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Generation of Novel, Biologically Active Harvey Sarcoma Viruses via Apparent Illegitimate Recombination

MITCHELL P. GOLDFARB† AND ROBERT A. WEINBERG*

Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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NIH 3T3 cells transfected with Harvey sarcoma virus (HSV) DNA may acquire deleted proviruses (Goldfarb and Weinberg, J. Virol. 38:125-135, 1981). Such proviruses lack the right end of the wild-type HSV DNA genome corresponding to the 3'-proximal portion of the viral RNA. As expected, the RNA transcripts of these deleted HSV (delHSV) proviruses lacked sequences normally found at the 3' end of wild-type HSV RNA. Since frequently these delHSV RNA transcripts were longer than wild-type HSV RNA, we suggest that transcription proceeded through the deleted provirus and continued into flanking nonviral sequences. When delHSV-transformed cells were infected with Moloney murine leukemia virus (M-MLV), delHSV RNA was pseudotyped into new virus particles, demonstrating that the 3'-proximal sequences of wild-type HSV RNA are not essential for virion RNA encapsidation. Cells which carried a delHSV genome and were infected with M-MLV helper released very low titers of highly transmissible sarcoma virus. The inability to rescue high titers of sarcoma virus from these cells reflected the necessary presence of the deleted 3'-terminal sequences for normal efficient transmission of the sarcoma virus genome (Goldfarb and Weinberg, J. Virol. 38:125-135, 1981). The small amount of highly transmissible sarcoma virus rescuable from delHSV-transformed cells originated via genetic recombination between delHSV and the M-MLV helper used for the sarcoma virus rescue. The recombinant sarcoma virus genomes reacquired a competent 3' genomic end from the parental M-MLV genome, which restored efficient transmissibility. The locations of sites for recombination between the delHSV and M-MLV genomes appeared to be nonrandom. These sites were in genomic regions where the parental genomes bore no detectable sequence homology. Structural mapping of these recombinant sarcoma virus genomes indicated that the HSV transformation gene lies within 2.0 kilobases of the RNA 5' end. Based upon our genetic recombination studies, we suggest a model to explain how leukemia viruses can recombine with cellular sequences to generate novel defective viruses.

Harvey sarcoma virus (HSV) is a retrovirus which can induce solid tumors in rodents and can cause morphological transformation of mouse fibroblasts cultured in vitro. The HSV genome codes for a 21,000-dalton phosphoprotein which is responsible for induction and maintenance of cell transformation in vitro (32, 33). Although the HSV genome is not known to specify any additional proteins, there are genomic sequences outside the transforming gene which play critical roles in the life cycle of this virus. A sequence carried by HSV RNA allows the viral RNA to be packaged selectively and efficiently into newly synthesized virions formed in a cell which is coinfected with HSV and a replication-competent helper virus (14). Furthermore, faithful reverse transcription of HSV RNA within newly infected cells appears to depend upon specific nucleotide sequences at the 5' and 3' termini of the viral RNA (11, 13).

We have sought to map the sequences on the HSV genome which play necessary roles in mediating cell transformation and cell-to-cell virus transmission. In the accompanying article (16), we show that NIH 3T3 fibroblasts transfected with HSV DNA sometimes acquire an incomplete HSV provirus. Cells bearing the deleted HSV (delHSV) genome become transformed, but infection with Moloney murine leukemia virus (M-MLV) helper results in the rescue of only very low levels of wild-type sarcoma virus. Since the proviral DNA in these transfected

† Present address: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

cells lacks the right end of the HSV provirus (corresponding to the 3′ end of the viral RNA), we concluded that the transforming gene of HSV is located in the central or left-end portion of the HSV provirus (corresponding to the middle or 5′ end of the viral RNA). We also concluded that the inefficient transmissibility of these deleted viral genomes was probably correlated with the absence of the right end of the provirus (16). Such a conclusion is consistent with the findings of others that indicate the vital role of 3′-proximal RNA sequences in several steps of viral DNA synthesis (13).

These findings and conclusions do not explain the apparently wild-type behavior of a small amount of sarcoma virus which was rescuable from these cells after helper virus infection. We speculated that the RNA genome of the rescued wild-type sarcoma virus reacquired biological activity through the restoration of a functional 3′ genomic end. This restoration could have resulted from one of the following mechanisms: (i) the extreme left end of the delHSV provirus, which was derived originally from reverse transcription of a viral RNA 3′ end, may have been the source of the reacquired 3′-terminal RNA sequence, or (ii) genetic recombination between delHSV and the M-MLV used for rescue of sarcoma virus could have generated a novel sarcoma virus genome bearing a newly acquired 3′-terminal sequence of M-MLV origin.

In the experiments described here, we analyzed the size and structure of the RNA transcribed from delHSV proviruses and of the RNA genomes of wild-type sarcoma virus rescued from delHSV-transformed cells. This analysis defined the mechanism by which wild-type sarcoma virus was regenerated. It also led to a more precise functional mapping of the HSV genome.

MATERIALS AND METHODS

Preparation of cytoplasmic RNA. Cytoplasmic RNA was extracted by the urea-sodium dodecyl sulfate method, as described elsewhere (20, 29). When necessary, cytoplasmic RNA was subjected to two rounds of polyadenylic acid selection by chromatography on oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc.) (3).

Preparation of ^{32}P-labeled virion RNA. Virus-infected cell cultures were incubated for 2 h in medium containing 1 of mCi $^{32}P_i$ (New England Nuclear Corp.) per 10-cm culture dish. Virus particles were harvested from the cells during a subsequent 5-h chase period, and [^{32}P]RNA was extracted from virions as described elsewhere (31).

RNA gel electrophoresis. Electrophoresis of RNA through 1% agarose gels containing methylmercuric hydroxide was conducted as described elsewhere (4), except that the electrophoresis buffer was 5 mM $Na_2B_4O_7$–10 mM Na_2SO_4–1 mM EDTA (no boric acid was added, and the solution had a pH of 9.0 to 9.2).

When gels were to be autoradiographed directly, they were soaked in 0.5 M ammonium acetate for 30 min, soaked twice in methanol, and dried onto a glass plate by overlaying with paper towels.

Preparation of ^{32}P-labeled cDNA and nick-translated double-stranded DNA. The synthesis of rat sequence-enriched HSV ^{32}P-labeled complementary DNA (cDNA) and the ^{32}P nick translation of HSV- and M-MLV-plasmid DNA hybrids were by previously described methods (15, 25).

RNA diazo-derivatized filter hybridization. RNA in methylmercury agarose gels was transferred to diazo-derivatized filter paper by the method of Alwine et al. (1). The RNA on these filters was hybridized to ^{32}P-labeled cDNA or denatured nick-translated [^{32}P]DNA (5 to 10 ng/ml) in a 50% formamide solution at 42°C (6).

Molecularly cloned retrovirus DNA. The recombinant pBR322 plasmid bearing the 3.2-kilobase pair (kbp) rat-specific HSV DNA fragment (see Fig. 2E) was derived by methods described in the accompanying article (16).

pBR322 plasmid bearing *Hin*dIII-permuted M-MLV 8.8-kbp DNA (see Fig. 2A) and plasmid bearing the *Hin*dIII-*Sac*I 3′-end-specific M-MLV 3.3-kbp fragment (see Fig. 2D) were the kind gifts of E. Gilboa.

All plasmids were propagated in *Escherichia coli* χ1776 cells.

^{32}P-labeling of plasmid DNA in vivo. A 50-ml volume of plasmid-bearing bacteria which were grown to a cell density of 0.5 to 0.8 unit of absorbance at 550 nm in low-phosphate medium was supplemented with 40 μg of chloramphenicol per ml and 5 mCi $^{32}P_i$, and the plasmid was allowed to amplify for 5 h at 37°C. ^{32}P-labeled plasmid DNA was extracted by methods described elsewhere (22). DNAs prepared in this manner had specific activities of 3×10^5 to 10×10^5 dpm/μg.

S1 nuclease mapping of RNA-DNA heteroduplexes. ^{32}P-labeled recombinant plasmid DNA was cleaved with the restriction endonuclease required to separate the retrovirus DNA insert from the pBR322 DNA, and the insertion DNA was purified by agarose gel electrophoresis and recovered by the sodium iodide glass powder method (35). When necessary, insert DNA was further cleaved by additional restriction endonucleases, and in some cases, fragments were gel purified before use in heteroduplex studies.

Cytoplasmic RNA (not selected on oligodeoxythymidylic acid-cellulose) was hybridized to [^{32}P]DNA by the method of Berk et al. (5). Reaction mixtures (40 μl) contained 0.4 M NaCl, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.1), 1 mM EDTA, 80% formamide, 1 mg of RNA per ml, and 0.17 pmol of DNA fragments per ml (e.g., 1 μg of M-MLV 8.8-kbp DNA per ml). Hybridizations with M-MLV [^{32}P]DNA were conducted at 56.5°C, and hybridizations with HSV [^{32}P]DNA were conducted at 53°C. These temperatures were 4 to 6°C above the respective DNA-DNA melting temperatures. Heteroduplex samples were digested with S1 nuclease (Boehringer Mannheim Corp.) as described in the original procedure (5), and the samples were subjected to alkaline electrophoresis through 2% agarose gels for 10 to 16 h

at 1.3 V/cm. After electrophoresis, gels were soaked twice in methanol and dried onto glass plates before autoradiography.

Southern filter hybridization of recombinant sarcoma virus unintegrated linear DNA. Procedures for the purification of X1:V2 recombinant sarcoma viral DNA (see below), cleavage with restriction endonucleases, and detection of viral DNA fragments by Southern filter hybridization directly paralleled procedures used to map HSV 6.0-kbp unintegrated linear DNA (15).

Sarcoma virus rescue from nonproducer transformed cells. The rescue of sarcoma virus from nonproducer transformed cells by infection with murine leukemia virus (MLV), the focus assay for infectious sarcoma virus, and the XC plaque assay for MLV were all performed as previously described (2, 21, 26).

RESULTS

Sizes of RNA species transcribed from delHSV proviruses. We initially studied the RNA transcripts transcribed from HSV proviruses lacking right genomic ends. These deleted proviruses are missing 1.3 kbp or more of the viral sequence. The transcripts of these delHSV proviruses were identified by using RNA gel electrophoresis and filter blot hybridization (1). Polyadenylated cytoplasmic RNA was prepared from three transformed cell lines (clones X1, 16-1, and 16-7), each of which carried a delHSV genome. In addition, as control reagents, we prepared RNAs from two transformants which bore complete HSV proviruses (clones 14 and 16) (16). The RNA samples were subjected to agarose gel electrophoresis in the presence of methylmercuric hydroxide, and the RNA contents of the gel were transferred and affixed to diazo-derivatized filter paper. The filter paper was incubated with a ^{32}P-labeled HSV cDNA sequence probe which had been depleted of sequences homologous to the M-MLV genome (16).

Figure 1 shows that cells transformed by wild-type HSV contained a single prominent species of HSV-specific polyadenylated cytoplasmic RNA (lanes d and e), which was the same size as HSV 5.4-kilobase (kb) virion RNA (lanes g and h). In contrast, each of the three cell lines transformed by delHSV contained two species of HSV-specific polyadenylated RNA (lanes a, b, c, and f). These three cell lines did not share viral RNA species of common sizes. The masses of the larger RNA transcripts in the delHSV-transformed cells were at least 5.4 kb. If the initiation of transcription of the delHSV proviruses was identical to the initiation of transcription of wild-type HSV proviral DNA, then the synthesis of RNA transcripts larger than 5.4 kb would have required transcription to proceed through the length of the delHSV provirus and continue into the adjacent nonviral DNA sequences. Less certain was the structure of the smaller RNA transcripts which were, at most, slightly longer than the delHSV proviruses from which they originated.

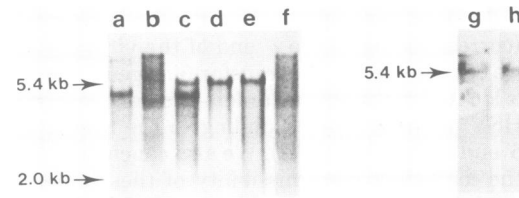

FIG. 1. *Filter hybridization analysis of cytoplasmic RNAs from delHSV-transformed cells. Polyadenylated cytoplasmic RNAs were prepared from lines of cells transformed by delHSV (clones X1, 16-1, and 16-7) and wild-type HSV (clones 14 and 16). The RNA samples were subjected to electrophoresis through methylmercury-1% agarose slab gels. The gel contents were blotted onto diazobenzyloxymethyl cellulose filter paper, and the filter was hybridized with rat sequence-enriched HSV ^{32}P-labeled cDNA. The polyadenylated RNAs were from clone X1 (lane a), clone 16-7 (lane b), clone 16-1, (lane c), clone 16 (lane d), and clone 14 (lanes e and g). Lane f is a longer exposure of lane b; lane h contained HSV virion RNA. The position of ribosomal 2-kb RNA was detected by ethidium bromide staining of the gel before RNA transfer.*

S1 heteroduplex mapping of sequence components in RNA transcribed from delHSV DNA. We wished to identify those segments of the wild-type HSV genome which were retained in the RNA transcripts of delHSV proviruses. Toward this end, we used the S1 heteroduplex mapping technique of Berk et al. (5). [^{32}P]DNAs from defined segments of the HSV or M-MLV genome were annealed to total cytoplasmic RNA from delHSV-transformed cells. The RNA-DNA heteroduplexes generated were digested with S1 nuclease to remove unhybridized DNA molecules and single-stranded tails extending from heteroduplex molecules. The sizes of the S1-resistant DNA segments were determined by alkaline agarose gel electrophoresis. The appearance of a discrete DNA fragment in these gels indicated that the annealed RNA molecules bore a sequence complementary to this DNA segment which was present in an uninterrupted form in the RNA molecule (5).

Since the HSV strain was derived originally after recombination between rat sequences and the M-MLV genome (27), certain portions of HSV viral RNA retain homology with the M-MLV genome. The M-MLV homologous regions are found at the genomic ends of the HSV RNA genome. Other investigators have reported that

1 kb of the sequence at the 3′ end of wild-type HSV RNA is derived from the 3′ portion of the M-MLV RNA genome. In addition, approximately 100 nucleotides of the 5′ end of HSV RNA is derived from sequences present at the 5′ terminus of M-MLV RNA (8, 9).

We wished to confirm the presence of these M-MLV sequences in the wild-type HSV RNA genome and to ascertain which sequences, if any, were retained in the delHSV variant genomes. For the detection of these sequences common to the HSV and M-MLV genomes, we employed several M-MLV DNA sequence probes. The first probe contained the sequence of the entire M-MLV genome cloned into the bacterial plasmid pBR322. This clone was the gift of E. Gilboa. The 8.8-kbp M-MLV DNA in this clone was circularly permuted with respect to the sequence present in an M-MLV provirus. During the original cloning, circular M-MLV DNA was cleaved at one location by *Hin*dIII endonuclease, and the resulting linearized viral DNA was inserted into the *Hin*dIII site of the plasmid (Fig. 2A). This chimeric plasmid DNA was labeled in vivo with $^{32}P_i$, purified, and cleaved with *Hin*dIII, and the 8.8-kbp viral DNA was purified by gel electrophoresis before use. This 8.8-kbp M-MLV DNA was then cleaved with either *Sac*I or *Hpa*I endonuclease, generating two other probes (Fig. 2B and C). Finally, an M-MLV probe homologous to the 3′ end of M-MLV RNA was employed. This probe consisted of a 1-kbp *Hpa*I-*Sac*I fragment (Fig. 2D) isolated by gel purification from a subclone of M-MLV DNA (also a gift of E. Gilboa).

The four M-MLV probes were first annealed to cytoplasmic RNA from cells bearing a wild-type HSV provirus and producing HSV 5.4-kb RNA (clone 16 cells). S1 nuclease digestion of heteroduplexes formed by incubating cytoplasmic wild-type HSV 5.4-kb RNA with the M-MLV 8.8-kbp [^{32}P]DNA (Fig. 2A) yielded DNA fragments of 900, 700, 550, and 230 nucleotides (Fig. 3, lane b).

We suspected that the 900-base fragment resulted from hybridization of the 3′ region of the M-MLV DNA to the 3′ end of the HSV RNA. If this assumption was correct, then the region of M-MLV DNA homologous to the 3′ end of HSV RNA should have contained a *Sac*I cleavage site approximately 100 bases from the 3′ side of this homologous region (Fig. 2A). In addition, there should have been no *Hpa*I cleavage sites within this region. Indeed, hybridization of the *Sac*I-cleaved 8.8-kbp M-MLV DNA probe to wild-type HSV RNA generated an 800-nucleotide fragment (Fig. 3, lane z), whereas cleavage of the M-MLV DNA with *Hpa*I before hybridization generated the same 900-nucleotide fragment de-

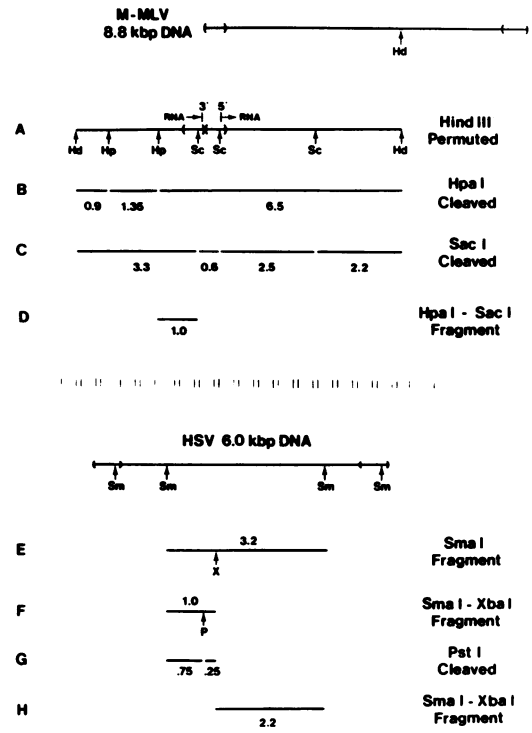

FIG. 2. *DNA fragments (probes) used in heteroduplex S1 mapping experiments. All [^{32}P]DNA retrovirus probes used in the heteroduplex analyses were derived from recombinant retrovirus-pBR322 plasmids labeled with ^{32}P in vivo. (A) HindIII-permuted M-MLV 8.8-kbp DNA (gift of E. Gilboa). The insertion was purified from pBR322 DNA by HindIII cleavage and gel electrophoresis. Terminal redundancies in the middle of the insertion are indicated in parentheses; the sites within these redundancies corresponding to the 5′ and 3′ ends of viral RNA are also indicated. Hd, HindIII; Hp, HpaI; Sc, SacI (11). (B) HindIII-permuted M-MLV 8.8-kbp DNA cleaved with HpaI before use. The sizes of the HpaI DNA fragments are given in kilobase pairs. (C) HindIII-permuted M-MLV DNA cleaved with SacI. (D) HpaI-SacI 3′-specific M-MLV 1-kbp DNA. HindIII-permuted M-MLV 8.8-kbp DNA was cleaved with SacI, and the 3.35-kbp fragment corresponding to the 3′-proximal RNA sequences was subcloned. This DNA subclone (gift of E. Gilboa) was labeled with ^{32}P and cleaved with HpaI, and the 1.0-kbp HpaI-SacI fragment was purified. (E) HSV rat-specific 3.2-kbp SmaI DNA fragment (16, 17). Sm, SmaI; X, XbaI. (F) The 1.0-kbp SmaI-XbaI HSV DNA fragment. The 3.2-kbp SmaI fragment was cleaved with XbaI, and the 1.0-kbp fragment was gel purified. P, Pst. (G) The 1.0-kbp SmaI-XbaI HSV DNA fragment cleaved with PstI before use. (H) The 2.2-kbp Sma-XbaI HSV DNA fragment. The 3.2-kbp SmaI fragment was cleaved with XbaI, and the 2.2-kbp fragment was gel purified. (A) through (H) correspond to probes A through H, respectively, which are referred to in Table 1 and the legends to Fig. 3 and 4.*

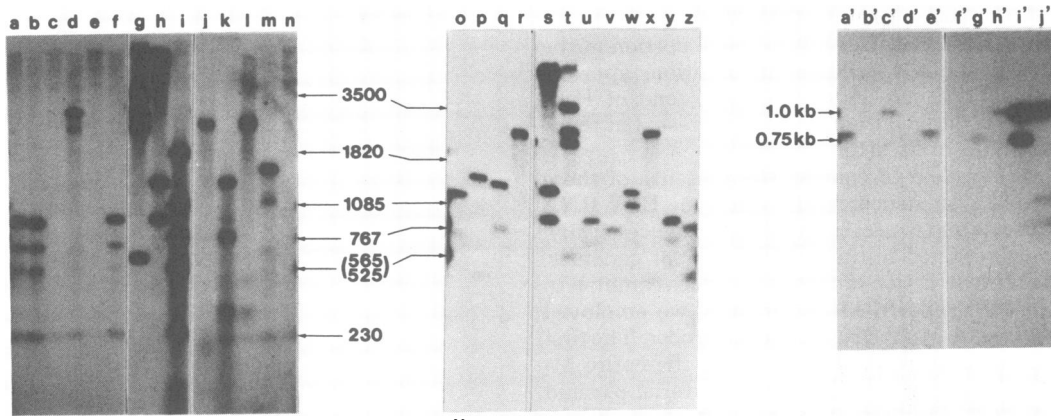

FIG. 3. *Electrophoresis of RNA-M-MLV [^{32}P]DNA hybrids digested with S1 nuclease. Cytoplasmic RNA from cells transformed by HSV, delHSV, or recombinant sarcoma virus was hybridized to one of the four M-MLV ^{32}P-labeled DNA probes (Fig. 2A through D). Hybrids were digested with S1 nuclease and subjected to electrophoresis through alkaline 2% agarose gels. Gels were dried and autoradiographed. Lane a, Clone 16 RNA, probe A; lane b, same as lane a, but threefold-higher S1 concentration; lane c, 16-1 RNA, probe A; lane d, 16-1:V1 RNA, probe A; lane e, 16-7 RNA, probe A; lane f, 16-7:V1 RNA, probe A; lane j, X1:V1 RNA, probe C; lane k, X1:V1 RNA, probe B; lane l, X1:V1 RNA, probe A; lane m, X1:V2 RNA, probe A; lane n, X1 RNA, probe A; lane o, X1:V2 RNA, probe B; lane p, X1:V2 RNA, probe C; lane q, X1:V1 RNA, probe B; lane r, X1:V1 RNA, probe C; lane u, 16-7:V1 RNA, probe B; lane v, 16-7:V1 RNA, probe C; lane w, 16-1:V1 RNA, probe B; lane x, 16-1:V1 RNA, probe C; lane y, 16 RNA, probe B; lane z, 16 RNA, probe C; lane a', 16 RNA, probe D; lane b', 16-1 RNA, probe D; lane c', 16-1:V1 RNA, probe D; lane d', 16-7 RNA, probe D; lane e', 16-7:V1 RNA, probe D; lane f', X1 RNA, probe D; lane g', X1:V1 RNA, probe D; lane h', X1:V2 RNA, probe D; lanes g and t, probe C without S1 digestion; lanes h and s, probe B without S1 digestion; lane j', probe D without S1 digestion; lane i', HSV DNA probes F and G without S1 digestion; lane i, simian virus 40 Hinf-cleaved [^{32}P]DNA, which was also run in other gel lanes (data not shown). Molecular weight standards (in bases) included simian virus 40 Hinf fragments, the 3.35-kb fragment of probe C, the 1.0-kb fragment of probes D and E, and the 0.75-kb fragment of probe G.*

tected in the previous experiment when uncleaved DNA was used (Fig. 3, lane y). To confirm this alignment further, we utilized the 3'-specific *Hpa*I-*Sac*I M-MLV DNA fragment (Fig. 2D) as a probe. The use of this probe yielded the expected 800-nucleotide fragment (Fig. 3, lane a). We concluded that the 3' end of HSV RNA contains a 900-nucleotide, contiguous sequence derived from the 3' end of the M-MLV genome.

When the 8.8-kbp M-MLV DNA probe was hybridized to RNAs prepared from the three delHSV-transformed cell lines (clones X1, 16-1, and 16-7), only a 230-base fragment of DNA could be protected after S1 digestion (Fig. 3, lanes c, e, and n). The 900-base heteroduplex fragment specific for the 3' end of wild-type HSV RNA was absent. Therefore, as expected, the RNA transcripts of delHSV proviruses lacked the M-MLV-derived sequences at the 3' end of HSV RNA. The presence of the protected 230-nucleotide DNA fragment in heteroduplexes with RNAs from delHSV transformants indicated that the 5' end of delHSV RNA transcripts contained a 230-base sequence derived from the 5' end of the M-MLV RNA genome. In addition, the presence of this fragment in heteroduplexes with wild-type HSV RNA indicated that the 230 nucleotides at the 5' end of wild-type HSV RNA were derived from the 5' end of M-MLV RNA. Thus, the 5' ends of wild-type HSV and delHSV RNAs were identical. Our size determinations for the 5'- and 3'-terminal HSV-M-MLV homologies were similar to the values previously reported by others (8, 9).

Besides the above-mentioned fragments, we detected a series of other DNA fragments in the lanes of these gels. Thus, two additional DNA segments of 550 and 700 nucleotides were detected after S1 nuclease digestion of the complexes formed between wild-type HSV RNA and 8.8-kb M-MLV DNA. These extra fragments appeared to result from annealing of the 3' end of HSV RNA to the right M-MLV DNA terminal repeat in the *Hind*III-permuted M-MLV clone (Fkg. 2A) (generating a heteroduplex of ~525-bases) and the additional hybridization of the 5' end of the RNA to DNA sites adjacent to the 525-base pair heteroduplex, giving an additional 150 nucleotides of protection from S1 nuclease digestion. Such bands have been observed by others in analyses of heteroduplexes of M-MLV DNA and Abelson MLV RNA. (The genomic ends of Abelson MLV are also derived

from M-MLV [30].)

To determine whether other wild-type HSV sequences were retained in the transcripts of delHSV genomes, we used as a probe the 3.2-kbp *Sma*I fragment of HSV DNA derived from cloned HSV DNA (17), which was a gift of D. Lowy and E. M. Scolnick. This *Sma*I fragment is derived from the middle of the wild-type HSV genome and contains only sequences of rat origin. The *Sma*I fragment was subcloned into pBR322 plasmid, as described previously (16) (Fig. 2E). ^{32}P-labeled 3.2-kbp viral DNA was used as a probe directly. Alternatively, the 3.2-kbp fragment was cleaved with *Xba*I, and the resulting 2.2- and 1.0-kbp fragments were purified by gel electrophoresis before use as probes (Fig. 2F and H).

When wild-type HSV RNA was annealed to the 3.2-kbp HSV DNA probe, the expected 3.2-kb fragment was not observed upon analysis of the products of S1 digestion. Rather, four smaller species of differing intensities were observed (Fig. 4, lane c). These smaller fragments arose as a consequence of sequence mismatches between the DNA and RNA molecules. In one case the mismatch was recognized efficiently by the S1 nuclease, generating 2.5- and 0.7-kb fragments. In the other case the mismatch was detected only occasionally by the nuclease, causing a low yield of 1.4- and 1.1-kb DNA fragments. These mismatches between the DNA and RNA reflect mutations which accumulated in the genomes of the HSV substrains which were analyzed in these experiments. We used the DNA of one substrain for transfection, whereas others used the DNA of a second substrain to generate the molecular clones of the HSV genome. Although these alterations appear to have no functional significance for the HSV genome, they did provide useful landmarks for the present analysis.

The use of the 3.2-kb HSV DNA probe to study the delHSV RNAs resulted in incomplete protection of various portions of the probe (Fig. 4, lanes d, g, and i). The use of the left 1.0-kb portion of this probe (in the form of a *Sma*I-*Xba*I fragment [Fig. 2F]) indicated the presence of this entire 1-kb region in all three delHSV RNA genomes (Fig. 4, lanes p, r, and v). Thus, the delHSV genomes all terminated between the *Xba*I site (Fig. 2E) and the *Sma*I site to its right.

The results of all of these heteroduplex experiments (Table 1), along with the results of the previously described RNA filter hybridization experiments, enabled us to construct the maps of the delHSV RNA transcripts shown in Fig. 5.

M-MLV pseudotyping of RNA transcribed from delHSV proviruses. M-MLV infection of delHSV-transformed cell lines is followed by the release of large quantities of infectious helper virus, but far less infectious sarcoma virus (16). We determined the sizes of the RNA molecules in the virions released from two of these M-MLV-infected, delHSV-transformed lines. The two cell lines (clones X1 and 16-1) were infected with M-MLV at a multiplicity of infection of more than 1 and incubated 2 days later in medium containing $^{32}P_i$. Virus was harvested from the cultures, and viral RNA was prepared. The viral RNA was subjected to elec-

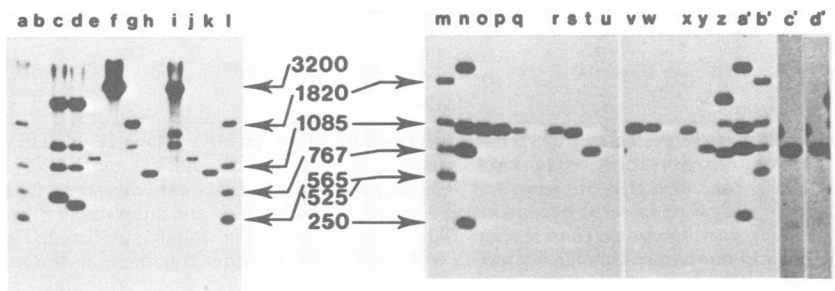

FIG. 4. *Electrophoresis of RNA-HSV rat-specific [^{32}P]DNA hybrids digested with S1 nuclease. The same cytoplasmic RNAs analyzed in Fig. 3 were hybridized to one of the four HSV rat-specific ^{32}P-labeled DNA probes (Fig. 2E through H). Lane b, NIH 3T3 RNA, probe E; lane c, clone 16 RNA, probe E; lane d, 16-1 RNA, probe E; lane e, 16-1:V1 RNA, probe E; lane g, 16-7 RNA, probe E; lane h, 16-7:V1 RNA, probe E; lane i, X1 RNA, probe E; lane j, X1:V1 RNA, probe E; lane k, X1:V2 RNA, probe E; lane o, 16 RNA, probe F; lane p, 16-1 RNA, probe F; lane q, 16-1:V1 RNA, probe F; lane r, 16-7 RNA, probe F; lane s, 16-7:V1 RNA, probe F; lane t, 16-7:V1 RNA, probe G; lane u, NIH 3T3 RNA, probe F; lane v, X1 RNA, probe F; lane w, X1:V1 RNA, probe F; lane x, X1:V2 RNA, probe F; lane y, X1:V2 RNA, probe G; lane z, 16 RNA, probe H; lane c', long exposure of lane s; lane d', long exposure of lane x; lane f, probe E, no S1 digestion; lanes n and a', probes F, G, and H, no S1 digestion; lanes a, l, m, and b', simian virus 40 Hinf-cleaved [^{32}P]DNA. Molecular weight standards (in bases) included simian virus 40 Hinf fragments and 3.2-kb probe E.*

TABLE 1. *S1 nuclease digestion of hybrids formed between wild-type HSV, delHSV, or recombinant sarcoma virus RNAs and ^{32}P-labeled HSV or M-MLV DNAs*[a]

Cytoplasmic RNA from clone:	[^{32}P]DNA probe[b]	Size(s) of S1-resistant DNA fragment(s) (kb)	Detected on:[c]	
			Figure	Lane(s)
16 (wild-type HSV)[d]	A	0.9,0.23,(0.7),(0.55)[e]	3	a and b
	B	0.9,(0.7),(0.55)	3	y
	C	0.8,(0.6),(0.4)	3	z
	D	0.8	3	a′
	E	2.5,0.7–0.75,(1.4),(1.1)	4	c
	F	1.0	4	o
	H	1.4–1.5,0.7,(1.0–1.1),(0.4)	4	z
16-1 (delHSV)	A	0.23	3	c
	D		3	b′
	E	2.5,0.65,(1.4),(1.1)	4	d
	F	1.0	4	p
16-7 (delHSV)	A	0.23	3	e
	D		3	d′
	E	1.8–1.9,(1.4),(0.5)	4	g
	F	1.0	4	r
X1 (delHSV)	A	0.23	3	n
	D		3	f′
	E	3.1,(1.6),(1.4)	4	i
	F	1.0	4	v
16-1:V1 (recSV)[f]	A	2.6,0.23,(2.2),(0.7),(0.55)	3	d
	B	1.3,1.1,(0.7),(0.55)	3	w
	C	2.4–2.5,(0.4)	3	x
	D	1.0–1.05	3	c′
	E	1.2	4	e
	F	1.0	4	q
16-7:V1 (recSV)	A	0.9–0.95,0.23,(0.7),(0.55)	3	f
	B	0.9,(0.7),(0.55)	3	u
	C	0.8,(0.6),(0.4)	3	v
	D	0.8–0.85	3	e′
	E	0.95–1.0	4	h
	F	0.95–1.0	4	s
	G	0.75,0.20	4	t and c′
X1:V1 (recSV)[g]	A	2.3,0.3,0.23	3	l
	B	1.35,0.75–0.8,0.3,0.23	3	k and q
	C	2.3,0.23	3	j and r
	D	0.75–0.8	3	g′
	E	1.2	4	j
	F	1.0	4	w
X1:V2 (recSV)	A	1.5,0.23,(1.1),(0.7),(0.55)	3	m
	B	1.1,(0.7),(0.55)	3	o
	C	1.4,(0.6),(0.4)	3	p
	D	1.0–1.05	3	h′
	E	0.95–1.0	4	k
	F	0.95–1.0	4	x
	G	0.75,0.2–0.25	4	y and d′

[a] Cytoplasmic RNAs were extracted from cells transformed by wild-type HSV (clone 16), delHSV (clones 16-1, 16-7, and X1), and recombinant sarcoma viruses (clones 16-1:V1, 16-7:V1, X1:V1, and X1:V2). The RNAs were hybridized to M-MLV and HSV rat-specific [^{32}P]DNA probes, the hybrids were digested with S1 nuclease, and [^{32}P]DNA fragments were detected by alkaline agarose gel electrophoresis and autoradiography of the dried gel. The [^{32}P]DNA fragments visualized in the autoradiograms shown in Fig. 3 and 4 are listed. The [^{32}P]DNA fragments present in less-than-equimolar quantities with respect to the other fragments in the same lane are given in parentheses.

[b] The M-MLV-specific DNA probes A, B, C, and D and the HSV rat-specific DNA probes E, F, G, and H are described in the text and illustrated in Fig. 2.

[c] The figures and gel lanes where DNA fragments were detected.

[d] The HSV genome in the recombinant DNA plasmid used as the probe diverged slightly from the HSV genome in transformed clone 16 cells. Hybrids between clone 16 HSV RNA and HSV 3.2-kb [^{32}P]DNA were mismatched and, hence, fully S1 sensitive at a site 0.7 kb from the right end of the [^{32}P]DNA fragment and partially S1 sensitive at a site 1.8 kb from the right end of the [^{32}P]DNA fragment. These sites corresponded to 4.0 and 2.9 kbp on the HSV 6-kbp DNA physical map. The delHSV 16-1 and 16-7 genomes, which were derived by transfection with clone 16 cell-linked HSV DNA, contained mismatches identical to mismatches in the recombinant HSV DNA-pBR322 plasmid.

[e] We assigned a measurement of 230 bases to the 0.23-kb fragment because it comigrated with the 230-base simian virus 40 *Hinf* DNA fragment in the adjacent lane.

[f] recSV, Recombinant sarcoma virus.

[g] X1:V1 recombinant sarcoma virus acquired 2.6 kb of the M-MLV 3′-terminal RNA sequence. However, the acquired M-MLV sequence differed from M-MLV RNA at a site about 300 bases from the 3′ terminus, rendering X1:V1 RNA–M-MLV DNA heteroduplexes S1 sensitive at this site.

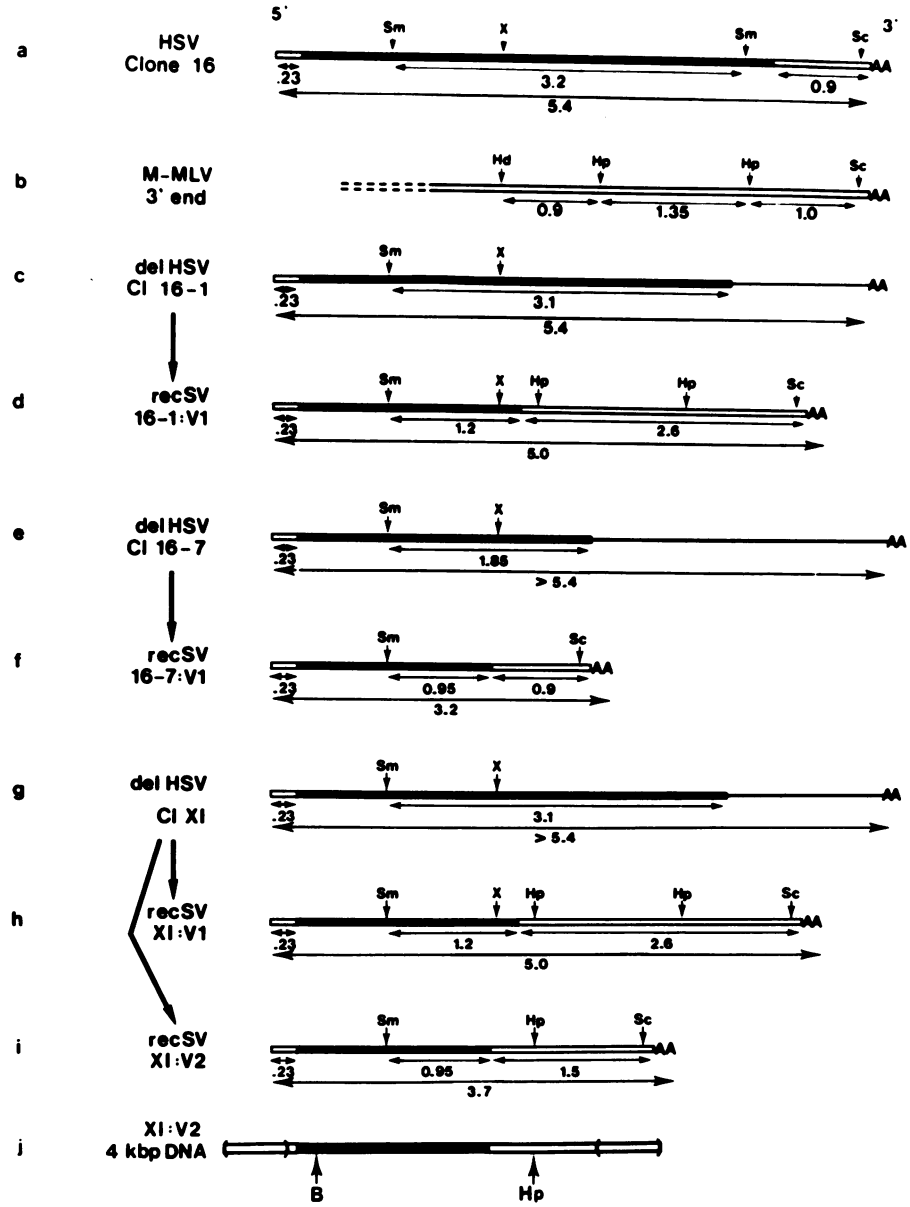

FIG. 5. *Structural maps of HSV RNA, delHSV RNA transcripts, and recombinant sarcoma viral (recSV) RNA genomes. The gel electrophoresis-RNA filter hybridization data (Fig. 1 and 7) and the S1 heteroduplex mapping data (Fig. 2 and 3 and Table 1) were used to construct maps of delHSV RNA transcripts and the recombinant sarcoma viral RNA genomes. For all of the RNAs, rat-specific sequences are indicated by thick black lines, M-MLV-derived sequences are indicated by white lines, and sequences of nonviral origin are indicated by thin black lines. Certain restriction endonuclease cleavage sites on DNA homologous to the RNA genomes are indicated above each RNA map (12, 17). Hd, HindIII; Hp, HpaI; Sc, SacI; Sm, SmaI; X, XbaI. The lengths (in kilobases) of rat-specific and M-MLV-specific components of the RNA genomes, as determined by S1 mapping, are shown below the RNA maps, as are the total lengths of these genomes as determined by RNA filter hybridization. (a) Wild-type HSV 5.4-kb RNA in clone 16 cells. (b) The 3' end of M-MLV RNA. Distances between the HindIII, HpaI, and SacI cleavage sites on the corresponding viral DNA were determined from measurements of the viral DNA fragments in Fig. 3, lanes g and h. (c) Longer RNA transcript of delHSV provirus in clone 16-1 cells. (d) Recombinant 16-1:V1 sarcoma viral RNA rescued from clone 16-1 cells. (e) Longer transcript of 16-7 delHSV provirus. (f) Recombinant 16-7:V1 sarcoma viral RNA rescued from clone 16-7 cells. (g) Longer transcript of X1 delHSV provirus. (h) Recombinant X1:V1 sarcoma viral RNA rescued from X1 cells. (i) Second sarcoma viral RNA (X1:V2) rescued from X1 cells. (j) X1:V2 4-kbp DNA. The predicted structure of the viral DNA intermediate in X1:V2 virus replication showing terminal sequence redundancy (in parentheses), the HpaI cleavage site in the MLV-derived sequence, and the BamHI (B) cleavage site in 5'-proximal rat-specific sequences are shown.*

trophoresis through methylmercuric hydroxide-agarose gels. RNA was also extracted from virions released from NIH 3T3 cells infected only with M-MLV and from cell lines transformed by wild-type HSV and superinfected with M-MLV (clones 14 and 16). Figure 6, lane b, shows that wild-type HSV-transformed cells released the expected 8.2-kb M-MLV RNA and 5.4-kb wild-type HSV RNA. M-MLV-infected, delHSV-transformed clone X1 cells released virions containing M-MLV 8.2-kb RNA and an RNA smaller than HSV 5.4-kb RNA. The smaller species corresponded in size to an intracellular viral RNA transcript detected in clone X1 delHSV-transformed nonproducer cells (Fig. 1, lane a). Similarly, M-MLV-infected clone 16-1 cells (Fig. 6, lane m) released virus containing M-MLV 8.2-kb RNA and the two HSV-specific RNA species previously detected in the cytoplasm of clone 16-1 cells (Fig. 1, lane c). These experiments demonstrated that RNA transcribed from delHSV proviruses can be selectively pseudotyped into M-MLV-encoded virus particles. We conclude that the nucleotide sequences responsible for the packaging of HSV RNA into virions are retained in the delHSV RNA genomes.

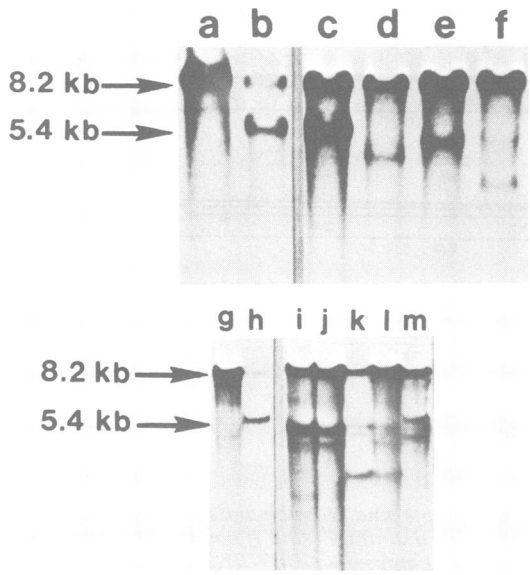

FIG. 6. *RNA in virus particles rescued from cells transformed by delHSV or recombinant sarcoma virus. Virus-producing cells were labeled with $^{32}P_i$, and ^{32}P-labeled RNA was extracted from newly synthesized virus particles. RNA samples were subjected to electrophoresis through methylmercury-1% agarose gels, and virion RNA was detected by autoradiography of the dried gels. All cell lines were infected with M-MLV before labeling with ^{32}P. Virion RNAs were from the following infected cell lines: lanes a and g, NIH 3T3; lanes b and h, clone 14; lane c, clone 16; lane d, clone X1; lane e, clone X1:V1; lane f, clone X1:V2; lane i, clone X1:V1; lane j, focus derived with clone X1:V1 virus; lane k, clone X1:V2; lane l, focus derived with clone X1:V2 serum; lane m, clone 16-1.*

Novel sizes of wild-type sarcoma virus genomes rescued from delHSV-transformed cells. The successfully encapsidated delHSV RNAs which were released after attempted M-MLV rescue were thought to be largely noninfectious as a consequence of their 3'-terminal deletions. Nevertheless, a small number of competent sarcoma virus genomes were also thought to be present in this virus stock, since upon titration a small amount of infectious sarcoma virus was detected. We suspected that these few competent genomes would differ in size and structure from the bulk of the viral transcripts present within the delHSV-transformed cells.

Virus released from M-MLV-infected, delHSV-transformed cells was introduced onto a monolayer culture of NIH 3T3 cells. Several of the foci which arose were independently picked and cultured. The expanded cultures derived from these foci released high titers of both M-MLV and sarcoma virus, confirming the presence of a transmissible sarcoma virus in each focus. High-titer virus stocks from each of the focus cultures were used in a second cycle of infection of NIH 3T3 cells (multiplicity of infection, less than 0.01), and the nonproducer foci which arose from this second cycle of virus passage were picked and cultured.

We analyzed the sarcoma virus RNA genomes in four of these independently isolated, nonproducer, transformed cell lines. Two of these nonproducer lines were derived from sarcoma virus rescued originally from clone X1 delHSV-transformed cells (nonproducer cultures X1:V1 and X1:V2). Another line was derived from particles rescued from delHSV-transformed clone 16-1 cells (culture 16-1:V1), and the last line arose after infection by particles derived from delHSV-transformed clone 16-7 cells (culture 16-7:V1). Cytoplasmic polyadenylated RNAs were prepared from these four nonproducer cultures, and the sizes of the sarcoma viral RNA species present in these RNAs were determined by the gel electrophoresis-filter blot hybridization method described above. As Fig. 7 shows, two of the nonproducer cell lines (16-1:V1 and X1:V1) had a single sarcoma virus-specific RNA species of ~5.0 kb (Fig. 7, lanes a and f). The X1:V2 nonproducer cells contained 3.7-kb sarcoma viral RNA (Fig. 7, lane b), whereas the 16-7:V1 nonproducer cells contained a single sarcoma viral RNA species of only 3.2 kb (lane g). These novel sarcoma viral RNAs also hybridized to M-MLV DNA (data not shown). These results

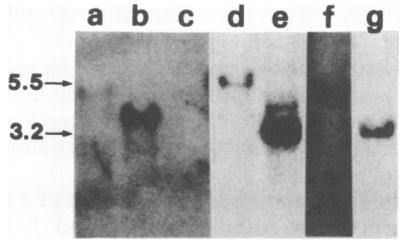

FIG. 7. *Filter hybridization analysis of cytoplasmic RNAs from cells transformed by recombinant sarcoma viruses. Polyadenylated cytoplasmic RNAs were prepared from NIH 3T3 cells and from lines of cells transformed by wild-type HSV (clone 16) and recombinant sarcoma viruses (clones 16-1:V1, 16-7:V1, X1:V1, and X1:V2). RNA samples were subjected to electrophoresis through a methylmercury-1% agarose gel, and the RNA contents of the gel were blotted onto a diazotized filter. The filter was hybridized with rat-specific HSV-pBR322 recombinant plasmid DNA (15, 16) labeled with ^{32}P by nick translation. Lane a, clone X1:V1 RNA; lane b, clone X1:V2 RNA; lane c, NIH 3T3 RNA; lane d, clone 16 RNA; lane f, clone 16-1:V1 RNA; lane g, clone 16-7:V1 RNA. The SmaI 3.2-kb HSV DNA fragment labeled with ^{32}P (lane e) served as a molecular weight marker, as did clone 16 wild-type HSV RNA (5.4 kb plus 100 to 200 bases of polyadenylic acid).*

show that the genomes of individual infectious sarcoma viruses rescued from a particular delHSV transformant (clone X1) can differ in size from one another and from the bulk of delHSV transcripts present in the cells from which they have just emerged. Moreover, sarcoma viruses rescued from different delHSV-transformed cells (clones X1 and 16-1) can have RNA genomes that are similar in size (5.0 kb).

Novel HSV genomes were transmitted stably. Once created, the novel HSV genomes described above appeared to be passaged stably through multiple rounds of infection. For example, the X1:V1 and X1:V2 transformed lines were infected with M-MLV, and the RNAs of the rescued virus particles were analyzed as described above. In addition, the virus from these stocks was used to generate new foci, whose rescuable virus was analyzed in turn. Figure 6 shows that virus rescued from X1:V1 cells contained 8.2-kb helper M-MLV RNA and a second RNA species just smaller than HSV 5.4-kb RNA (Fig. 6, lanes e and i). This second species corresponded in size to the 5.0-kb virus-specific RNA present in the X1:V1 nonproducer cells. Upon passage of this X1:V1 sarcoma virus, the mass of the sarcoma viral RNA present in a subsequent cycle of passage remained 5.0 kb (Fig. 6, lane j). The sarcoma viral RNA genome in the virus particles rescued from X1:V2 cells was substantially smaller than HSV 5.4-kb RNA (Fig. 6, lanes f and k) and corresponded in size to the 3.7-kb sarcoma virus-specific RNA in X1:V2 cells. The small size of this sarcoma viral genome was preserved upon serial virus passage (lane 1). There was also an RNA species of 5.0 kb released from X1:V2 cells (Fig. 6, lane f), which did not appear to be infectious and which we did not characterize further. In conclusion, novel sarcoma virus genomes of 5.0 and 3.7 kb were transmitted faithfully from cell to cell, as would be expected of wild-type sarcoma viruses.

Recombination between M-MLV and delHSV generated wild-type sarcoma viruses. The novel wild-type sarcoma virus RNAs identified above were examined by S1 heteroduplex mapping to determine their M-MLV and HSV rat-specific sequence components. Cytoplasmic RNAs were prepared from the nonproducer transformed cell lines carrying the novel HSV genomes. The RNAs were first annealed to ^{32}P-labeled, *Hin*dIII-permuted 8.8-kbp M-MLV DNA (Fig. 2A). As Fig. 3 shows, all four of the novel sarcoma viral RNA genomes had the same 5′ end found on the RNA transcripts of delHSV and wild-type HSV genomes. This 5′ end was detected as a 230-nucleotide segment derived from the 5′ end of the M-MLV RNA genome (Fig. 3, lanes d, f, l, and m). In addition, the four novel sarcoma viral RNAs protected segments of M-MLV DNA ranging in length from 0.9 to 2.6 kb. These data indicated that novel sarcoma viral genomes that were generated after M-MLV infection of delHSV-transformed cells acquired helper M-MLV sequences via genetic recombination.

Further S1 heteroduplex analysis of hybrids formed between the four novel sarcoma virus RNAs and the other three M-MLV [^{32}P]DNA probes (Fig. 2B through D) demonstrated that the new leukemia viral sequences acquired by the recombinants were all derived from the extreme 3′ end of M-MLV RNA. For example, 16-1:V1 viral RNA contained a 2.6-kb segment of the M-MLV genome (Fig. 3, lane d). This viral RNA completely protected the 3′-specific *Hpa*I-*Sac*I 1.0-kbp M-MLV DNA probe (Fig. 3, lane i). Furthermore, cleavage of the 8.8-kbp M-MLV DNA with *Sac*I before hybridization shortened the 2.6-kb heteroduplex DNA fragment to between 2.4 and 2.5 kb (Fig. 3, lane x). Cleavage of the 8.8-kbp DNA with *Hpa*I before annealing with RNA yielded heteroduplex DNA fragments of 1.1 and 1.3 kb (Fig. 3, lane w). A summary of the maps of the newly acquired M-MLV sequences is presented in Fig. 5d, f, h, and i.

The acquisition of M-MLV RNA 3′-terminal sequences by the recombinant sarcoma viral genomes readily explains why these novel genomes behaved indistinguishably from wild-type HSV genomes. These recombinant sarcoma viral ge-

nomes reacquired sequences which were common to both HSV and M-MLV and played critical roles in viral DNA synthesis and, hence, in virus transmissibility.

We next determined which rat-specific HSV sequences were still present in the novel recombinant sarcoma virus genomes. Cytoplasmic RNAs from the four nonproducer recombinant virus-transformed cell lines were annealed individually to the ^{32}P-labeled 3.2-kbp DNA probe, whose sequence spanned from 1.5 to 4.7 kbp on the HSV DNA map (Fig. 2E). RNAs from both X1:V1 and 16-1:V1 cells protected a 1.2-kb DNA fragment (Fig. 4, lanes e and j), whereas RNAs prepared from X1:V2 and 16-7:V1 cells protected a 0.95-kb fragment (Fig. 4, lanes h and k). Hybridization of these various RNAs to restriction endonuclease-derived segments of the 3.2-kbp DNA (Fig. 2F and G) showed that all four recombinant viral RNAs contained sequences homologous to the extreme left end of the 3.2-kbp rat-specific DNA fragment. The simplest interpretation of these results, which is substantiated below, is that the 5′ portions of the novel, transmissible HSV RNAs were derived from the 5′ ends of the parental delHSV RNA genomes.

The S1 heteroduplex analysis and RNA filter hybridization analysis of the recombinant sarcoma viral RNAs enabled us to construct physical maps of the recombinant sarcoma virus genomes (Fig. 5). Although the S1 mapping experiments did not include the use of a DNA probe homologous to the 800 bases of the rat-specific sequences nearest the 5′ end of HSV RNA, we assumed that these sequences were present in all of the recombinant sarcoma viral genomes, because (i) all of these genomes contained the HSV-derived sequences immediately adjacent to both ends of this 800-base region, and (ii) in each case, the sum of the lengths of the viral genome segments detected by S1 mapping plus ~100 to 200 bases of 3′ polyadenylic acid was ~800 bases less than the total genome length determined by RNA filter blot hybridization. Below we describe detailed DNA physical mapping studies which confirmed that one of the recombinant sarcoma viral genomes contains these 5′-proximal rat-specific sequences.

Multiple nonrandom sites for delHSV-M-MLV genetic recombination. The sites of recombination between the genomes of delHSV and M-MLV were clearly nonrandom. For example, X1:V1 and 16-1:V1 sarcoma viral RNA genomes were both 5 kb long, both contained 2.6 kb of sequences derived from the 3′ end of M-MLV RNA, and both contained 2.25 kb of sequences derived from the 5′ end of delHSV RNA (Fig. 5). In addition, the X1:V2 and 16-7:V1 sarcoma virus genomes contained 2.0 kb of delHSV 5′ RNA sequences, but different amounts of M-MLV-derived sequences. The sensitivity of measurement afforded by gel electrophoresis allowed us to conclude that delHSV-M-HLV recombination occurred at preferred regions (each less than 100 bases long) along the parental genomes.

Physical map of a DNA genome of a novel sarcoma virus. Although we showed that each novel sarcoma viral RNA bore the 5′ portion of the HSV RNA and the 3′ portion of the M-MLV RNA, we have not yet demonstrated that the newly acquired 3′ M-MLV sequence components were located at the 3′ ends of the recombinant sarcoma viral RNA genomes, as is presumed in the maps of Fig. 5. We sought to verify this configuration by constructing a restriction endonuclease map of the provirus of the novel recombinant X1:V2 DNA genome. Given the assumed orientation of the sequences in the X1:V2 3.7-kb RNA (Fig. 5i), this genome should be reverse-transcribed to generate a double-stranded DNA of ~4 kbp, which bears at its ends the terminally redundant sequence found at the ends of both HSV 6.0-kbp DNA and M-MLV 8.8-kbp DNA. Figure 5i shows that this 4-kbp DNA genome should bear an *Hpa*I cleavage site in its M-MLV-derived right end. In addition, the 4-kbp DNA should have a *Bam*HI cleavage site within the 800 bases of leftward rat-specific sequences not previously characterized in the S1 mapping studies.

Virus rescued from X1:V2 cells by M-MLV superinfection was used to infect JLS V9 BALB/c cells, and low-molecular-weight DNA was extracted from these cells 24 h later by the method of Hirt (19). From this DNA, we prepared a fraction containing the unintegrated linear double-stranded DNA form of the X1:V2 viral genome free of contaminating M-MLV DNA by using methods described previously (15). Samples of this DNA fraction were cleaved with one restriction endonuclease or with combinations of two restriction endonucleases. The digests were subjected to gel electrophoresis-Southern filter hybridization analysis, using representative HSV ^{32}P-labeled cDNA as a nucleic acid probe. Wild-type HSV 6-kbp DNA was digested and analyzed in parallel to provide molecular weight standards. The results of these filter hybridizations are summarized in Table 2. Figure 8 shows the deduced physical map of X1:V2 viral DNA. The mass of this viral DNA was 4.0 kbp (Table 2), as predicted from Fig. 5. Furthermore, the map of this DNA verifies our orientation of the sequences in X1:V2 viral RNA (Fig. 5i and j).

All MLV-infected delHSV-transformed cells could release recombinant sarcoma virus. We wished to determine whether all

TABLE 2. *Restriction endonuclease digestions of X1:V2 recombinant sarcoma viral DNA*[a]

Restriction endonuclease(s)	Size(s) of detected viral DNA fragment(s) (kbp)[b]
None	4.0
*Eco*RI	4.0
*Xba*I	3.4
*Pst*I	2.25, 1.85
*Pst*I + *Xba*I	1.9, 1.55
*Sma*I	2.5, 1.0
*Sma*I + *Xba*I	2.35, 1.0
*Hpa*I	2.9, 1.2
*Bam*HI	3.2

[a] Low-molecular-weight linear DNA extracted from cells recently infected with the X1:V2 sarcoma virus–M-MLV complex was subjected to Southern filter hybridization analysis, using an HSV representative ^{32}P-labeled cDNA probe. Viral DNA species of about 4 and 9 kbp were detected, and only the 4-kbp species would hybridize to the HSV rat-specific 3.2-kbp [^{32}P]DNA probe (data not shown). The 4-kbp sarcoma viral DNA was freed from contaminating M-MLV 9-kbp DNA by agarose gel electrophoresis, and samples of gel-purified DNA were cleaved with restriction endonucleases and subjected to gel electrophoresis-Southern filter hybridization analysis, using a representative HSV ^{32}P-labeled cDNA probe. Low-molecular-weight DNA containing wild-type HSV 6-kbp DNA was also subjected to restriction endonuclease-Southern blot analysis to provide molecular weight standards.

[b] Fragments smaller than 1 kbp were not detected in these experiments.

delHSV-transformed cells infected by M-MLV released low levels of novel recombinant sarcoma virus or whether a small subpopulation of the infected cells released most of the infectious sarcoma virus particles. delHSV-transformed clone 16-7 cells were infected with M-MLV at a high multiplicity of infection (more than 2), and single-cell clones were derived from this infected culture. Three such clones were assayed for release of infectious sarcoma and leukemia viruses. As Table 3 shows, all three clones released low levels of sarcoma virus (ratio of focus-forming units to PFU, 4×10^{-4}), which were comparable to the yield of sarcoma virus recovered from the M-MLV-infected clone 16-7 culture

TABLE 3. *Infectious sarcoma virus released from cloned cells transformed by delHSV and infected with M-MLV*[a]

Cell line	Ratio of focus-forming units to PFU
delHSV 16-7 (M-MLV infected)	6×10^{-4}
Clone 1	4×10^{-4}
Clone 2	4×10^{-4}
Clone 3	4×10^{-4}

[a] delHSV-transformed clone 16-7 cells were infected with M-MLV at multiplicity of infection of >2, and then three single-cell clones were derived from the infected culture. Cultures derived from these clones, as well as the original infected culture, were assayed for the release of infectious sarcoma and leukemia viruses by the focus and XC plaque assays.

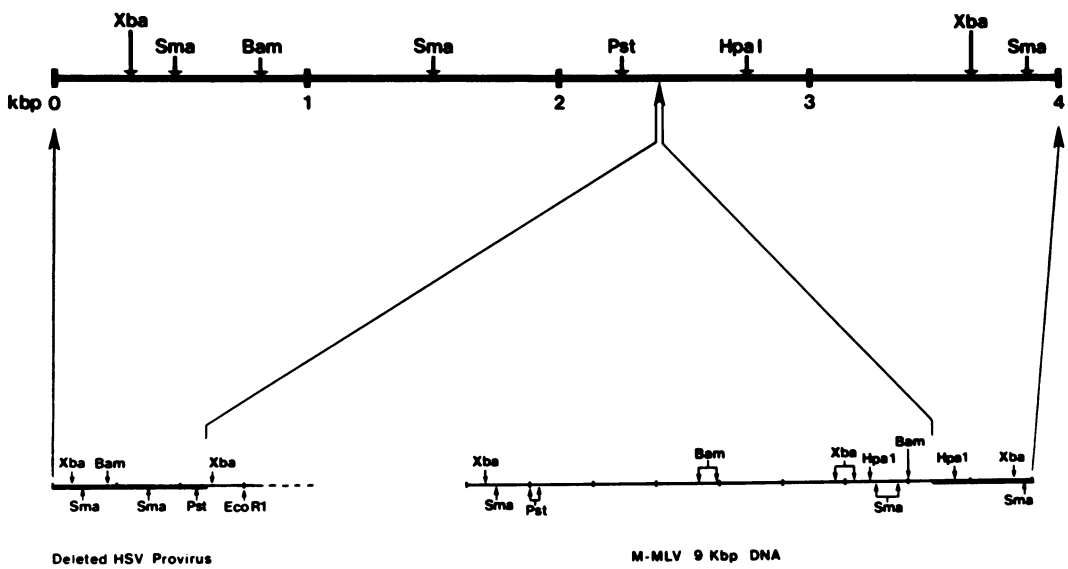

FIG. 8. *Physical map of X1:V2 recombinant sarcoma virus DNA. The restriction endonuclease digests of X1:V2 4.0-kbp linear DNA (Table 2) and the physical maps of HSV and M-MLV linear DNAs (12, 14) were used to construct a physical map of X1:V2 4.0-kbp DNA. The components of this genome derived from the delHSV clone X1 and M-MLV parental genomes are indicated on the figure.*

described above. Therefore, production of small quantities of recombinant sarcoma virus was a property of all three delHSV-transformed cell lines tested here and probably a property of all cells in this population.

DISCUSSION

The genome of nontransmissible delHSV lacking wild-type HSV 3′-terminal RNA sequences can recombine with M-MLV helper RNA to generate phenotypically wild-type sarcoma viruses. The recovered transmissibilities of these novel sarcoma viruses result from reacquisition of competent 3′-terminal genomic RNA sequences from the leukemia helper virus. Infection of delHSV-transformed cells with another murine leukemia virus (AKV) also results in the release of small quantities of phenotypically wild-type sarcoma virus, presumably derived by delHSV-AKV genetic recombination (unpublished data).

We analyzed the genomes of four independently derived delHSV-M-MLV recombinant sarcoma viruses. Two of these genomes apparently had identical contributions from both delHSV and M-MLV sequences, whereas the other two recombinant genomes had indistinguishable delHSV sequence components but differed markedly in the sizes of the acquired M-MLV sequences (Fig. 5). The nonrandom recombination events suggested by these results resemble similarly nonrandom recombination events which originally gave rise in vivo to the HSV and Kirsten sarcoma virus (KiSV) isolates. Both HSV and KiSV arose during MLV infections of rats (18, 23), and both sarcoma viruses originated by recombination between an MLV genome and rat nucleotide sequences which appeared to be largely retroviral in nature (27, 28). The 3′ portions of both HSV RNA and KiSV RNA consist of ~1 kb of an MLV-derived 3′-terminal sequence linked to the same rat-specific sequences (9). The MLV-rat sequence recombination joints in the HSV and KiSV genomes are so similar that HSV RNA-KiSV DNA heteroduplex molecules have no detectable lack of homology in the region spanning the recombination joints (9). In this regard, it is striking that the RNA genome of one recombinant sarcoma virus described here (clone 16-7:V1) has virtually the same contribution of 3′-terminal MLV RNA as found in the original HSV and KiSV RNA genomes.

In many biological systems, genetic recombination between two genomes requires sequence homology between the genomes near or at the site of recombination. It is unclear whether sequence homology plays a role in genetic recombination between delHSV and M-MLV. Electron micrographs of HSV-M-MLV nucleic acid heteroduplexes show no detectable homology between the HSV and M-MLV genomes at the sites of genetic recombination reported here (9), although neither electron microscopy nor S1 heteroduplex mapping, as described here, would reveal very short regions of homology. Thus, at the level of resolution presently available, these recombination events appear illegitimate. It is conceivable that homology or identity of the 5′ ends of the delHSV and M-MLV genomes was required to achieve an alignment of the genomes before recombination. It would be of interest to determine whether delHSV can recombine with a less-related retrovirus, such as feline leukemia virus, to generate transmissible sarcoma viruses.

The study of delHSV-M-MLV recombination offers some prospects for elucidating retrovirus recombination mechanisms. We found that three randomly picked M-MLV-infected delHSV-transformed cell lines all released low levels of recombinant sarcoma virus. This finding indicates that delHSV-M-MLV recombinant genomes are not generated shortly after M-MLV infection of delHSV-transformed cells by occasional integration of newly synthesized M-MLV DNA within the delHSV provirus. A consequence of recombination via integration would be the production of high levels of recombinant sarcoma virus from a very small percentage of M-MLV-infected delHSV-transformed cells and no sarcoma virus production from the vast majority of cells. Therefore, this mechanism is excluded by the findings described here.

There are two other mechanisms which might explain delHSV-M-MLV recombination. delHSV and M-MLV RNA might occasionally be packaged together to create heterodimeric RNA genomes within virus particles. If this heterodimeric RNA were subsequently reverse-transcribed, a polymerase could transcribe portions of both MLV and delHSV genomes via copy choice (10). Alternatively, an NIH 3T3 cell might be coinfected simultaneously with an M-MLV particle and a delHSV particle. Recombination could occur between the two reverse-transcribed DNA genomes before their integration. This latter mechanism predicts that infection of NIH 3T3 cells with virus rescued from delHSV-transformed cells should generate foci with two-hit kinetics, whereas the former mechanism predicts that focus induction should occur with one-hit kinetics. Our present experiments have not resolved this issue.

Functional map of the HSV genome. This study of the delHSV and recombinant sarcoma viral genomes has suggested biological roles of different segments of the HSV genome.

(i) Transformation gene. Based upon the

structural maps of the delHSV and X1:V2 recombinant sarcoma viral genomes (Fig. 5c and i), we conclude that the transforming gene of HSV lies within 2.0 kb of the 5′ end of HSV RNA. Recently, Chang et al. (7) have localized the HSV transforming gene with great precision in this region of the HSV RNA genome. This 2.0-kb region consists predominantly of rat cell-derived sequences, with the 5′-most 230 nucleotides being of M-MLV origin (Fig. 5).

(ii) Sequence signal for RNA packaging. Viral RNAs are packaged into virions at far greater efficiency than nonviral cytoplasmic RNAs (14). We have shown that deleted HSV RNA transcripts lacking the 3′-terminal nucleotide sequences of M-MLV origin can be packaged selectively into virus particles. In addition, transmissible delHSV-M-MLV recombinant viral genomes may lack the rat-specific HSV sequences located farther than 2.0 kb from the HSV RNA 5′ end. We conclude that the signal sequence for the encapsidation of HSV RNA lies within 2.0 kb of the 5′ end. Using a deletion mutant of avian sarcoma virus, other authors have concluded that one sequence necessary for packaging lies close to the 5′ end of the genomic RNA (24).

(iii) Sequences required for HSV replication. We have shown that the presence of 900 nucleotides of a M-MLV-derived sequence at the 3′ end of HSV RNA appears to be associated with competence for genome replication. This finding was not unexpected, since others have shown that the 3′ ends of retrovirus genomes play critical roles in RNA reverse transcription (10, 13).

(iv) Nonessential sequences. The RNA genome of the 16-7:V1 recombinant sarcoma virus lacks 2.5 kb of the rat-specific RNA sequences which extend from within 2.0 kb of the HSV RNA 5′ end to approximately 900 bases from the RNA 3′ end. Despite this deletion, 16-7:V1 sarcoma virus can transform NIH 3T3 cells without the aid of helper leukemia virus, and the titer of infectious sarcoma virus attainable from these transformed cells after M-MLV rescue is comparable to the titers observed upon infection with wild-type HSV (16). It is possible that certain phenotypes of cell transformation other than morphological transformation are not induced in cells infected with 16-7:V1 sarcoma virus. However, we conclude that the region from 2.0 to 4.5 kb on the HSV RNA genome is not essential for morphological transformation in vitro and for virus transmission.

We also note that the 16-7:V1 sarcoma virus genome, which lacks the nonessential sequences of HSV, is presently the smallest known transmissible mammalian retrovirus genome (length of genomic RNA, 3.2 kb). The existence of this genome implies great flexibility in the size of efficiently packaged mammalian retroviral RNA (from 3 to 9 kb).

Model for the generation of novel defective transforming viruses. The results presented here suggest a two-stage model for the generation of novel transforming genomes. The first step in this process is the coincidental integration of an MLV genome adjacent to a cellular gene whose expression leads to a transformed phenotype. Because of occasional errors in transcriptional regulation or lesions in the provirus, transcripts arise which are initiated within the viral genome and continue into the adjacent cellular sequences. For example, such transcripts might arise by failure to terminate at the right end of the provirus or by utilization of the right terminal redundancy as a transcriptional promoter. The consequence of such cotranscription would be an RNA molecule whose 5′-proximal sequences would be of MLV origin and whose remaining sequences would be of cellular origin.

Many such hybrid molecules would be structurally similar to the transcripts derived from the delHSV proviruses studied here. They would then be able to participate in the second event, namely recombination with a competent MLV helper genome which exists in the same infected cell and with which these genomes share 5′-proximal homologies. This latter step occurs with surprisingly high frequency in the delHSV-transformed cells studied here and might rapidly create a transmissible transforming virus once an integration event has generated the proper juxtaposition of viral and host cell sequences.

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