into 1 ml of a solution containing 0.25 M NaCl, 30 mM sodium acetate (pH 4.5), 2 mM $ZnSO_4$, and 5% glycerol. A 0.2-ml amount of each diluted mixture was set aside, and the remainder was digested with S1 nuclease (Boehringer Mannheim) at 45°C for 1 h. S1-resistant [^{32}P]DNA was monitored by trichloroacetic acid precipitation and filtration.

Sarcoma virus rescue from nonproducer transformed cells. The rescue of sarcoma virus from nonproducer transformed cells by infection with M-MLV, the focus assay for infectious sarcoma virus, and the XC plaque assay for murine leukemic virus were all performed as previously described (2, 19, 23).

RESULTS

Strategy for locating HSV proviral DNA within transfected cells. We first wished to determine whether a transformed cell derived by transfection with unintegrated HSV DNA contained a provirus integrated into one or many sites in the cellular genome. This analysis was made possible by exploitation of the Southern gel-filter transfer (blot) procedure and by the use in this procedure of a sequence probe specific for the HSV genome. Such work also depended upon knowledge of the physical map of HSV (Fig. 1), which has been derived by us and by others (11, 15). An important landmark on this map is the centrally located *Eco*RI endonuclease site.

A cell acquiring an HSV provirus may carry this provirus in a configuration which is colinear and nonpermuted with respect to unintegrated linear HSV DNA (18). In this case, the single HSV *Eco*RI site (Fig. 1) is situated precisely in the middle of the 6-kbp provirus. The provirus in turn is flanked on both sides by cellular DNA sequences containing *Eco*RI sites at unknown distances. Thus, *Eco*RI-cleaved DNA from such a transformed cell includes two DNA fragments larger than 3 kbp, each of which contains 3 kbp of HSV sequences linked to cellular sequences. If the mouse cell chromosomes contain multiple sites for HSV DNA integration, a second independently transformed cell will contain a HSV provirus flanked by different cellular sequences, so that *Eco*RI-cleaved DNA from this cell includes two HSV-specific DNA fragments that differ in size from the HSV-specific *Eco*RI DNA fragments present in the first cell.

DNA fragments containing HSV sequences can be detected by subjecting the DNAs to gel electrophoresis and Southern filter hybridization, using a nucleic acid probe specific for HSV sequences. Radioactively labeled HSV RNAs or cDNA's are not suitable probes for use in the filter hybridization just proposed. Portions of the HSV genome are derived from M-MLV sequences. When present in a probe, these M-MLV sequences result in the detection of many endogenous viral genomes in the DNAs of normal mouse cells (27). The detection of this large number of endogenous viral sequences in turn obscures analysis of the HSV DNA sequences of interest.

This problem can be circumvented by using an HSV subgenomic probe which contains HSV sequences of rat origin but lacks all sequences

homologous to M-MLV (5). We generated such a probe by the molecular cloning of a large, rat-specific fragment of HSV DNA into the bacterial plasmid pBR322. This 3.2-kbp fragment, which was produced by endonuclease *Sma*I, spans the region from 1.5 to 4.7 kbp on the HSV physical map (Fig. 1). The entire HSV DNA genome, cloned into the coliphage vector λgtWES:λB, was kindly provided by D. Lowy and E. M. Scolnick. The HSV DNA within this clone originated from an HSV 6.0-kbp closed circular DNA molecule which had been linearized by cleavage with *Eco*RI before its introduction into the λ cloning vector (15). We prepared the λ:HSV hybrid phage, cleaved its DNA with endonuclease *Eco*RI, and purified the HSV 6-kbp DNA insert by sucrose gradient sedimentation. The purified HSV DNA was circularized with T4 ligase and then cleaved with endonuclease *Sma*I to generate fragments of 3.2, 1.2, 1.0, and 0.6 kbp. Decanucleotide linkers containing endonuclease *Bam*HI cleavage sites were ligated to the ends of the *Sma*I fragments, and after cleavage with *Bam*HI, the fragments were ligated to *Bam*HI-cleaved pBR322 DNA. This ligated DNA was used to transform *E. coli* χ1776 cells, and we selected a clone of plasmid-containing bacteria which contained the desired 3.2-kbp HSV DNA insertion.

HSV DNA-transfected cells generally acquire a complete provirus flanked by a unique set of nonviral DNA sequences. Large numbers of foci were observed upon transfection of NIH 3T3 mouse fibroblast monolayers with DNA extracted from cells recently infected with HSV. The DNA used in this fraction was prepared from the Hirt supernatant fraction of these recently infected cells. This DNA fraction was highly enriched for unintegrated HSV DNA, and the transformation-inducing capacity of this DNA preparation was associated with unintegrated HSV 6.0-kbp DNA (11). Of the many foci which resulted from transfection of this DNA, three foci were picked, single-cell cloned, and expanded into cell lines (clones 14, 16, and X3). All three of these cell lines were negative for the release of sarcoma virus particles. However, upon infection with M-MLV helper, all of the cultures released high titers of HSV (data not shown). This demonstrated that these cells contained transmissible HSV genomes. In this respect, these transformed cells were phenotypically identical to cells transformed by HSV infection.

The HSV proviral DNA sequences within these three transformed cell lines were analyzed by gel electrophoresis and Southern filter hybridization, using as a probe the cloned rat-specific subgenomic HSV DNA fragment labeled with $^{32}PO_4$ by nick translation with DNA polymerase. As a negative control for this experiment, DNA from nontransfected NIH 3T3 cells was analyzed in parallel. As shown in Fig. 2, lanes a and r, the HSV-specific probe hybridized weakly to several *Eco*RI cleavage fragments of NIH 3T3 DNA, demonstrating that the NIH 3T3 Swiss mouse genome contains several sequences that are weakly homologous to the rat-specific portion of the HSV genome. (The area of radioactivity at the top of lane a represents a hybridization artifact.)

*Eco*RI-cleaved DNA from each of the three transformed cell lines exhibited at least two novel, HSV-specific fragments (Fig. 2, lanes b, f, and j); the DNAs from clones 16 and X3 had two virus-specific fragments, whereas the DNA from clone 14 cells exhibited three such fragments. The *Eco*RI HSV-specific DNA fragments from these three cell lines were all larger than the 3-kbp fragments observed upon analysis of *Eco*RI-cleaved, unintegrated HSV DNA (Fig. 2, lane h). In addition, all seven of these virus-specific fragments differed in size from one another. These results demonstrate that cells transfected with HSV DNA acquired copies of HSV DNA linked to nonviral DNA sequences. Furthermore, each independently derived transformed cell line contained an HSV genome linked to a different set of nonviral DNA sequences.

The covalent linkage of HSV proviral DNA to nonviral DNA sequences within transfected cells did not directly prove that the HSV DNA in these cells was integrated into DNA sites which were chromosomally associated. Therefore, we termed the proviral DNA within transfected cells "cell-linked HSV DNA."

Cell-linked HSV DNA can induce transformation of NIH 3T3 cells upon transfection. The above-described experiments confirm previous work (20, 25) demonstrating that unintegrated retrovirus DNA can be transfected easily into recipient cells. We wished to determine whether the resulting cell-linked HSV DNA could in turn induce transformation of NIH 3T3 cells upon a further cycle of transfection. DNAs from the three nonproducer HSV-transformed cell lines described above (clones 14, 16, and X3) were applied to NIH 3T3 cell cultures. As Table 1 shows, all three DNAs could induce focal transformation of the recipient cells. A total of 25 foci arising from these transfections were picked and cultured from further study. All of these transformants were negative for sarcoma virus production. When the 25 cultures were infected with M-MLV helper, 22 of them released high titers of sarcoma virus. The remaining three cultures released low but detectable levels of sarcoma virus (see below). We

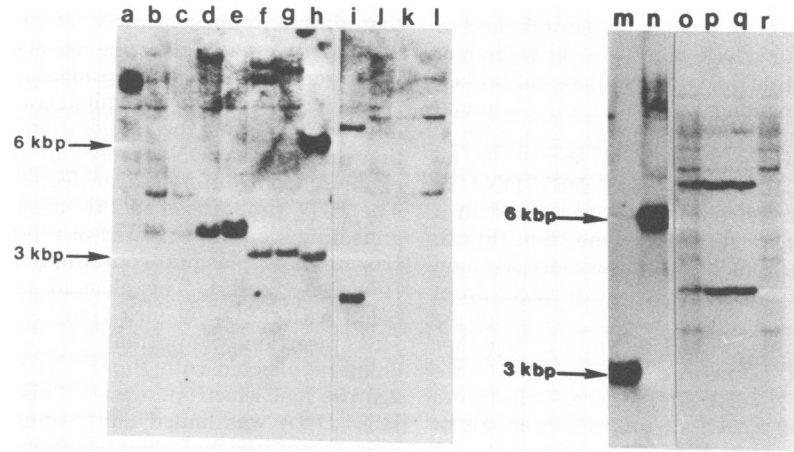

FIG. 2. *Southern filter hybridization analysis of DNAs from cells transformed by HSV DNA transfection. EcoRI-cleaved DNAs from cell lines derived by transfection of NIH 3T3 cells with unintegrated and cell-linked HSV DNA were subjected to electrophoresis through neutral 1% agarose slab gels. The gel contents were blotted onto nitrocellulose filters, and the filters were hybridized with denatured ^{32}P-labeled HSV pBR322 plasmid bearing the 3.2-kilobase rat-specific SmaI DNA fragment. Clones 14, 16, X1, and X3 were derived from four independent foci induced by transfection with low-molecular-weight DNA from cells recently infected with HSV. Clone 14-2 was derived from a focus induced by transfection with clone 14 cellular DNA, clones 16-1, 16-6, and 16-7 were from three foci induced by clone 16 DNA, and clones X3-1 and X3-4 were from foci induced by clone X3 DNA. EcoRI-cleaved DNAs were from clones 16 (lane b), 16-6 (lane c), 16-1 (lane d), 16-7 (lane e), 14 (lane f), 14-2 (lane g), X1 (lane i), X3 (lanes j and o), X3-1 (lanes k and p), and X3-4 (lanes l and q). Lanes a and r contained EcoRI-cleaved NIH 3T3 DNA. Unintegrated HSV 6.0-kbp and EcoRI-cleaved unintegrated HSV 3.0-kbp DNA fragments were the molecular weight standards used (lanes h, m, and n).*

TABLE 1. *Transformation of NIH 3T3 cells by transfection with cell-linked HSV DNA*[a]

Expt	DNA from cell line:	Amt of DNA transfected (μg)	No. of foci obtained
1	14	45	11
	16	45	2
	X3	30	1
2	14	75	35
	16	150	13
	X3	150	9

[a] DNA was extracted from three cell lines (clones 14, 16, and X3) that were transformed by HSV DNA transfection and were known to contain rescuable HSV proviruses. The DNA samples were used to transfect NIH 3T3 cell cultures, and transformed foci were scored 14 to 17 days later.

conclude that transformed cell lines derived by transfection with cell-linked HSV DNA generally contain complete and transmissible proviruses.

Cells transfected with cell-linked HSV DNA acquire the HSV genome and its flanking donor cellular sequences. Upon transfection of NIH 3T3 cells with cell-linked HSV DNA, the HSV DNA may remain attached to its flanking cellular sequences. The recipient cell would thereby acquire a large fragment of donor DNA containing both the HSV provirus and linked nonviral sequences. In this case, the *Eco*RI cleavage sites in the flanking cellular sequences would probably be cotransferred together with the HSV provirus upon transfection. Southern filter hybridization would show that one or both of the HSV-containing *Eco*RI fragments in transfected recipient cell DNA are the same as the HSV-containing *Eco*RI fragments in the donor cell DNA. Alternatively, upon transfection of NIH 3T3 cells with cell-linked HSV DNA, the HSV DNA genome might become dissociated from its flanking nonviral cell sequences before establishment within the recipient cells. In this case, the *Eco*RI cleavage sites on both sides of the provirus within the recipient cells would be positioned differently from the flanking *Eco*RI sites in the donor DNA.

Figure 2 shows a Southern filter hybridization analysis of *Eco*RI-cleaved DNAs from single-cell cloned focus cultures derived by transfection with cell-linked HSV DNA. Transfection of NIH 3T3 cells with DNA from HSV-transformed clone X3 cells gave rise to clone X3-1 and clone X3-4 transformed cell lines. *Eco*RI-cleaved DNA from these two recipient cell lines contained two HSV-specific DNA fragments (Fig. 2, lanes p and q), which were identical in size to

the virus-specific fragments in *Eco*RI-cleaved clone X3 donor DNA (lane o). This finding demonstrates that in two instances cell-linked HSV DNA remained associated with flanking nonviral donor sequences upon transfection. This conclusion was extended to show that the *Eco*RI sites flanking cell-linked HSV DNA in clone 16 donor cells were also preserved in a transformed recipient clone (clone 16-6) (Fig. 2, lanes b and c). Finally, we observed that upon transfection of NIH 3T3 cells with DNA from HSV-transformed clone 14 cells, the resulting clone 14-2 acquired the HSV provirus together with nonviral sequences bearing the more closely linked *Eco*RI site, whereas the more distantly linked *Eco*RI site on the other side of the provirus was lost (Fig. 2, lanes f and g).

In summary, we found that cells transfected with cell-linked HSV DNA acquired a DNA fragment bearing the HSV provirus together with closely linked nonviral DNA sequences.

Provirus within transfected cells undergoes amplification. Transformed cells derived via transfection with HSV DNA contain a complete HSV provirus linked to a unique set of nonviral DNA sequences. This configuration appears to resemble the configuration observed after infection of cells with retrovirus particles (28), in which each provirus is present at a unique location in the cellular genome and present in a copy number (probably one) which is independent of its integration site. An analysis of the DNAs of such infected cells yields a series of radioautographic bands of equal intensity, each representing a provirus associated with a unique cellular DNA sequence (28).

An analysis of the DNAs of HSV-transfected cells indicated the presence of bands of radioactivity of greatly varying intensities. For example, the HSV-specific fragments in the DNA from clone 14 cells hybridized far more intensely (Fig. 2, lane f) than did the HSV fragments in the DNA from clone 16 cells (lane b). In addition, the virus-specific bands in *Eco*RI-cleaved clone X3-4 DNA (lane q) were far more intense than the identically sized virus-specific fragments in clone X3 DNA (lane o). This behavior stands in marked contrast to that observed upon analysis of proviruses derived from infection of cells by M-MLV (28). The unequal hybridization intensities of different DNA samples were not due to unequal amounts of DNA applied to different channels of the Southern blot gel, since the total DNA present in each lane was controlled internally by the intensities of hybridization between the rat sequences of the HSV probe and some weakly homologous sequences in the mouse cell background.

The above-described observations suggested that different lines of HSV DNA-transfected cells carry greatly differing numbers of HSV DNA genomes per cell. We measured the provirus copy number in individual transfected cell lines by calculating the acceleration of reassociation rate of HSV DNA caused by the presence of DNA from different transfected cells lines. The HSV probe used in this measurement of annealing kinetics consisted of the DNA of the recombinant plasmid containing the 3.2-kbp HSV DNA insert. The plasmid DNA was labeled with $^{32}PO_4$ by nick translation, and the HSV insertion was purified by cleavage of the chimeric plasmid with *Bam*HI and preparative agarose gel electrophoresis. This ^{32}P-labeled HSV DNA was boiled and reannealed in the presence of various cellular DNAs, and hybridized [^{32}P]DNA was detected by resistance of this probe to digestion by single-strand-specific S1 nuclease.

Figure 3 shows the results of such hybridization kinetic experiments. When the ^{32}P-labeled HSV DNA probe was mixed with a 2×10^6-fold excess of NIH 3T3 cellular DNA, the reannealing of labeled DNA was 50% completed at a C_0t value of about 2×10^{-3} mol·s/liter (Fig. 3) (C_0t = [concentration of viral DNA nucleotides in moles per liter] [time in seconds]) (3). The ^{32}P-labeled HSV DNA probe was also incubated in the presence of an equal mass of unlabeled HSV 3.2-kbp rat-specific DNA plus a 2×10^6-fold excess of NIH 3T3 DNA. This reconstruction allowed calibration of the kinetics expected from an analysis of the cellular DNA bearing a single HSV genome per diploid DNA complement. This ratio of DNAs was chosen because rodent genomes are 10^6-fold more complex (3×10^9 nucleotides) than the HSV DNA fragment (21). This reconstructed mixture of cellular and unlabeled viral DNAs, which simulated the behavior of a single copy of HSV DNA per diploid cell genome, accelerated the annealing of the ^{32}P-labeled HSV DNA probe approximately twofold, as expected (Fig. 3).

The DNAs from cells transformed by transfection with HSV DNA accelerated the reannealing of ^{32}P-labeled HSV DNA far more dramatically than expected of DNA containing one provirus per diploid genome. For example, clone X3 cellular DNA accelerated [^{32}P]DNA hybridization by 5- to 15-fold (Fig. 3), implying that clone X3 cells have 5 to 15 copies of HSV DNA per diploid genome. Clone 16 DNA accelerated probe reannealing (Fig. 3) to a similar extent, indicating a similar copy number. The acceleration provided by clone X3-1 DNA (Fig. 3) and clone X1-1 DNA (data not shown) was even far greater than that provided by the above-mentioned clone X3 and 16 DNAs. Readjusting the

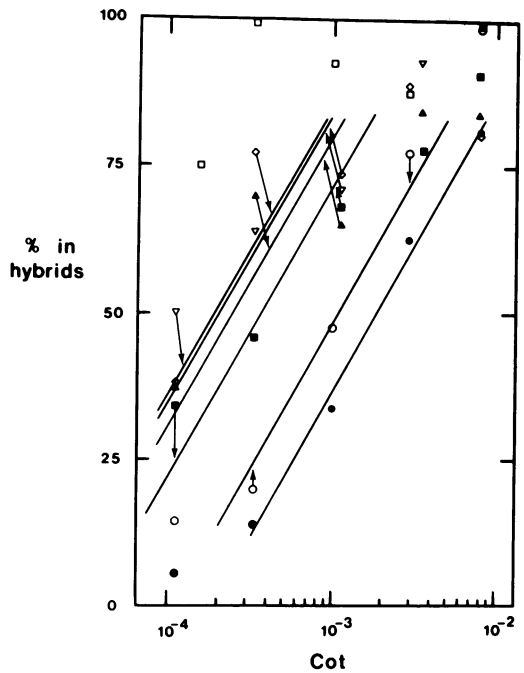

FIG. 3. *Reannealing of HSV [^{32}P]DNA in the presence of DNA from transfected cells. The 3.2-kbp rat-specific HSV DNA fragment (see text) was labeled with ^{32}P by nick translation. HSV [^{32}P]DNA (2 ng/ml) was denatured and allowed to reanneal in the presence of cellular DNA (4 mg/ml) for 1 to 72 h in a buffer containing 0.75 M sodium ions. Reaction mixtures were digested with S1 nuclease to determine the percentages of ^{32}P in hybrids. This probe reannealed to an endpoint maximum of 40%, and the data are plotted as a percentage of maximal hybridization. Cot values (Cot = [concentration of ^{32}P-labeled DNA in moles of nucleotide per liter] × [time in seconds]) were corrected for the elevated sodium ion concentration (3). [^{32}P]DNA was reannealed in the presence of 4 mg of NIH 3T3 DNA per ml (●), 4 mg of NIH 3T3 DNA per ml plus 2 ng of unlabeled HSV 3.2-kbp rat-specific DNA per ml (○), 4 mg of clone X1 DNA per ml (▲), 4 mg of clone 16 DNA per ml (▽), 4 mg of clone X3-1 DNA per ml (□), 0.4 mg of clone X3-1 DNA per ml plus 3.6 mg of NIH 3T3 DNA per ml (■), and 0.4 mg of clone X1-1 DNA per ml plus 3.6 mg of NIH 3T3 DNA per ml (◇).*

ratio of cell DNA to probe to a 2×10^5-fold excess resulted in a 4- to 10-fold increase in reannealing rate (Fig. 3). Therefore, clone X3-1 and X1-1 cells have 40 to 100 copies of HSV DNA per diploid genome.

The relative HSV provirus copy numbers for the different transformed cell lines correlated with the relative intensities of the HSV-specific DNA bands observed in Southern filter hybridizations. For example, clone X3 DNA yielded a relatively weak band upon blot analysis (Fig. 2, lane o) and contained 5 to 15 HSV proviruses per diploid DNA complement. Clone X3-1 DNA, which yielded far more intense radioautographic bands (Fig. 2, lane p) carried 40 to 100 HSV genomes per diploid DNA complement.

The association of HSV proviruses with a unique set of cellular sequences in each of these lines appears to conflict with the large number of HSV genomes present in each of the cells of these lines. This paradox can only be resolved by assuming that upon transfection, an unintegrated HSV DNA becomes associated with a unique set of cellular sequences and that this cell-virus array is then amplified in many of the cells descended from the initial transfectant. This same process is repeated upon introduction of cell-linked HSV DNA, in which there is once again a great amplification of cell-virus DNA arrays.

Rescue of sarcoma virus from cells transfected with HSV DNA. Most nonproducer transformed cell lines derived by transfection with HSV DNA release high titers of HSV after M-MLV infection. Table 2, experiment 1, shows two examples of HSV DNA-transfected cell lines from which we recovered more than 10^6 focus-forming units (FFU) of sarcoma virus particles per ml of culture fluid after M-MLV infection. The level of sarcoma virus released from these cells exceeded the plaque-forming titer of M-MLV particles being released (i.e., ratio of FFU to PFU, >1). Of 52 transformed cell cultures, each originating from a focus derived by transfection with unintegrated or cell-linked HSV DNA, 48 released similarly high titers of sarcoma virus after M-MLV infection (data not shown). In contrast, the remaining four transformed cultures released only low levels of sarcoma virus (ratio of FFU to PFU, 10^{-4} to 2×10^{-3}) after M-MLV infection (Table 2, experiment 2).

This inability to recover higher levels of sarcoma virus could not be attributed to poor replication of the helper virus within these cultures, since the cells released high titers of M-MLV helper. These four transfected cell lines were termed "poorly rescuable." One of the poorly rescuable cell lines was derived by transfection of NIH 3T3 cells with unintegrated linear HSV DNA, and the other three poorly rescuable transformants were derived by transfection with cell-linked DNA.

The DNAs extracted from poorly rescuable transformed cells were able to induce focal transformation of NIH 3T3 cells upon transfection (Table 3). Foci derived from these transfections were cultured independently and then infected with M-MLV in an attempt to rescue sarcoma virus. These focus-derived cultures were once again poorly rescuable (Table 2, experiment 3).

TABLE 2. *Quantitative analysis of virus recovered from sarcoma virus-transformed cells*[a]

Expt[b]	Cell line	No. of FFU/ml	No. of PFU/ml	Ratio of FFU to PFU
1	14	5×10^7	1×10^6	50
	16	1.5×10^7	2×10^6	8
2	X1	5×10^3	2×10^7	3×10^{-4}
	16-1	2.5×10^3	1.5×10^6	1.5×10^{-3}
	16-7	5×10^2	9×10^5	6×10^{-4}
3	X1-1	4×10^4	5×10^7	8×10^{-4}
	16-1-1	1.5×10^4	7.5×10^6	2×10^{-3}
	16-1-2	1×10^4	4×10^6	2.5×10^{-3}
	16-7-1	4×10^2	3.5×10^6	1×10^{-4}
	16-7-2	4.5×10^3	5×10^6	9×10^{-4}
4	X1:V1	5×10^6	1.5×10^6	3
	X1:V2	1.5×10^6	9×10^5	1.5
	16-1:V1	2×10^4	2×10^4	1
	16-1:V2	2×10^6	1×10^6	2
	16-7:V1	5×10^6	1×10^6	5
	16-7:V2	7×10^6	9×10^5	8

[a] Each transformed cell line was infected with M-MLV at a multiplicity of infection of >1. After 2 days, virus-containing media from the cultures were clarified of cells and cell debris, and the virus samples were serially diluted and assayed for infectious sarcoma and leukemia viruses. The data are presented as the number of FFU of sarcoma virus per milliliter, the number of PFU of leukemia virus per milliliter, and the ratio of sarcoma virus FFU to leukemia virus PFU.

[b] Experiment 1, transformed cell lines derived by transfection with HSV DNA and bearing a highly rescuable provirus; experiment 2, poorly rescuable transformed cell lines derived by transfection with full-length HSV DNA; experiment 3, transformed cell lines derived by transfection with the DNA from the cells in experiment 2; experiment 4, transformed cell lines derived by infection with the sarcoma virus rescued from the cells in experiment 2.

These findings demonstrated that the phenomenon of poor rescuability was due to a stable genetic element tightly linked to the HSV provirus within the DNA of the poorly rescuable cells.

To determine whether poorly rescuable cells released large amounts of poorly transmissible HSV or small amounts of wild-type, highly transmissible HSV, NIH 3T3 cells were infected with the low-titer sarcoma virus released from poorly rescuable cells. Six foci which arose from these infections were individually picked, cultured, and assayed for release of infectious sarcoma and leukemia viruses. All six of these cultures produced high titers of sarcoma virus (ratio of FFU to PFU, 1) (Table 2, experiment 4). We conclude that M-MLV infection of poorly rescuable cells induced release of a small amount of a sarcoma virus which behaved like highly transmissible, wild-type HSV.

TABLE 3. *Transformation of NIH 3T3 cells by transfection with DNA from poorly rescuable transformed cells*[a]

Expt	DNA from cell line:	Amt of DNA transfected (μg)	No. of foci obtained
1	14	45	11
	X1	38	6
2	14	100	19
	16-1	100	14
	16-7	100	4
	X1	100	2
3	14	75	161
	16-7	100	11
	16-1	96	44

[a] DNAs were extracted from highly rescuable clone 14 cells and from poorly rescuable transformed cell lines (clones 16-1, 16-7, and X1). These DNAs were used to transfect NIH 3T3 cells, and cultures were scored for transformed foci 14 to 17 days later.

Poorly rescuable transformed cells contain incomplete HSV genomes. DNA from one poorly rescuable cell line, clone X1, was cleaved with restriction endonuclease *Eco*RI and subjected to Southern filter hybridization analysis (Fig. 2, lane i). The *Eco*RI-cleaved DNA contained two virus-specific DNA fragments. Unexpectedly, one of these HSV-specific fragments was smaller than 3 kbp. This blot analysis of clone X1 DNA suggested that poorly rescuable clone X1 cells must contain deleted HSV proviruses.

Deletions in the HSV DNAs of this and two other poorly rescuable cell lines were determined with further restriction endonuclease mapping experiments. DNAs from these lines (clones X1, 16-1, and 16-7) were digested with either *Xba*I or *Sma*I plus *Eco*RI and then subjected to Southern filter hybridization analysis by using as a probe HSV ^{32}P-labeled cDNA which had been partially depleted of the sequences homologous to M-MLV (see above). Figure 4, lanes a through e, show the analysis of *Xba*I-cleaved DNAs. Whereas the Hirt supernatant unintegrated linear HSV DNA (lane e) and the DNA from clone 14 highly rescuable transfected cells (lane d) had both 3.2- and 2.2-kbp viral DNA fragments, the *Xba*I-cleaved DNAs from the three poorly rescuable transformed cell lines (lanes a, b, and c) contained only the 2.2-kbp viral fragment derived from the left side of the DNA genome (Fig. 1). Figure 4, lanes f through j, show the analysis of DNA samples cleaved with both *Sma*I and *Eco*RI. Whereas the unintegrated HSV DNA (lane i) and the clone 14 cell DNA (lane j) had four viral

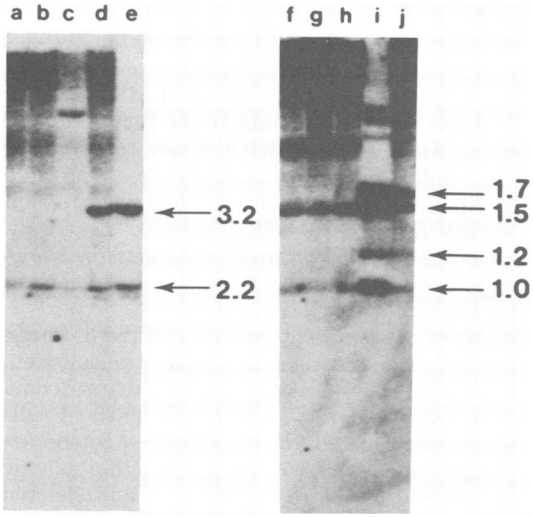

FIG. 4. *Restriction mapping of deleted HSV proviruses. The DNAs from poorly rescuable HSV nonproducer transformed cell lines (clones X1, 16-1, and 16-7) and from highly rescuable clone 14 cells were cleaved with restriction endonucleases and then subjected to agarose gel electrophoresis and Southern filter hybridization analysis, using rat sequence-enriched HSV ^{32}P-labeled cDNA. Lanes a through e, DNA samples cleaved with XbaI and run through a 1.4% agarose gel; lanes f through j, DNA samples cleaved with EcoRI plus SmaI and run through a 2% agarose gel. Lanes a and g contained clone 16-1 DNA, lanes b and h contained clone 16-7 DNA, lanes c and f contained clone X1 DNA, and lanes d and j contained clone 14 DNA. Gel-purified unintegrated HSV 6.0-kbp DNA was cleaved with the same restriction endonucleases (lanes e and i) to provide molecular weight standards (whose sizes are indicated in kilobase pairs).*

DNA fragments (1.7, 1.5, 1.2, and 1.0 kbp), the cleaved DNAs from all three poorly rescuable transformants (lanes f, g, and h) contained only the 1.5- and 1.0-kbp viral DNA fragments derived from the left side of the DNA genome.

The Southern filter hybridization data in Fig. 4 were used to construct a physical map of the deleted HSV proviral DNAs in the three poorly rescuable transformed cell lines (Fig. 5). In all three cases, a portion of the right side of the HSV DNA genome was missing (corresponding to the 3'-proximal portion of the viral RNA), with the deletions extending in from the right end at least 1.3 kbp, but less than 3.0 kbp. The region of these deletions spanned the entire right end of the HSV genome which was homologous to M-MLV sequences (Fig. 1).

These deleted proviruses conferred an unusual phenotype on the cells carrying them; the cells were highly transformed but did not yield infectious HSV particles in high titer upon attempted virus rescue. We tentatively concluded that the residual portion of the HSV provirus present in these cells allowed expression of virus-induced transformation, whereas deleted portions of the provirus were required for the efficient transmission of HSV genomes during helper-mediated rescue. Unresolved by these conclusions was the observation that these cell lines, whose HSV proviruses were partially deleted, were nevertheless able to release small amounts of particles which behaved like infectious HSV. The resolution of this paradox and a more precise characterization of the deleted proviruses are given in the accompanying report (12).

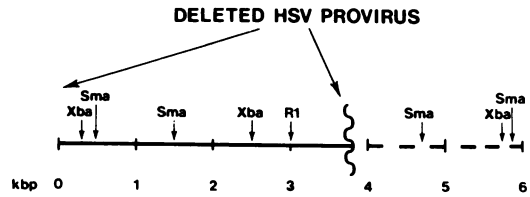

FIG. 5. *Physical map of deleted HSV proviral DNA. The proviral DNA in poorly rescuable clone X1, 16-1, and 16-7 cell lines lacked the SmaI and XbaI sites located at 4.7, 5.7, and 5.9 kbp along HSV 6-kbp DNA, but contained restriction endonuclease cleavage sites located in the left half of HSV 6-kbp DNA. Each deleted HSV provirus was between 3.0 and 4.7 kbp long.*

Origin of deleted HSV genomes. The origin of these deleted HSV proviruses is most readily explained by random degradation or shearing of the DNA during the transfection process. This is supported by Fig. 2, which shows an analysis of the DNAs of poorly rescuable clones 16-1 and 16-7, both of which were derived via transfection of NIH 3T3 cells with cell-linked HSV DNA from clone 16 cells. The *Eco*RI-cleaved DNAs from clone 16-1 and 16-7 cells contained a 3.5-kbp HSV-specific DNA fragment (Fig. 2, lanes d and e) that was also present in *Eco*RI-cleaved clone 16 DNA (lane b). This observation implies that in clone 16-1 and 16-7 cells, the left portion of the HSV provirus was conserved upon clone 16 DNA transfection, together with the adjacent, leftward lying cellular DNA sequences. The right end of the HSV provirus and the rightward lying cellular sequences were lost in both cases. Thus, a process of nonspecific degradation may provide an explanation for the generation of the deleted genomes present in some transfected cells.

DISCUSSION

Upon transfection of NIH 3T3 cells with unintegrated HSV DNA, the viral DNA becomes joined to nonviral DNA sequences within the

recipient cells. In contrast, upon transfection with cell-linked HSV DNA, the viral DNA within the recipient cells usually retains its linkage to the flanking nonviral donor sequences. Whether unintegrated or cell-linked HSV DNA is employed in transfection, the viral DNA and its linked nonviral sequences undergo sequence amplification within the transfected recipient cells to between 6 and 100 copies per diploid cell. We suspect that the fate of transfected retroviral DNA molecules differs from the fate of viral DNA molecules synthesized within a cell after retrovirus infection. Other investigators have shown that nonproducer transformed cells derived by sarcoma virus infection can have as few as one copy of proviral DNA per haploid genome (8).

The establishment of HSV DNA within transfected cells may follow the same process by which other types of transfected DNA molecules become expressed within recipient cells. Other investigators have shown that upon transfection of the thymidine kinase gene into cells lacking thymidine kinase, this gene became joined nonspecifically to other transfecting DNA molecules. This novel DNA structure bearing the thymidine kinase gene could persist as an extrachromosomal entity for many cell generations (M. Wigler, personal communication). We suggest that transfected HSV DNA becomes linked to other cotransfected, nonviral DNA molecules within the recipient cells to generate a self-replicating DNA structure. This structure containing the HSV genome could undergo amplification by unequal segregation at mitosis or by replication at a rate faster than once per cell generation. It is conceivable that the increase in HSV genome copy number within a cell may confer a selective growth advantage upon that cell.

The amplification of viral DNA sequences after transfection of cells with sarcoma viral DNA may make these transfected cells excellent agents for the production of virus stocks having high ratios of sarcoma virus to leukemia virus. When the transfected cells are infected with leukemia virus, there would be a great excess of sarcoma proviruses compared with leukemia proviruses within the cells and presumably a great excess of sarcoma viral RNA compared with leukemia viral RNA. In support of this speculation, we note that M-MLV infection of clone 14 transformed cells (derived by transfection with HSV DNA) results in the release of 5×10^7 FFU of HSV per ml and only 1×10^6 PFU of M-MLV per ml (Table 1).

Some cells transfected with HSV DNA acquired a deleted HSV provirus lacking between 1.3 and 3.0 kbp of the right end of full-length HSV DNA. Cells bearing the deleted provirus were transformed morphologically, but the deleted HSV genome was, at best, poorly transmissible when rescued with M-MLV helper. These experiments demonstrated that the 3′ end of the HSV genomic RNA is not essential for virus-induced transformation, whereas the same 3′ sequences are critical for efficient transmission of HSV from cell to cell. A more precise localization of the HSV transforming gene to the 5′ region of the viral RNA genome has been reported recently by others (4).

Given the apparent necessity of the 3′-terminal HSV RNA sequences for virus transmission, it was unclear how any transmissible sarcoma virus could be rescued from transformed cells containing deleted HSV proviruses. In the accompanying article (12), we report that genetic recombination between M-MLV and deleted HSV can regenerate phenotypically wild-type sarcoma viruses.

ACKNOWLEDGMENTS

We thank D. Lowy and E. Scolnick for the gift of a molecular clone of HSV DNA. We are also grateful to D. Steffen for helpful discussions.

M.P.G. was supported by Public Health Service predoctoral training grant from the National Institutes of Health and by a Johnson and Johnson Fellowship. R.A.W. was a fellow of the Rita Allen Foundation. This research was supported by Public Health Service grant CA-17537 from the National Cancer Institute and by Public Health Service core grant CA-14051 from the National Cancer Institute to S. E. Luria.

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