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Molecular cloning of Moloney murine sarcoma virus: Arrangement of virus-related sequences within the normal mouse genome

(recombinant DNA/transforming retrovirus/bacteriophage λ/heteroduplex analysis)

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ABSTRACT **The unintegrated circular DNA form of Moloney murine sarcoma virus (MSV) has been cloned in bacteriophage λ. Discrete deletions in the viral genome were shown to occur during propagation of recombinant phage in *Escherichia coli*. Heteroduplex and restriction enzyme analyses indicated the deletion of tandemly repeated sequences within certain of the cloned MSV DNA inserts. Cloned MSV DNA was used to prepare a probe composed of its acquired cellular (*src*) sequences, shown previously to be necessary for MSV transformation. Analysis of *Eco*RI digests of normal mouse cellular DNA revealed the presence of a single 14-kilobase-pair fragment containing these sequences which lacked contiguity with endogenous type C helper viral information of the same cells. Thus, the sarcoma virus-specific sequences of MSV are represented within the normal mouse genome in a manner analogous to that of a cellular gene.**

Investigations of RNA-containing sarcoma viruses have shown promise in the elucidation of cellular genetic alterations crucial to the initiation and maintenance of the malignant state. Accumulating evidence indicates that sarcoma viruses contain some nucleotide sequences of a type C RNA virus as well as some genetic information derived from the host cell (1–5). Thus, sarcoma viruses appear to have arisen in nature by a mechanism involving genetic recombination between type C helper viruses and host cell nucleotide sequences.

In the analysis of the replication of competent avian sarcoma virus, a combination of genetic and molecular studies has made it possible to conclude that the cell-derived sequences of avian sarcoma virus are essential for its transforming function (4–6). With a different approach, the cell-derived sequences of the replication-defective mammalian sarcoma virus, Moloney murine sarcoma virus (MSV), have been localized (7, 8). Investigation of the transforming activity of restriction fragments of the double-stranded linear MSV DNA molecule has revealed an essential role of its cell-derived sequences in transformation (9–11).

The recent development of molecular cloning techniques (12) made it feasible to approach analysis of the fine structure of Moloney MSV and the relationship of its cell-derived nucleotide sequences to genetic information of the normal cell. Here we report the molecular cloning of the supercoiled form of the Moloney MSV genome and demonstrate the occurrence of specific deletions of viral information in the recombinant DNA molecules during amplification in *Escherichia coli*. We have utilized specific fragments of the cloned molecule as probes to study the arrangement of the helper virus and cell-derived sequences of Moloney MSV within the DNA of normal mouse cells.

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METHODS

Cells and Viruses. Moloney MSV was originally isolated from a solid tumor of a BALB/c mouse inoculated with Moloney mouse leukemia virus (MuLV) (13). The MSV-124 strain (14) of Moloney MSV contains a 20-fold excess of MSV to MuLV (15). In the present studies, MSV-124 was propagated in NIH/3T3 cells (16).

Preparation of Viral and Cellular DNAs. Unintegrated MSV proviral DNA was isolated from NIH/3T3 cells by the Hirt procedure (17) at ≈12 hr after infection. The low molecular weight DNA fraction was subjected to CsCl equilibrium density gradient centrifugation in the presence of ethidium bromide (18). Fractions containing closed circular DNA were pooled, extracted with isobutanol to remove ethidium bromide, dialyzed, and then precipitated with alcohol. High molecular weight DNA was isolated from cultured cells by lysis with sodium dodecyl sulfate and proteinase K followed by phenol extraction and alcohol precipitation.

Restriction Enzyme Analysis. Restriction endonucleases were purchased from New England Biolabs and used according to the conditions suggested by the supplier. Intact and endonuclease-digested DNAs were analyzed on agarose gels, and MSV-containing sequences were detected by the Southern blotting technique (19) as modified (10).

Molecular Cloning. Charon 21A strain of λ phage was propagated as described by Blattner *et al.* (11, 20), and DNA was purified from CsCl-banded phage by the method of Enquist *et al.* (21). The phage DNA was circularized by hybridizing the cohesive ends. Phage and target DNAs were independently treated with *Hin*dIII followed by endonuclease inactivation as described by Blattner *et al.* (20). Vector and target DNAs were mixed in equimolar ratios and treated with DNA ligase. The resultant recombinant DNA was packaged *in vitro* into phage particles as described (22). Typical packaging efficiencies for Charon 21A DNA subsequent to *Hin*dIII digestion and ligation were 5×10^5–1×10^6 plaque-forming units/μg (untreated λ DNA was packaged with an efficiency of 5×10^6–2×10^7 plaque-forming units/μg). The resultant phage was plated onto *E. coli* K-12 DP50supF, and plaques containing recombinant phage were located by the plaque filter hybridization technique (23). Plaques giving positive hybridization reactions were picked and replated as necessary until the number of recombinant phage in the population was greater than 95%. All procedures involving bacterial strains and phage were those supplied by F. Blattner (11, 20). P2/Ek2 containment conditions were used as described in the *Federal Register*, Dec. 22, 1978, Part VII.

Use of Cloned MSV DNA Fragments as Molecular Probes. Cloned MSV DNA was excised from the vector DNA by using

Abbreviations: MSV, murine sarcoma virus; MuLV, murine leukemia virus; kbp, kilobase pair(s).

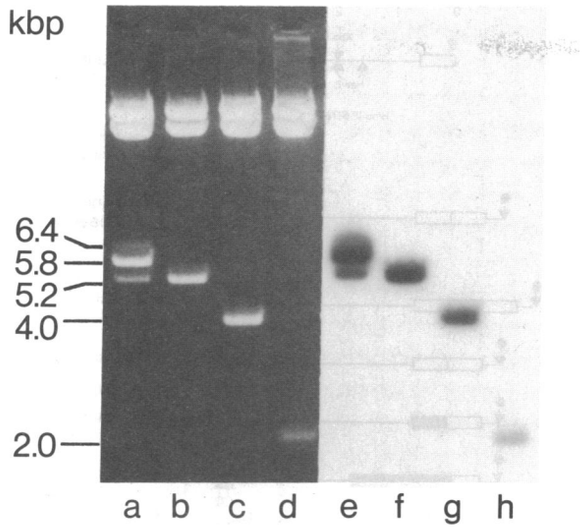

FIG. 1. Electrophoretic analysis of λ–MSV recombinant DNAs. DNAs extracted from plaque-purified phages were digested with *Hin*dIII, electrophoresed on agarose gels, and stained with ethidium bromide (lanes a–d). The fragments containing MSV sequences were identified by blotting and hybridization analysis (lanes e–h). Fragments of *Hin*dIII-digested λ DNA served as molecular weight standards.

*Hin*dIII and was redigested with appropriate restriction enzymes to generate smaller fragments. The fragments were purified on preparative agarose gels, electroeluted, and labeled to high specific activity with ^{32}P by the nick/translation method (24).

Electron Microscopy. Recombinant phage DNAs were digested with *Hin*dIII. Equal amounts of DNAs (2–5 μg) were mixed, denatured with 30 mM NaOH, neutralized with 0.1 M Tris·HCl at pH 8.0, and then allowed to hybridize in the presence of 50% formamide at 25°C for 4–6 hr. Samples were spread onto a distilled water hypophase and prepared for electron microscopy according to the method of Davis *et al.* (25). Uranyl acetate-stained grids were rotary shadowed with platinum/palladium and examined in a Siemens 101 electron microscope.

RESULTS

Molecular Cloning of MSV. We approached the molecular cloning of MSV-124 by a strategy involving the use of unintegrated closed circular viral DNA isolated from newly infected cells. The vector used in these experiments was Charon 21A λ phage developed by Blattner and his colleagues (11). MSV DNA was cleaved with *Hin*dIII to form full-length linear molecules that were subsequently ligated to *Hin*dIII-digested λ phage DNA. The *in vitro* packaging technique (22) was applied to enhance the infectivity of the resulting recombinant DNA molecules. Around 2% of the resulting plaques registered as containing MSV sequences by the filter hybridization method (23) utilizing MSV cDNA as a probe.

After plaque purification, phage DNA was prepared, digested with *Hin*dIII, and analyzed by electrophoresis on agarose gels. Fig. 1 shows the resulting patterns for the DNA of phages, representative of the results of analysis of more than 20 individual recombinants. Of the fragments observed after ethidium bromide staining, the two uppermost bands [24 and 17 kilobase pairs (kbp)] represented the λ phage DNA arms (Fig. 1, lanes a–d). A total of five different inserts, 6.4, 5.8, 5.2, 4.0, and 2.0 kbp, were shown to contain MSV DNA as determined by blotting analysis followed by hybridization to MSV cDNA (Fig. 1, lanes e–h).

During the propagation of the recombinant phages, we noticed that, despite being derived from a single plaque, the progeny phage population in several cases contained MSV inserts of several sizes: λ–MSV-1 DNA contained MSV inserts of 6.4, 5.8, and 5.2, kbp (Fig. 1,e) and λ–MSV-4 DNA contained inserts of 4.0 and 2.0 kbp. Restriction enzyme analysis showed that this phenomenon was not due to tandem insertions of MSV DNAs but to the rise of different phage populations during growth (data not shown). Although continued passage of some plaques, such as λ–MSV-1, did not change the composition of different MSV inserts in the population, passage of other phage stocks resulted in accumulation of phage containing the smaller MSV inserts. In studies to be presented elsewhere, all five MSV DNA inserts were shown to cause transformation upon transfection of NIH/3T3 cells. However, only foci induced by the three largest inserts contained rescuable MSV.

Structure of MSV Inserts. In order to determine the relationships between the different size classes of MSV inserts, heteroduplex and restriction enzyme analyses were performed. λ–MSV DNAs were digested with *Hin*dIII, denatured, allowed

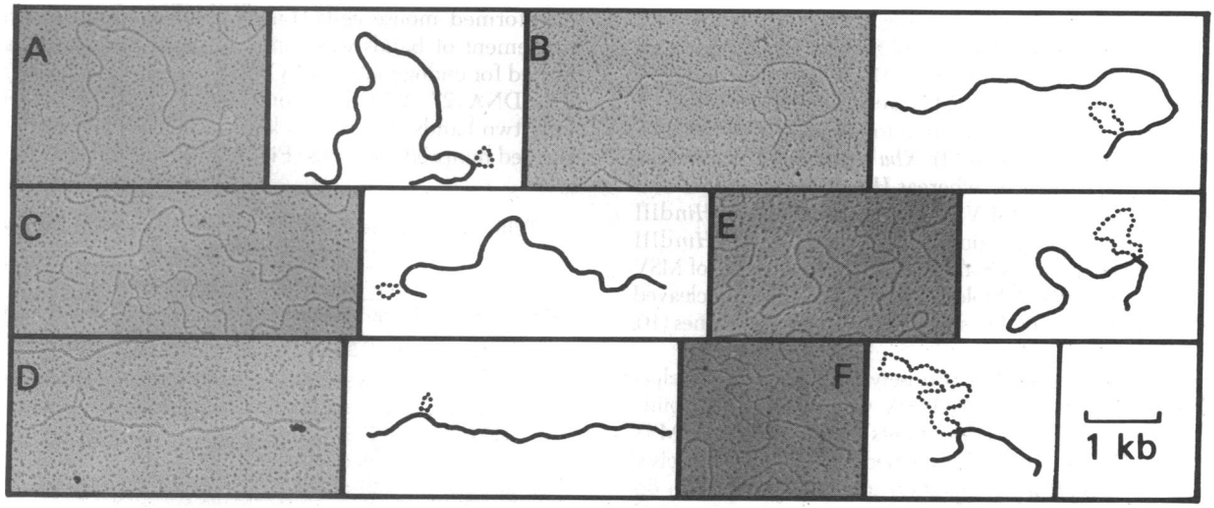

FIG. 2. Electron microscopy of heteroduplexes formed between different cloned MSV DNAs. Heteroduplexes were formed as follows: (*A*) between inserts of 6.4 and 5.8 kbp; (*B*) 6.4 and 5.2 kbp; (*C*) 5.8 and 5.2 kbp; (*D*) 5.8 and 5.2 kbp inserts cleaved with *Bgl* II; (*E*) 5.8 and 4.0 kbp; and (*F*) 5.8 and 2.0 kbp.

to form heteroduplex structures, and spread for observation in the electron microscope. All heteroduplex molecules contained a single-stranded region of nonhomology, flanked on both sides by homologous double-stranded regions.

The 6.4-kbp insert hybridized to the 5.8- and 5.2-kbp molecules to form structures with single loops of 0.6 and 1.2 kbp, respectively (Fig. 2 *A* and *B*). The positions of the loops on individual molecules significantly varied and averaged a distance of 0.7 kbp from one end of the 6.4–5.8 kbp duplexes and a distance of 0.4 kbp from one end of the 6.4–5.2 kbp duplexes. Similarly, heteroduplexes between the MSV inserts of 5.8 and 5.2 kbp showed a 0.6-kb loop of variable location, an average distance of 0.4 kbp from one end of the molecule (Fig. 2*C*). In a second series of experiments, the 5.8-kbp insert was hybridized with the two smallest inserts (4.0 and 2.0 kbp) to give single-stranded loops of nonhomology of 1.8 and 3.8 kb, respectively (Fig. 2 *E* and *F*). The location of these loops on the molecules appeared to be less variable and averaged 0.7 kb from one end. The lengths of the heteroduplex molecules are summarized in Table 1.

We next determined the orientation of the different deletion loops with respect to the known physical map of MSV DNA. The 5.8–5.2 kbp hybrids were digested with *Bgl* II and viewed in the electron microscope. *Bgl* II cleaves the terminal 1300 nucleotides of both molecules (4.5–5.8 kbp in Fig. 3). Thus, if the 0.6-kb loop were localized to the area of 0.4–1.0 kbp on the cloned DNA, the long homology region in the *Bgl* II-cleaved heteroduplex should be only 3.4 kbp. Indeed, such molecules were observed (Fig. 2*D*). By an analogous approach, the deletion loops in the 6.4–5.8 and 6.4–5.2 kbp heteroduplexes were oriented (data not shown). The results localized the nonhomologous regions in each of these three inserts to 0.4–1.0 kbp on the map of the cloned MSV genome (Fig. 3).

Orientation of the 4.0- and 2.0-kbp MSV inserts with respect to the linear DNA was accomplished by restriction enzyme analysis. The 4.0-kbp insert was cleaved with *Bgl* I into fragments of 2.8, 0.7, and 0.5 kbp and by *Bgl* II endonuclease into fragments of 2.7 and 1.3 kbp (Fig. 3). Thus, the segment of the MSV genome containing these cleavage sites was retained in the 4.0-kbp MSV DNA insert. Similar analysis of the 2.0-kbp MSV species revealed that it had lost the *Bgl* II site together with one *Bgl* I site but had retained the *Bgl* I site at 5.0 kbp. The retention, in both 4.0- and 2.0-kbp MSV inserts, of at least 800 nucleotides mapping at 5.0–5.8 kbp on the viral genome (*Hin*dIII to *Bgl* I fragment) oriented the loop in each case at the opposite end of the molecule (Fig. 3).

Arrangement of MSV-124 *src* and Helper Viral Sequences Within Normal Mouse Cellular DNA. By heteroduplex techniques, the segment of the MSV genome extending 3.4–4.9 kbp from the 5′ end has been found to consist entirely of nonhelper viral sequences (7). *Bgl* II, *Xba* I, and *Bgl* I cleave near the 5′ end of the sequences, whereas *Hin*dIII cuts at the 3′ end (15). Because Moloney MuLV DNA does not contain a *Hin*dIII site in the corresponding region of its genome (26), the *Hin*dIII site is likely to be included in the cell-derived sequences of MSV DNA. On the other hand, Moloney MuLV and MSV are cleaved by *Bgl* II, *Bgl* I, and *Xba* I in similar areas of their genomes (10, 26).

In order to determine more precisely the 5′ junction of helper viral and *src* sequences of MSV DNA, we compared the ability of a number of MSV DNA fragments to hybridize with MSV and MuLV cDNAs. The 5.2-kbp cloned MSV DNA was excised from the vector by *Hin*dIII digestion and then cleaved with *Bgl* II, *Xba* I, or *Bgl* I. The two fragments produced by *Bgl* II digestion (0–4.4 and 4.5–5.8 kbp) as well as the *Xba* I fragment (0–4.8 kbp) and the *Bgl* I fragments (0–4.3 and 4.3–5.0 kbp)

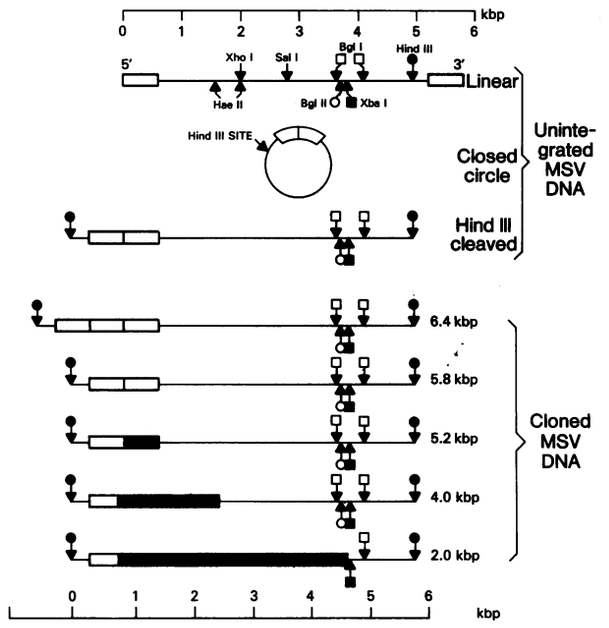

FIG. 3. Restriction enzyme analysis of cloned MSV DNAs. The physical map of the 5.8-kbp MSV-124 linear DNA has been reported (14). The darkened blocks represent deleted sequences as determined by heteroduplex and restriction enzyme analysis. Open blocks represent the 0.6-kbp terminal redundancies of MSV DNA.

hybridized to both MuLV and MSV probes. In contrast, the *Xba* I/*Hin*dIII fragment (4.8–5.8 kbp), and the *Bgl* I/*Hin*dIII fragment (5.0–5.8 kbp) hybridized to MSV but not to the MuLV probe (unpublished observations). These results demonstrated that the segment of the cloned viral genome spanning 4.8–5.8 kbp (*Xba* I/*Hin*dIII) did not contain detectable helper viral sequences and, therefore, comprised the *src* region.

To study the arrangement of MSV-related information in the DNA of normal mouse cells, we purified, from cloned MSV DNA, fragments extending 0–4.8 kbp (*Xba* IA) and 4.8–5.8 kbp (*Xba* IB) on the MSV map (Fig. 3). These fragments were radiolabeled by the nick/translation method (24) for use as probes. After *Eco*RI digestion, DNAs of BALB/3T3 and MSV-transformed nonproducer BALB/3T3 lines were fractionated by agarose gel electrophoresis, transferred to nitrocellulose sheets, and hybridized with each probe. The *Xba* IA probe detected several bands within the DNAs of both normal and MSV-transformed mouse cells (Fig. 4*A*, lanes a and b). This arrangement of bands was consistent with that previously reported for endogenous type C viral sequences in BALB/c cellular DNA (27). In striking contrast, the *Xba* IB probe detected only two bands (14 and 7.6 kbp) in the DNA of MSV-transformed nonproducer cells (Fig. 4*A*, lanes c and d). Moreover,

Table 1. Measurements of heteroduplexes formed between different MSV inserts

MSV inserts, kbp	Length, kbp*		
	Long-homology region	Loop	Short-homology region
6.4 and 5.8	5.13 ± 0.23	0.57 ± 0.05	0.67 ± 0.3
6.4 and 5.2	4.77 ± 0.2	1.15 ± 0.25	0.42 ± 0.3
5.8 and 5.2	4.75 ± 0.28	0.62 ± 0.07	0.45 ± 0.3
5.8 and 4.0	3.20 ± 0.21	1.89 ± 0.23	0.67 ± 0.09
5.8 and 2.0	1.21 ± 0.23	3.77 ± 0.32	0.70 ± 0.09

* At least 15 molecules of each combination were measured. Lengths were determined by using ϕX174 DNA as standard (5.386 kbp). Results are shown as mean ±SD.

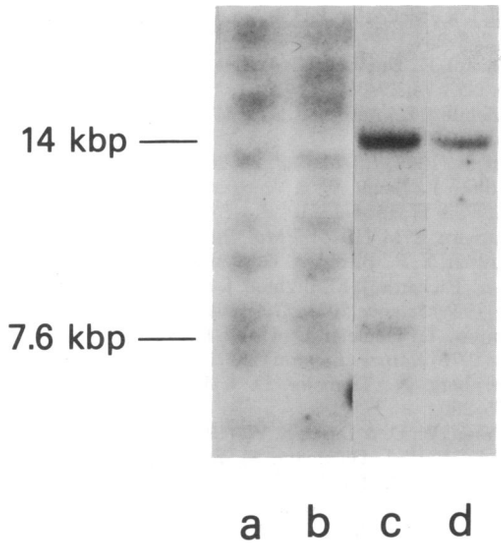

FIG. 4. Identification of sequences homologous to the helper viral (*Xba* IA) and *src* (*Xba* IB) information of MSV in BALB/c mouse cell DNA. High molecular weight DNAs isolated from normal (lanes a and c) or MSV-transformed (lanes b and d) BALB/3T3 cells were cleaved with *Eco*RI, fractionated by agarose gel electrophoresis, and blotted onto nitrocellulose filters. The blots were hybridized with *Xba* IA (lanes a and b) or *Xba* IB (lanes c and d) radiolabeled MSV fragments as described in the text. Molecular weight standards were those described in the legend to Fig. 1.

there was only a single band (14 kb) detected in DNA of BALB/3T3 cells (Fig. 4A, lane d). This band failed to correspond with any of the cellular bands detected with the *Xba* IA probe, composed mainly of the helper viral sequences of the MSV genome. When cellular DNAs of several other mouse strains were analyzed by the same techniques, each was found to contain a single 14-kbp *Eco*RI fragment possessing *src*-related information (Fig. 5). These results provided strong evidence that the 14-kb band contained normal mouse genetic information related to the *src* sequences of the MSV genome.

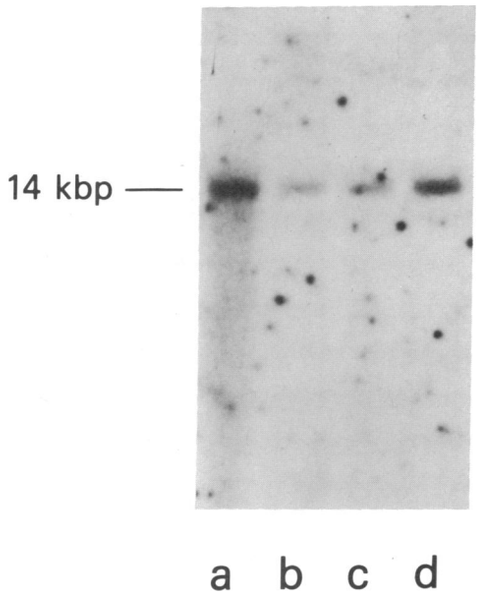

FIG. 5. Detection of MSV-*src* related information sequences in the DNAs of different mouse strains. High molecular weight DNA cleaved with *Eco*RI was fractionated on agarose gels, blotted onto nitrocellulose filters, and hybridized with nick-translated *Xba* IB (*src*) MSV DNA fragment. Sources of DNA were livers of BALB/c (lane a), NIH/Swiss (lane b), C57BL (lane c), and AKR (lane d) mice.

DISCUSSION

The advantages inherent in having quantities of viral DNA far in excess of those obtainable from *in vivo* synthesized MSV DNA led us to attempt the molecular cloning of the MSV genome. Utilizing recombinant DNA techniques, we have obtained a number of independently cloned molecules from the unintegrated circular form of MSV DNA.

Redundant sequences of 0.6 kbp have been shown to be present at the termini of Moloney MuLV (26) and Moloney MSV DNA (28). Molecular cloning of the *Hin*dIII-cleaved circular MSV molecule permutates the viral genome with a resultant tandem arrangement of the two terminal repeats in the area of 0.2–1.4 kbp on the cloned MSV DNA. Heteroduplexes between such a cloned molecule with another containing only a single repeat would be expected to show structures with a loop of 0.6 kb located between 200 and 800 nucleotides from the end of the molecule. Similarly, hybridization of an MSV molecule with three tandem repeats to one with a single repeat should yield a loop of 1200 nucleotides appearing in the same area of the genome. Indeed, these two structures correspond to the forms observed in the analysis of the 5.8–5.2 kbp and 6.4–5.2 kbp MSV DNA inserts, respectively. The sizes of the loops were constant, but their location was variable, providing direct evidence for tandem duplication (29). Thus, we conclude that the 6.4-, 5.8-, and 5.2-kbp inserts are MSV genomes with three, two, and one repeat, respectively.

The 6.4- and 5.2-kbp MSV DNA clones were probably derived by recombination in the bacterial host from the 5.8-kbp species, which is the major MSV DNA species *in vivo* (15). However, that they existed in the infected cell and were cloned as such cannot be excluded. Hager *et al.* (30) cloned a closed circular DNA form of Harvey MSV in λ phage and observed inserts that differed in size by steps of 0.65 kbp. Their studies also suggested that the cloned Harvey MSV DNAs undergo deletions of tandemly arranged 0.65-kbp redundant sequences. In the present studies, we also found two smaller MSV inserts, 4.0 and 2.0 kbp, that were derived from the larger molecules during propation of the λ–MSV in bacteria. The single deletion in each was shown to arise by excision of a continuous segment of DNA starting at about 0.6 kbp and ending at 2.4 (for the 4.0-kbp insert) or 4.4 kbp (for the 2.0-kbp insert) on the map of cloned MSV. Observation of these smaller inserts in several different λ–MSV clones suggests a nonrandom mechanism for their formation. Thus, additional direct repeats probably exist in the MSV genome.

The essential role in transformation of the cell-derived insertion sequences of Moloney MSV has recently been established by analysis of the transforming activity of restriction fragments of unintegrated MSV DNA obtained from newly infected cells (10). The availability of cloned MSV DNA made it possible to prepare specific fragments for use as molecular probes to analyze the arrangement of MSV-related sequences within normal mouse cell DNA. By agarose gel electrophoresis and Southern blotting of *Eco*RI-cleaved mouse cell DNA, we demonstrated that the cell-derived MSV genetic information was present in a single 14-kbp fragment in cellular DNAs of several different mouse strains. These sequences were not contiguous with endogenous type C helper viral information of the same cells. Thus, our results demonstrate that sequences related to the *src* region of MSV are represented within the normal mouse genome in a manner analogous to that of a cellular gene.

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Note Added in Proof. Vande Woude *et al.* (31) have recently reported the cloning of the integrated form of MSV.

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