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Structure and Functions of the Kirsten Murine Sarcoma Virus Genome: Molecular Cloning of Biologically Active Kirsten Murine Sarcoma Virus DNA

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The unintegrated closed circular form of viral DNA prepared from NIH3T3 cells infected with Kirsten murine sarcoma virus was cloned into bacterial plasmid pBR322. The closed circular DNA, which consisted of two different-sized populations, was enriched from the virus-infected cells, linearized with *Bam*HI, and inserted into pBR322 DNA. Four different recombinant DNAs (clones 2, 4, 6, and 7) were obtained, and a physical map of each was constructed by using various restriction enzymes. Clone 4 DNA had the largest insertion, corresponding to a complete copy of the linear DNA. This suggested that this insertion contained two copies of the 0.55-kilobase pair long terminal redundant sequence. Clone 2 and clone 6 insertion DNAs had deletions of 0.2 and 0.5 kilobase pair, respectively, which mapped near the right end (3′ side of viral RNA) of the linear DNA. Clone 7 DNA appeared to have a deletion of a single copy of the large terminal redundant sequence. Transfection of BALB3T3 cells with the clone 4 DNA insertion showed that this DNA had transforming activity. The efficiency of transfection with clone 4 Kirsten murine sarcoma virus DNA was enhanced eightfold by inserting *Eco*RI-cleaved viral DNA into the *Eco*RI site of pBR322. The *Eco*RI-inserted DNA produced foci with single-hit kinetics, suggesting that a single molecule of Kirsten murine sarcoma virus DNA can induce transformation. Results of transfections with *Eco*RI-inserted Kirsten murine sarcoma virus DNA cleaved with various restriction enzymes suggested that the first 3.3-kilobase pair region at the left end of the linear DNA is important for the initiation of transformation or maintenance of transformation or both.

The genomes of murine sarcoma viruses (MSVs) contain sequences derived from host cell genomes and from portions of helper murine leukemia virus genomes (5, 7, 10, 22, 30). Thus, these viruses are replication defective. Accumulating evidence suggests that the transforming sequences of the sarcoma viruses reside in the genome portions derived from host cells (2, 3, 20, 33).

When replication-defective Harvey MSV or Moloney MSV infects mouse cells, one linear DNA and two closed circular viral DNAs are synthesized (12, 14, 29), as reported for other replication-competent retroviruses (28). The linear DNA has two copies of a long terminal redundant (LTR) sequence approximately 0.6 kilobase pair (kbp) long, which have the same orientation at each end of the molecule. One of the circular molecules has the same structure as a linear molecule joined head to tail, i.e., two adjacent LTR sequences. The other circular molecule has only one copy of the LTR sequence, and thus its molecular weight is less by the molecular weight of the LTR sequence.

Kirsten MSV (Ki-MSV) is one of the replication-defective MSVs (17). We have identified the linear and closed circular forms of viral DNA in Ki-MSV-infected mouse cells (31). In the work reported here, we further characterized the closed circular DNA and found two different-sized circular DNA species. To investigate the fine structure of the circular DNAs and to elucidate the functions of each segment of the Ki-MSV genome, the circular molecules were cloned into bacterial plasmid pBR322 at a unique restriction site. We isolated four different cloned DNA molecules, one of which appeared to have a complete copy of the linear DNA. We used this molecule to determine which segment was responsible for viral transformation of fibroblasts.

MATERIALS AND METHODS

Cells and viruses. The mouse cell line 58-2T, which continuously produces more than 40 times as much Ki-MSV as helper murine leukemia virus, was

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the source of Ki-MSV used to infect NIH3T3 cells (31). BALB3T3 clone A31 (1) was used for transfection experiments with Ki-MSV DNA. All cells were grown in Eagle minimal essential medium containing 10% fetal bovine serum and 20 μg of gentamicin per ml.

Preparation of closed circular Ki-MSV viral DNA. NIH3T3 cells were grown in 300 1-liter culture bottles to one-third confluency. Cells were infected with Ki-MSV at 5 focus-forming units per cell in the presence of 2 μg of Polybrene per ml. At 20 h postinfection, the fraction containing unintegrated Ki-MSV DNA was prepared by a modification of the procedure of Hirt (15, 31). The Hirt supernatant fractions were centrifuged for 13 h at 18°C and 23,000 rpm in a Beckman SW27 rotor in 5 to 20% sucrose gradients containing NTE buffer (0.01 M Tris-hydrochloride, pH 7.0, 0.1 M NaCl, 10 mM EDTA) and 0.2% sodium dodecyl sulfate. After centrifugation, fractions (approximately 3 ml) were collected from the bottom of each gradient, and the DNA was ethanol precipitated in the presence of 50 μg of yeast RNA per ml at −20°C overnight. Precipitates were dissolved in E buffer (40 mM Tris-acetate, pH 7.5, 0.005 M sodium acetate, 0.001 M EDTA) for electrophoresis. A small sample of each fraction was electrophoresed in a 1% agarose gel, and Ki-MSV-specific DNA was detected by Southern filter hybridization, using ^{32}P-labeled Ki-MSV RNA as a probe (31). Fractions containing the closed circular form of Ki-MSV DNA were pooled and electrophoresed in a 1% preparative agarose gel at 1 to 2 V/cm for 10 to 20 h. *Hin*dIII-digested λ DNA fragments were run at the same time in the lanes at the two ends of each gel. After electrophoresis, the lanes containing marker DNAs were stained with ethidium bromide (1 μg/ml) for 30 min. The area corresponding to the 4.3- to 3.0-kbp region was determined from the positions of marker DNA fragments, and this area was cut out from each sample lane. The gel was dissolved in NaI, and the DNA was adsorbed to glass beads and eluted as described previously (32).

Bacteria and bacterial plasmid pBR322. The EK-2 certified host *Escherichia coli* χ1776 and HB101 carrying plasmid pBR322 were generously provided by P. Curtis and R. Weinmann, respectively, of the Wistar Institute. *E. coli* χ1776 was grown either in liquid L broth (10 g of tryptone [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract, and 5 g of NaCl per liter supplemented with 100 μg of diaminopimelic acid per ml and 100 μg of thymidine per ml or on L-broth plates containing 1% agar.

Preparation of plasmid DNA. Bacteria containing plasmids were grown to an absorbance at 650 nm of 0.9 in L broth supplemented with diaminopimelic acid and thymidine at 37°C with aeration. Chloramphenicol (12.5 μg/ml) was then added, and the cells were incubated for an additional 5 h. Cells were collected by centrifugation, and a cleared lysate was prepared by the method of Katz et al. (16). The lysate containing plasmid DNA was treated with 10 μg of RNase A per ml for 30 min at 37°C and then with 100 μg of proteinase K per ml for another 30 min at 37°C. This lysate was extracted with 2 volumes of a phenol-chloroform mixture (1:1 [vol/vol] mixture of water-saturated phenol and chloroform-isoamyl alcohol [24:1, vol/vol]). The DNA was then ethanol precipitated after 0.1 M (final concentration) NaCl was added. The precipitate was dissolved in TE buffer (0.01 M Tris-hydrochloride, pH 7.0, 0.01 M EDTA) and then centrifuged with an SW41 rotor at 18°C for 4.5 h at 37,000 rpm in a 5 to 20% sucrose gradient in NTE buffer containing 0.2% sodium dodecyl sulfate. Ribosomal 18S and 28S RNAs were centrifuged in a parallel gradient. After centrifugation, fractions (1 ml) were collected from the bottom of each gradient. The fractions corresponding to 15S to 40S were pooled, and the DNA was ethanol precipitated. The precipitate was dissolved in TE buffer, and closed circular DNA was further purified by cesium chloride-ethidium bromide centrifugation as described previously (31).

Restriction enzyme digestion. All restriction enzymes used in these experiments were purchased from New England Biolabs, Boston, Mass., Bethesda Research Laboratories, Rockville, Md., or Boehringer Mannheim Co., Indianapolis, Ind. DNAs were digested according to the instructions of the suppliers.

Molecular cloning of circular DNA. All manipulations were carried out in compliance with the National Institutes of Health Guidelines for Recombinant DNA Research in a P2 physical containment facility, using the EK-2 host-vector system and *E. coli* χ1776-pBR322.

The closed circular pBR322 DNA was cleaved with *Bam*HI. This DNA was extracted with 2 volumes of the phenol-chloroform mixture, treated with ethylether, ethanol precipitated, and dissolved in a small volume of 0.01 M Tris-hydrochloride (pH 8.0). The phosphate at the 5′ end of the pBR322 DNA was removed by treatment with bacterial alkaline phosphatase, as described previously (31).

The Ki-MSV closed circular DNA preparation was also cleaved with *Bam*HI, extracted with the phenol-chloroform mixture, treated with ethylether, ethanol precipitated, and dissolved in a small volume of 0.1 mM EDTA. Ligation was performed at 8°C for 24 h in a 100-μl reaction mixture containing 66 mM Tris-hydrochloride (pH 7.5), 6.6 mM $MgCl_2$, 0.2 mM ATP, 50 μg of bovine serum albumin per ml, 10 mM dithiothreitol, 4 μg of *Bam*HI-cleaved, bacterial alkaline phosphatase-treated pBR322 DNA per ml, 4 μg of the *Bam*HI-cleaved circular Ki-MSV DNA preparation per ml, and 4 U of ligase (Bethesda Research Laboratories). The ligated DNA was then transformed to *E. coli* χ1776 as described previously (9), except that the transformation buffer was 30 mM sodium acetate (pH 5.6)–70 mM $MnCl_2$–30 mM $CaCl_2$. The cells were then spread onto plates of solid L broth containing 35 μg of ampicillin per ml. Colonies were picked up with toothpicks, inoculated into pairs of plates, and incubated at 37°C overnight. One plate of each pair was used for colony hybridization as described by Grunstein and Hogness (13), using ^{32}P-labeled Ki-MSV RNA as a probe. The preparation of the [^{32}P] RNA probe and filter hybridization have been described elsewhere (31). Colonies positive for hybridization were picked up from the other plates and purified by three cycles of single-colony isolation.

Agarose gel electrophoresis and Southern filter hybridization. Electrophoresis of DNA in a 1% neutral agarose slab gel, staining with ethidium bromide, transfer to a membrane filter, hybridization with the [^{32}P]RNA probe, and exposure of the filter to X-ray film have been described previously (26, 31). For

the detection of closed circular DNA, the gel was first treated with 1 N acetic acid for 30 min. The DNA in the gel was then denatured and processed as described above. Unlabeled and ^{32}P-labeled λ DNAs cleaved with *Hin*dIII were prepared and electrophoresed as size markers, as described elsewhere (31).

Infectivity assay of cloned DNA. Recombinant DNA was cleaved with a restriction enzyme, extracted with the phenol-chloroform mixture, ethanol precipitated, and dissolved in 0.1 mM EDTA. When the DNA was cleaved with two enzymes, the ethanol precipitate of the first digest was dissolved in the buffer for the second restriction enzyme, digested with the enzyme, and treated as described above.

Focus-forming activity of the digested DNA was assayed on BALB3T3 cells by using a modification of the calcium precipitation method described by Lowy et al. (18). At 1 day before the transfection, 3×10^5 BALB3T3 cells were seeded onto 35-mm plates containing Eagle minimal essential medium supplemented with 10% fetal bovine serum and 10 μg of gentamicin per ml. The enzyme-digested recombinant DNA was mixed with 20 μg of salmon sperm DNA per ml in 0.5 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline. Then DNA was precipitated with 0.05 volume of 2.5 M $CaCl_2$. After 20 to 30 min at room temperature, 0.2-ml samples of the mixture were directly applied to two plates, each containing 2 ml of fresh medium. The plates were incubated for 4 h at 37°C in a CO_2 incubator. The cells were treated with 20% glycerol in Tris-saline buffer for 1 min, washed once with this buffer, and refed with the fresh medium as described previously (11). Foci were counted 10 to 14 days after transfection.

RESULTS

Characteristics of circular Ki-MSV DNA used for molecular cloning. We have reported elsewhere (31) that the linear and closed circular forms of Ki-MSV DNA are synthesized in NIH3T3 cells recently infected with Ki-MSV and that the two forms are detected as 16S and 22 to 24S molecules, respectively, by neutral sucrose gradient centrifugation. To characterize the closed circular DNA further, each fraction of a neutral sucrose gradient was examined for Ki-MSV-specific DNA by agarose gel electrophoresis and by Southern filter hybridization, using ^{32}P-labeled Ki-MSV RNA as a probe. As Fig. 1 shows, a peak of two faster-migrating Ki-MSV DNA species about 4 kbp long was detected in fraction 6 of the sucrose gradient, corresponding to 22S to 24S in sedimentation value; 21S ^{3}H-labeled simian virus 40 form I DNA sedimented between fractions 6 and 7 (data not shown). A linear 7.0-kbp DNA species peak was detected at fraction 8, corresponding to 16S. To determine whether the faster-migrating species were circular Ki-MSV DNA, the DNA species from pooled fractions were recovered from the agarose gel and treated with *Eco*RI, which cleaves linear Ki-MSV DNA only once (31). The two faster-migrating species were converted by *Eco*RI to linearized DNA species that were 7 and about 6.5 kbp long (Fig. 2, lane 3), whereas the linear 7.0-kbp DNA was converted to 4.0- and 3.0-kbp fragments (Fig. 2, lane 1). These

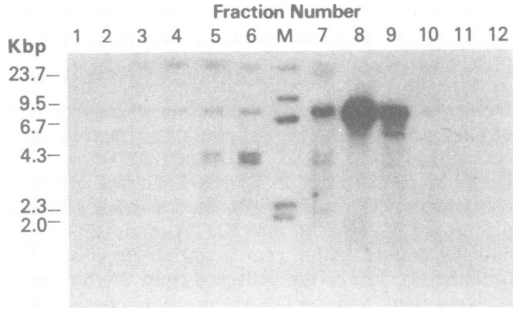

FIG. 1. *Detection of Ki-MSV DNA species in sucrose gradient fractions by Southern filter hybridization. Hirt supernatant fractions (0.33 mg) from Ki-MSV-infected cells were centrifuged in a 5 to 20% sucrose gradient. In a parallel gradient, ^{3}H-labeled simian virus 40 form I DNA was centrifuged. A sample of each fraction was electrophoresed, the DNA was transferred to a membrane filter and hybridized to ^{32}P-labeled Ki-MSV RNA, and the filter was exposed to a X-ray film as described in the text. [^{32}P]DNA digested with HindIII (λ DNA markers) was electrophoresed in lane M and treated as described above.*

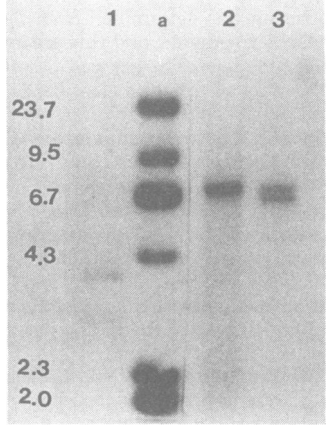

FIG. 2. *Southern filter hybridization of EcoRI-digested linear and closed circular Ki-MSV DNAs. The linear and closed circular Ki-MSV DNAs were extracted from a gel as described in the text, digested with EcoRI, electrophoresed together with ^{32}P-labeled λ DNA markers (lane a) in an agarose gel, transferred to a membrane filter, and treated as described in the legend to Fig. 1. Lane 1, Linear DNA cleaved with EcoRI; lane 2, undigested linear DNA; lane 3, closed circular DNA digested with EcoRI. The numbers at the left indicate lengths (in kilobase pairs).*

results indicated that two size classes of circular Ki-MSV DNA were synthesized in almost equal amounts soon after virus infection; one was indistinguishable in size from the linear DNA (Fig. 2, lane 2), and the other was approximately 0.5 kbp shorter than the linear DNA.

Molecular cloning of the circular form of Ki-MSV DNA. To clone circular Ki-MSV DNA into plasmid pBR322, a large amount of closed circular Ki-MSV DNA was prepared from infected cells and enriched first by Hirt extraction, then by sucrose gradient centrifugation, and finally by neutral agarose gel electrophoresis. During electrophoresis most of the nonsupercoiled DNA, which cosedimented with circular Ki-MSV DNA in the sucrose gradient centrifugation, migrated more slowly than the circular Ki-MSV DNA (data not shown). Ki-MSV circular DNA enriched in this way was treated with *Bam*HI, which cleaves linear Ki-MSV DNA once (31), and was ligated to the unique *Bam*HI site of pBR322 DNA. The ligated DNA was then used to transform *E. coli* χ1776. Approximately 7,000 colonies were obtained and screened for the presence of Ki-MSV DNA sequences by using ^{32}P-labeled viral RNA as a probe. Four colonies (clones 2, 4, 6, and 7) positive for hybridization with the probe were selected and analyzed further.

Size of the insertion. Circular recombinant DNA was prepared from each clone, cleaved with *Bam*HI, electrophoresed in a 1% agarose gel, and stained with ethidium bromide. As Fig. 3A shows, each recombinant DNA contained 4.4 kbp of pBR322 DNA and one of the following three different-sized insertions: 7.0, 6.8, and about 6.5 kbp. The sizes of the largest and smallest insertions were indistinguishable from the sizes of large and small circular DNAs, respectively. When the *Bam*HI digest of recombinant DNA was transferred from the gel to a nitrocellulose filter and hybridized with the ^{32}P-labeled Ki-MSV RNA probe, only the insertion DNA was hybridized (Fig. 3B). Thus, clone 2, 4, 6, and 7 recombinant DNAs contained insertion DNAs 6.8, 7.0, about 6.5, and about 6.5 kbp long, respectively, which had sequences related to the Ki-MSV genome.

Restriction endonuclease maps of cloned recombinant DNAs. To construct restriction endonuclease maps, recombinant circular DNAs were digested with one or two restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, *Sma*I, *Xba*I, *Kpn*I), the cleavage sites of which were determined on linear Ki-MSV DNA (31). The digests were electrophoresed in a 1% agarose slab gel, and the fragments produced were detected either by ethidium bromide staining or by Southern filter hybridization, using ^{32}P-labeled

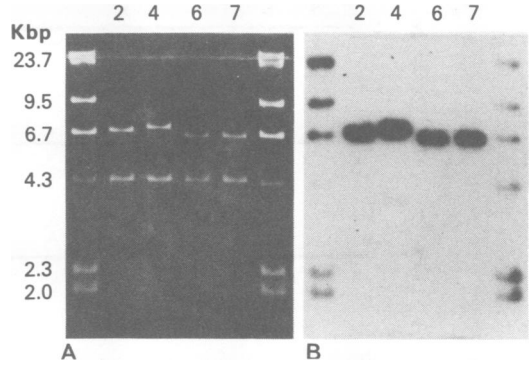

FIG. 3. *Agarose gel electrophoresis and Southern filter hybridization of BamHI-digested recombinant DNAs. Recombinant DNA (0.035 μg) was digested with BamHI, electrophoresed together with unlabeled and ^{32}P-labeled λ DNA markers in an agarose gel, and stained with ethidium bromide, and the DNA fragments were visualized by UV fluorescence (A). The DNA fragments in the gel were transferred to a membrane filter and treated as described in the legend to Fig. 1 (B). The numbers at the top indicate the recombinant DNA clones tested.*

Ki-MSV RNA as a probe. The sizes of the fragments were estimated, and restriction endonuclease maps were constructed (Fig. 4).

Since cloned insertion Ki-MSV DNAs were obtained from circular molecules that had been linearized at the *Bam*HI site, the restriction endonuclease maps of the inserted Ki-MSV DNAs were expected to be permutations of the linear DNA. The restriction endonuclease analysis showed that the largest clone 4 insertion met all of the predictions made from the permutated map of Ki-MSV linear DNA (Fig. 4A, B, and D). The restriction endonuclease maps of the clone 2, 6, and 7 insertions were constructed (Fig. 4C, E, and F, respectively) like the clone 4 insertion map. The clone 2, 6, and 7 insertions were found to be 0.2, about 0.5, and about 0.5 kilobase (kb), respectively, smaller than the clone 4 insertion. The clone 2 insertion met all of the predictions made from the permutated map of the linear DNA, except for the 0.2-kb deletion inside the 1.2-kb *Sma*I fragment of the linear DNA (Fig. 4A). When clone 2 DNA was digested with *Sma*I or with *Sma*I plus *Bam*HI and the fragments produced were compared with the clone 4 DNA digest, the 1.2-kb *Sma*I fragment was replaced by a 1.0-kbp fragment in the clone 2 DNA digest. Similarly, the 0.5-kbp deletion of clone 6 insertion DNA was located within the 1.2-kbp *Sma*I fragment (Fig. 4E). This was suggested by the results of a size analysis of recombinant circular DNA digested with *Sma*I. Interestingly, the *Eco*RI site was absent

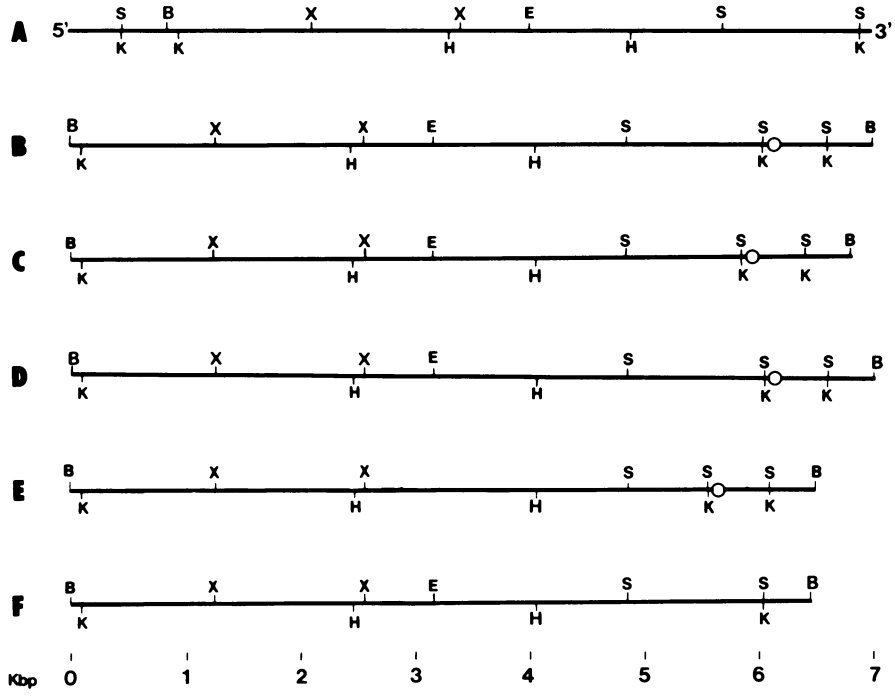

FIG. 4. *Restriction endonuclease maps of Ki-MSV insertions. (A) Unintegrated linear Ki-MSV DNA. The left and right ends correspond to the 5' and 3' polarities, respectively, of viral RNA. (B) Linear Ki-MSV DNA permutated at the unique BamHI site. (C) Ki-MSV insertion of clone 2 recombinant DNA. (D) Ki-MSV insertion of clone 4 recombinant DNA. (E) Ki-MSV insertion of clone 6 recombinant DNA. (F) Ki-MSV insertion of clone 7 recombinant DNA. The restriction endonuclease maps of cloned insertion DNAs were constructed from the results of the size analyses of restriction endonuclease fragments. The cleavage sites of EcoRI (E), HindIII (H), SmaI (S), KpnI (K), BamHI (B), and XbaI (X) are shown. The open circles indicate the putative junction points of the left and right ends of the linear DNA.*

in the clone 6 insertion. *Eco*RI digestion of recombinant DNA resulted in only a single species of about 11 kbp, which could be cleaved further into fragments about 6.5 and 4.4 kbp long with *Bam*HI (data not shown). The finding that the 1.6-kbp fragment containing the *Eco*RI site was produced by *Hin*dIII digestion of clone 6 DNA suggested that the clone 6 insertion had a point mutation, an addition, or a deletion of a short segment in the *Eco*RI recognition sequence. A comparison of the clone 7 recombinant DNA digested with *Sma*I or *Kpn*I and the digested fragments of clone 4 DNA indicated that clone 7 insertion DNA lacked the 0.55-kbp *Sma*I or *Kpn*I fragment (Fig. 4F). It was this fragment that contained sequences derived from both the left end and the right end of linear Ki-MSV DNA.

Transfection with recombinant Ki-MSV DNA. To determine whether clone 4 Ki-MSV, which was the same size as linear Ki-MSV DNA, was biologically active, circular Ki-MSV-pBR322 recombinant DNA was cleaved with *Bam*HI and was used to transfect BALB3T3 fibroblasts by the calcium precipitation method (19). When 0.15 μg of clone 4 Ki-MSV DNA was transfected, four to five foci were produced, whereas none was produced with pBR322 DNA (Table 1). This suggested that clone 4, in which Ki-MSV DNA was inserted at the *Bam*HI site, had transforming activity. To determine whether the site of insertion was critical for transforming ability (as it is for Harvey MSV DNA [4, 8]), we inserted Ki-MSV at another site (*Eco*RI) and recloned the molecule. Clone 4 DNA was digested with *Bam*HI and electrophoresed in an agarose gel, and the insertion was isolated from the gel. The insertion DNA was then circularized with ligase, cleaved with *Eco*RI, reinserted at the *Eco*RI site of pBR322 DNA, and used to transform *E. coli* χ1776. Recombinant DNA prepared in this way was designated clone 4(E) DNA. To ensure that Ki-MSV was inserted at the *Eco*RI site of pBR322 and that the insertion DNA retained the original *Bam*HI site, the circular recombinant DNAs were digested with *Eco*RI or with *Eco*RI plus *Bam*HI. The *Eco*RI-digested clone 4(E) DNA

TABLE 1. *Transfection of BALB3T3 cells with cloned Ki-MSV DNA*[a]

Source of recombinant DNA	Restriction enzyme	Amt of DNA (μg/plate)	Amt of KI-MSV DNA (μg/plate)[b]	No. of foci per plate[c]	No. of foci per μg of Ki-MSV DNA
Clone 4	*Bam*HI	0.25	0.15	4.5	30
Clone 4(E)	*Eco*RI	0.35	0.22	54.5	248
pBR322	*Bam*HI	0.25	0.0	<0.5	<3

[a] Recombinant DNA or pBR322 DNA was digested with a restriction enzyme and transfected to BALB3T3 cells, and the foci produced were counted as described in the text.

[b] Amount (in micrograms) of recombinant DNA × (molecular weight of Ki-MSV insertion DNA/molecular weight of recombinant DNA).

[c] Mean of duplicate plates.

produced 4.35-kbp pBR322 DNA and a 7.0-kbp Ki-MSV insertion that was cleaved further into 3.9- and 3.1-kbp species with *Bam*HI. Clone 4(E) DNA was cleaved with *Eco*RI and was used to transfect BALB3T3 cells (Table 1). The focus-forming ability of the clone 4 insertion increased eightfold after the reinsertion of Ki-MSV DNA at the *Eco*RI site, suggesting that the *Bam*HI site of Ki-MSV was important for the initiation of transformation or the maintenance of transformation or both.

To determine whether the transformation by Ki-MSV DNA molecules involved more than one of these molecules, BALB3T3 cells were transfected with different concentrations of *Eco*RI-digested clone 4(E) DNA, and the foci produced were counted (Fig. 5). The number of foci increased almost linearly with the concentration of Ki-MSV insertion DNA. Thus, the majority of the foci produced by clone 4(E) insertion DNA appeared to result from a single infectious molecule.

The sites of the Ki-MSV genome involved in transformation were studied further. Clone 4(E) recombinant DNA was digested with various restriction enzymes. More than 99% digestion was ensured by electrophoresing 0.5 μg of the digest in an agarose gel and staining with ethidium bromide; under these conditions, less than 0.005 μg of DNA was detected (data not shown). The digested DNA was then used to transfect BALB3T3 cells. As Fig. 6 shows, the number of foci produced by the transfection of *Hin*dIII-digested DNA was one-half the number of foci produced by *Eco*RI-digested DNA. However, when *Eco*RI-digested DNA was cleaved further with *Xba*I or with *Pvu*II and used to transfect BALB3T3 cells, no foci were produced. The restriction sites of *Hin*dIII, *Xba*I, and *Pvu*II were determined from size analyses of the restriction fragments and from the assumption that the restriction sites of the insertion DNA mapped as a permutation of the linear DNA at the *Eco*RI site. The results of the transfection experiment suggested that two *Hin*dIII sites which mapped at 2.1 and 3.7 kb from the right end of the linear DNA were not essential for the initiation and maintenance of viral transformation, but that at least one *Xba*I site and one *Pvu*II site were involved in the initiation of transformation or maintenance of transformation or both.

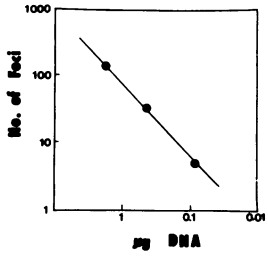

FIG. 5. *Titration kinetics for focus-forming activity of EcoRI-digested clone 4(E) recombinant DNA. Different concentrations of EcoRI-digested DNA were used to transfect, and foci were counted as described in the text. The abscissa shows the amount of Ki-MSV insertion DNA transfected, and the ordinate shows the number of foci formed.*

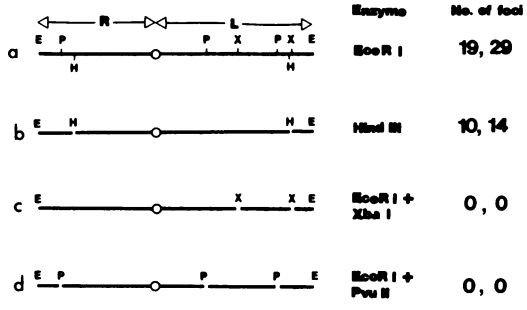

FIG. 6. *Focus-forming activity of clone 4(E) recombinant DNA cleaved with restriction enzymes. Clone 4(E) recombinant DNA (0.3 μg) was digested with one or two restriction enzymes and used to transfect BALB3T3 cells on duplicate plates, and foci were counted as described in the text. The restriction endonuclease sites on insertion DNA for EcoRI (E), XbaI (X), HindIII (H), and PvuII (P) are shown on line a. The open circles indicate the putative junction points of the left and right ends of the linear DNA. R and L indicate the right and the left fragments, respectively, of EcoRI-digested linear Ki-MSV DNA.*

DISCUSSION

We identified two size classes of Ki-MSV circular DNA in NIH3T3 cells recently infected with Ki-MSV. One of the circular DNAs was 7.0 kbp long and thus indistinguishable from linear Ki-MSV DNA, and the other circular DNA was about 0.5 kbp shorter. The circular DNA species were enriched from the infected cells by Hirt extraction, sucrose gradient centrifugation, and agarose gel electrophoresis and then linearized with *Bam*HI, inserted into plasmid pBR322, and transformed to *E. coli* χ1776. This procedure enabled us to isolate four clones carrying Ki-MSV-pBR322 recombinant DNA from 7,000 colonies.

Clone 4 DNA had the largest insertion, with a size indistinguishable from the size of a large circular DNA molecule and from the size of the linear Ki-MSV DNA synthesized in vivo. In addition, the results of a restriction enzyme analysis of clone 4 DNA agreed favorably with the results predicted from the permutated linear Ki-MSV DNA map. Thus, the clone 4 insertion was most probably derived from the large circular DNA, which was joined head to tail at the two ends of the linear DNA.

Clone 7 DNA had the 0.55-kbp deletion, and the size of the insertion was indistinguishable from the size of a small circular DNA synthesized in vivo. The restriction enzyme analyses showed that the 0.55-kbp *Sma*I or *Kpn*I fragment was absent. We conclude that clone 7 Ki-MSV DNA contains only one copy of the LTR sequence and that clone 4 DNA contains two copies. This conclusion is based on the finding that the 0.55-kbp *Sma*I or *Kpn*I fragment contains both the left end and the right end of the linear DNA and on other studies, which describe small and large circular DNA molecules containing one and two copies, respectively, of LTR sequences (14, 23, 28, 29, 34). The preliminary finding that both 1.2- and 0.55-kbp *Sma*I fragments produced 145-, 95-, and 45-base pair species after *Hpa*II digestion offers additional support for this view. Thus, it appears that the 1.2-kbp *Sma*I fragment contains a part of the LTR sequence and that the 0.55-kbp *Sma*I or *Kpn*I fragment contains a sequence corresponding to a single copy of the LTR sequence.

Clone 2 and 6 insertions had deletions of 0.2 and 0.5 kbp, respectively, inside the 1.2-kbp *Sma*I fragment. It is not yet clear whether these deletions are located inside or outside of the LTR sequence, as the structures of these sequences are similar to insertion sequences of transposons (6, 21, 25, 27) and a deletion is frequently observed next to an insertion sequence of a transposon (19, 27).

Clone 4 DNA induced foci when the DNA was used to transfect BALB3T3 cells. Preliminary results showed that all of the RNAs extracted from cells of several isolated foci that were induced by clone 4(E) DNA contained Ki-MSV-specific sequences, as judged by hybridization with Ki-MSV complementary DNA. Thus, the clone 4 Ki-MSV insertion is able to transform mouse cells. Results of transfections by the *Eco*RI-inserted Ki-MSV DNA cleaved with various restriction enzymes suggested that the 1.6-kbp *Hin*dIII fragment is not essential for the initiation and maintenance of transformation. Besides, the finding that *Xba*I, *Pvu*II, and *Bam*HI, which cleave within the 3.3-kbp *Hin*dIII left end fragment of linear Ki-MSV DNA, decrease or abolish the transforming activity of clone 4(E) DNA suggests that the region essential for transforming activity of Ki-MSV is probably located within the first 3.3-kbp region of the left end of linear DNA. The findings that the first 3.0-kb region from the 3′ end of the Harvey MSV genome is not essential for transforming activity (4, 8, 33) and that the first 3.7-kbp regions from the right ends of Ki-MSV and Harvey MSV linear DNAs are homologous also support this view.

The 3.3-kbp left end region of linear Ki-MSV DNA appears to contain the LTR sequence, the 0.7-kb sequence at the 5′ end of viral RNA which is homologous in the Ki-MSV and Harvey MSV genomes, and the region of nonhomology in these viral genomes (5, 31). The precise location of the region which encodes the transformation gene product(s) is not yet known. The p21 protein responsible for transformation by Ki-MSV (24) may be coded in either the 0.7-kb homologous region or the nonhomologous region or both.

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