Identification of Unintegrated Forms of Kirsten Murine Sarcoma Viral DNA and Restriction Endonuclease Cleavage Map of Linear DNA

NOBUO TSUCHIDA,^{1*} RYO KOMINAMI,² MASAKAZU HATANAKA,³ AND SEIICHIRO UESUGI[†]
Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104¹; Frederick Cancer Center, Biological Carcinogenesis Program, Frederick, Maryland 21701²; and Laboratory of Viral Carcinogenesis, National Institutes of Health, Bethesda, Maryland 20205³

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We detected unintegrated linear 7.0-kilobase pair DNA and covalently closed circular DNA species in NIH3T3 cells recently infected with Kirsten murine sarcoma virus. Using the linear DNA, we constructed a restriction endonuclease cleavage map and compared it with the map of Harvey murine sarcoma virus. The restriction endonuclease maps of two segments, one 1.2 kilobase pairs (*SmaI* site) to 3.7 kilobase pairs (*HindIII* site) from the right end (corresponding to the viral 3' side) and the other 0.5 kilobase pair (*SmaI* and *KpnI* sites) to 0.9 kilobase pair (*KpnI* site) from the left end, were identical in the two virus types.

Murine sarcoma viruses (MSVs) transform fibroblasts of a variety of animal cells in tissue culture and induce solid tumors (sarcomas) when injected into newborn mice (15, 18, 20). As is the case for most transforming or acute leukemia viruses, MSV replicates only in the presence of a helper nondefective leukemia virus (13). Two isolates of MSV, Kirsten MSV (Ki-MSV) and Harvey MSV (Ha-MSV), were isolated initially from sarcomas induced when the Kirsten strain of murine leukemia virus (MLV) and Moloney MLV, respectively, were injected into rats (15, 18). Both genomes contain at least two distinct sequences, one derived from the MLV genome and the other derived from the normal rat genome (5, 25, 29). A portion of the latter sequence is transcribed as 30S RNA molecules in normal rat cells, and this RNA is incorporated into virions of rat leukemia virus (35). The MLV-derived sequence has been detected in the first 50 to 200 bases at the 5' end and in the first 1,000 bases at the 3' end of 30S viral genome RNA (5, 29). In an attempt to compare the physical structures of the two genomes in detail, we extracted and identified by the Hirt method unintegrated linear and covalently closed circular forms of Ki-MSV DNA in infected NIH3T3 cells. These cells were infected recently with virus from mouse cells that produced much more Ki-MSV than helper MLV. We then constructed a physical map by using the linear form of Ki-MSV DNA for comparison with the published map of Ha-MSV (10, 12).

[†] Present address: Sapporo Medical College, Cancer Research Institute, Sapporo, Japan.

To identify the unintegrated forms of Ki-MSV DNA in recently infected cells, a cell line that favored the production of Ki-MSV over its helper virus was desirable. The Ki-MSV (MLV)-producing NRK rat cell line 58967 produces 10 to 100 times more MSV than helper virus (Kirsten MLV) (19). However, the virus particles produced by this line may contain rat endogenous 30S RNA, which has substantial homology with the 30S RNA of Ki-MSV (24, 35). Thus, an analysis of Ki-MSV genome DNA may be difficult. We chose the mouse cell line 58-2T, which produces Ki-MSV and a helper MLV that is closely related to Rauscher MLV (38), as the ratio of sarcoma virus to helper MLV in the culture medium was more than 100, as estimated from focus-forming units and XC plaque-forming units, respectively. The ratio of Ki-MSV-specific nucleotide sequence to helper-specific nucleotide sequence in the viral high-molecular-weight RNA was more than 40, as judged by hybridization kinetics (Table 1). The size of viral high-molecular-weight RNA and the size of viral RNA subunits were 50S to 55S and 30S, respectively (data not shown); these sizes are characteristic of defective MSVs (8, 19, 36). An examination of the incorporation of mouse endogenous 30S RNA into 58-2T virions (virus particles produced by 58-2T cells) revealed that, at most, 5% of the 58-2T complementary DNA hybridized to saturating amounts of the cellular RNA of cell line JLS-V9, in which mouse endogenous 30S RNA reportedly is present in relatively high amounts (2).

The presence of the linear and covalently

TABLE 1. *Sarcoma virus/helper virus ratio in 58-2T cells as determined by reassociation kinetics^a*

Complementary DNA	Viral high-molecular-weight RNA	$C_{t,1/2}$	Sarcoma virus/helper virus ratio ^b
58-2T	58-2T	1×10^{-2}	
Rauscher MLV	58-2T	$>4 \times 10^{-1c}$	
Rauscher MLV	Rauscher MLV	1×10^{-2}	>40
58-2T	Rauscher MLV	— ^d	

^a Viral ³H-labeled complementary DNA from 58-2T virus or Rauscher MLV was prepared by endogenous reaction of the reverse transcriptase of purified virions. The method described previously (38) was modified as follows. The reaction mixture contained 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.02 mM [³H]-TTP (40 to 50 Ci/mmol; New England Nuclear Corp., Boston, Mass.), 0.2 M glycine buffer (pH 8.0), 2 mM MnCl₂, 0.1% Nonidet P-40, 1 mg of DNase I-digested calf thymus DNA primer per ml (33), 50 μg of actinomycin D per ml, 10 mM dithiothreitol, and purified virions (0.5 to 1 mg of protein per ml). The mixture was incubated at 40°C for 16 to 18 h, and complementary DNA was purified as described previously (38). Hybridization analyses were carried out by using 58-2T and Rauscher MLV complementary DNAs and corresponding viral high-molecular-weight RNAs (1,000-fold excess of viral RNA over complementary DNA) as described previously (37, 39), except that the RNA and complementary DNA were hybridized at 50°C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 30% formamide. Maximum hybridization of a complementary DNA with the homologous viral high-molecular-weight RNA was 89% for 58-2T complementary DNA and 92% for Rauscher MLV complementary DNA. $C_{t,t}$ is the product of the initial concentration of sample RNA and time, as defined by Birnstein et al. (3).

^b For comparison, a sarcoma virus/helper virus ratio of 113 was determined by a biological assay (3.6×10^5 focus-forming units per ml, $3.2 \times 10^3 \times C$ plaques per ml). A 1-day-old culture fluid from 58-2T cells was used to infect BALB3T3 cells (1), and foci and XC plaques were assayed as described by Hartley and Rowe (14) and by Rowe et al. (22).

^c Did not reach plateau at $C_{t,t} = 8 \text{ mol} \cdot \text{s/liter}$.

^d At most, 5% of the 58-2T complementary DNA hybridized with Rauscher MLV RNA.

closed circular forms of viral DNA has been reported in cells recently infected with Moloney MSV and Ha-MSV, as well as with nondefective retroviruses (4, 9–12, 26, 28, 32). To identify forms of Ki-MSV DNA synthesized in recently infected cells, NIH3T3 cells were infected at a multiplicity of infection of 5 with 58-2T virus in the presence of 2 μg of Polybrene per ml (34), and at 16 to 24 h postinfection the DNA fraction was separated from chromosomal high-molecular-weight DNA by the Hirt extraction method

(17). The Hirt supernatant fraction was subjected to cesium chloride-ethidium bromide centrifugation, using simian virus 40 form I and II DNAs as density markers (Fig. 1). As Fig. 1A shows, less than 10% of the Ki-MSV-specific DNA banded at a density indicative of the covalently closed circular form; most of the Ki-MSV-specific DNA banded at a light density, where both the linear and open circular forms sediment (9). To distinguish between these two forms, the DNA banding at the light density was subjected to neutral agarose gel electrophoresis, in which a single species of virus DNA was detected at a molecular weight of 4.4×10^6 (7.0 kilobase pairs [kbp]) (Fig. 1B), and to neutral sucrose gradient centrifugation, in which a major peak of Ki-MSV DNA was found around 16S (Fig. 2A). For agarose gel electrophoresis, viral DNA was transferred to a membrane filter and hybridized with ³²P-labeled 58-2T viral RNA (30). No radioactivity was detected around 8.5 to 9 kbp, which reportedly is the size of a linear molecule synthesized in cells recently infected with a helper MLV (10). For the following reasons, we believe that most, if not all, of the Ki-MSV-specific DNA that banded at the light-density region after cesium chloride-ethidium bromide centrifugation was linear double-stranded DNA: (i) the molecular weight estimated from the gel electrophoresis was approximately consistent with the molecular weight estimated from the sedimentation analysis by using the formula $S_{20,w} = 0.034 \times MW^{0.404}$ (6), where $s_{20,w}$ is the sedimentation coefficient corrected to water at 20°C and MW is the molecular weight; (ii) the molecular weight estimated by both methods was approximately twice the molecular weight of Ki-MSV 30S virus RNA (19); and (iii) only a single species of viral DNA was detected by Southern filter hybridization. The viral DNA sedimenting in the heavy-density region was characterized further by analyses after both neutral and alkaline sucrose gradient centrifugations. As Fig. 2B and C show, the sedimentation coefficients were 22S to 24S in the neutral gradient and 55S to 60S in the alkaline gradient. These values are consistent with values for the closed circular form of the 16S linear DNA molecule (40). The presence of the linear and covalently closed circular forms of viral DNA in cells recently infected with Ki-MSV suggested that the mechanism of viral DNA synthesis in replication-defective Ki-MSV was similar to the mechanism in other nondefective retroviruses.

The 16S Hirt supernatant fractions of the infected cells were then used for physical mapping of the linear Ki-MSV DNA after centrifugation.

FIG. 1. Isopycnic centrifugation and agarose gel electrophoresis of unintegrated Ki-MSV DNA. (A) Nonchromosomal DNA was extracted by the Hirt method from 5×10^8 NIH3T3 cells 24 h after infection with Ki-MSV; 5 g of CsCl and 0.4 ml of an ethidium bromide solution (4.0 mg/ml) were added to 4.7 ml of Hirt supernatant fractions or to ^3H -labeled simian virus 40 DNA markers (Bethesda Research Laboratories, Rockville, Md.) in E buffer. The DNA was centrifuged for 36 h at 37,000 rpm and 20°C in a Beckman R50 rotor. Fractions (0.32 ml) were collected and extracted with isopropanol; 30 μl of each fraction was diluted fourfold with distilled water, and DNA was ethanol precipitated in the presence of yeast RNA. After treatment of DNA with 0.3 NaOH at 100°C for 20 min, the sample was neutralized with acetic acid, and the DNA was ethanol precipitated. The amount of Ki-MSV-specific DNA was estimated by hybridizing each fraction with 500 cpm of ^3H -labeled 58-2T complementary DNA for 48 h by using the modifications described in Table 1, footnote b. The arrows indicate the positions of ^3H -labeled simian virus 40 form I and II DNAs. Symbols: $\circ$, percentage of complementary DNA hybridized; $\diamond$, density. (B) Detection of Ki-MSV by Southern filter hybridization. After extraction with isopropanol, each

fraction, fraction 10 to fraction 15 (Fig. 1A) (3 μl each), was diluted with 200 μl of NTE buffer (0.01 M Tris-hydrochloride, pH 7.2, 0.2 M NaCl, 0.01 M EDTA) containing 10 μg of yeast RNA. Nucleic acids were ethanol precipitated, and the precipitate was dissolved in 50 μl of E buffer. The DNA was electrophoresed in a neutral 1% (wt/vol) agarose (medium EEO; Seakem, Rockland, Maine) slab gel with E buffer at 2 V/cm for 10 h (27). After electrophoresis, the gel was stained with 0.5 μg of ethidium bromide per ml at 25°C for 30 min and photographed under short-wave UV light. The DNA in the gel was transferred to a membrane filter as described by Southern (30). The filter was then baked for 2 h at 80°C in vacuo and prehybridized for 1 h in annealing mixture as described by Shank et al. (26). Hybridization with ^{32}P -labeled 58-2T viral RNA was performed by saturating filters with an annealing mixture containing 10^7 cpm of [^{32}P]RNA probe per ml and 100 μg of HOS (21) cellular RNA per ml. The [^{32}P]RNA probe was prepared as follows. Viral high-molecular-weight RNA was subjected to mild alkaline fragmentation by heating in 0.05 M NaCO_3 at 60°C for 15 min, and the solution was neutralized. The RNA was ethanol precipitated and dissolved in a small volume of water to a final concentration of 50 $\mu\text{g}/\text{ml}$. The 5' end of the RNA was labeled at 37°C for 3 h in a 30- μl reaction mixture containing 70 mM Tris-hydrochloride (pH 7.6), 10 mM MgCl_2 , 5 mM dithiothreitol, 0.04 nM [γ - ^{32}P]ATP (specific activity, 5,000 to 6,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), 1 μg viral RNA fragments, and 3 U of T4 polynucleotide kinase. The [^{32}P]RNA was purified by passage through a Sephadex G-50 column, extraction with phenol, and ethanol precipitation. The filter was wrapped with two sheets of Parafilm and incubated at 41°C for 16 to 36 h. Unhybridized [^{32}P]RNA was removed by washing three times (30 min each) in 35 ml of a solution containing 50% formamide and $2\times$ SSC at 37°C and then incubating the filter at 37°C for 20 min in 35 ml of $2\times$ SSC containing 10 μg of RNase per ml and 10 U of RNase T1 per ml. The filter was finally washed with $2\times$ SSC at 25°C for 10 min, air dried, and exposed for 1 to 7 days to Kodak X-Omat R film, using Dupont Cronex Lightning Plus intensifying screens. HindIII-cleaved λ DNA (New England Biolabs, Boston, Mass.) was used as a size marker and was treated with bacterial alkaline phosphatase, labeled with ^{32}P , electrophoresed, and treated as described above.

FIG. 2. Sucrose velocity sedimentation profile of linear and covalently closed circular Ki-MSV DNAs. (A) Centrifugation of linear DNA in a neutral sucrose gradient. After isopropanol extraction, fractions 11 to 14 (Fig. 1A) (60 μ l of each) were pooled and diluted with 3 volumes of water, and the DNA was ethanol precipitated in the presence of yeast RNA. The precipitate was dissolved in 200 μ l of NTE buffer. The sample was centrifuged with a Beckman SW41 rotor at 30,000 rpm for 13 h at 18°C in a 15 to 30% sucrose gradient containing NTE buffer. Fractions (0.55 ml) were collected from the bottom and processed as described in the legend to Fig. 1 by using 500 cpm of 3 H-labeled 58-2T complementary DNA (cDNA) as a probe. 3 H-labeled simian virus 40 DNA (forms I and II) was run in a parallel gradient. (B and C) Centrifugation of covalently closed circular DNA in neutral and alkaline sucrose gradients, respectively. The material remaining in fractions 6 through 8 after isopropanol extraction was pooled, and the DNA was ethanol precipitated after water and yeast RNA were added. The precipitate was dissolved in 0.4 ml of NTE buffer; 0.2 ml was centrifuged in a neutral 15 to 30% sucrose gradient containing NTE buffer and fractionated as described above, and the DNA was treated with an alkaline solution and processed as described in the legend to Fig. 1. The other 0.2 ml was centrifuged in an alkaline sucrose gradient containing 0.3 N NaOH, 0.7 M NaCl, and 5 mM EDTA at 36,000 rpm for 5 h at 15°C. Fractions (0.55 ml) were collected from the bottom, heated at 100°C for 20 min, and neutralized with acetic acid. The amounts of Ki-MSV-specific DNA were quantitated as described in the legend to Fig. 1. In both cases, 3 H-labeled simian virus 40 markers (forms I and II) were run in a parallel gradient.

the digested fragments (Fig. 3).

To determine 5'-to-3' orientation of Ki-MSV linear DNA in relation to viral RNA, the 3.3- and 2.1-kbp *Hind*III cleavage products (containing the left and right ends, respectively, of the 7.0-kbp linear DNA) were used to relate the restriction fragments to the 5'-to-3' orientation of viral RNA. *Hind*III fragments of linear Ki-MSV DNA were electrophoresed in a 1% agarose slab gel, transferred to a membrane filter, and hybridized with 32 P-labeled probe RNA in the presence of fivefold molar excesses of unlabeled viral RNAs of different lengths from the 3' polyadenylic acid [poly(A)] end of the RNA. If the 2.1-kbp fragment corresponded to the 3' end of viral RNA, this fragment should have competed with short poly(A)⁺ fragments more effectively than the 3.4-kbp fragment. As Fig. 4A shows, the intensity of the 2.1-kbp *Hind*III fragment was in fact drastically reduced when this fragment competed with 2.1-kilobase (kb) poly(A)⁺ RNA fragments and remained low even with longer poly(A)⁺ RNA fragments. The intensity of the 3.3-kbp *Hind*III fragment decreased gradually with increasing size of the poly(A)⁺ RNA fragment used to compete with the [32 P]RNA probe. These observations were confirmed by using a liquid scintillation counter to measure the radioactivities in the bands corresponding to the 2.1- and 3.3-kbp *Hind*III fragments. In Fig. 4B, the percentage of the radioactivity remaining in each band is plotted against the length of poly(A)⁺ RNA used to compete with the [32 P]RNA probe. These results suggest that the 3.3- and 2.1-kbp fragments correspond to 5' and 3' portions, respectively, of the viral RNA genome.

Extensive homology has been reported in nucleotide sequences of Ki-MSV and Ha-MSV, as determined by hybridization studies (36), heteroduplex analysis (5), and oligonucleotide fingerprint analysis of viral RNA (29). As Fig. 3 shows, there is a striking similarity in the cleavage sites of Ki-MSV and Ha-MSV (10, 12) between 1.3 and 3.7 kbp (*Sma*I at 1.3 kbp, *Hind*III at 2.1 kbp, *Eco*RI at 3.0 kbp, *Xba*I at 3.6 kbp, and *Hind*III at 3.7 kbp) in the right ends of both DNAs and between 0.5 and 0.9 kbp (*Sma*I at 0.5 kbp, *Kpn*I at 0.5 kbp, *Bam*HI at 0.8 kbp, and *Kpn*I at 0.9 kbp) in the left ends. Preliminary results for *Pst*I sites of Ki-MSV in the 1.3- to 3.7-kbp segment also coincide with the results for Ha-MSV (Tsuchida, unpublished data). These results are consistent with the previous report on analyses of heteroduplex formation between Ki-MSV complementary DNA and Ha-MSV RNA (5), in which extensive homology was found in the first 4.4 kb from the 3' end and in a 0.7-kb region at the 5' end of the viral

FIG. 3. Physical map of linear Ki-MSV DNA. The cleavage sites produced by KpnI, SmaI, BamHI, HindIII, XbaI, and EcoRI are shown. Linear DNA is oriented with respect to the 5' and 3' polarities of the viral RNA. The thick lines indicate regions in which the restriction maps of Ki-MSV and Ha-MSV coincide (10, 12). The XbaI cleavage site of Ki-MSV DNA that is not present in Ha-MSV linear DNA is underlined (10, 12).

FIG. 4. Orientation of the Ki-MSV DNA physical map in relation to viral RNA. HindIII was used to digest 16S Ki-MSV linear DNA. Samples were electrophoresed into eight slots of a neutral 1% agarose slab gel. After electrophoresis, the DNA in the gel was transferred to a membrane filter. The filter was cut into eight strips, and each strip was hybridized with 10^7 cpm of [32 P]RNA probe (specific activity, 7×10^7 cpm/ μ g) per ml in the presence of 5 M excesses of unlabeled viral poly(A)⁺ RNAs of different sizes (2.1, 2.7, 3.3, 4.1, 4.8, and 5.6 kb) or with 30S viral poly(A)⁺ RNA. Poly(A)⁺ RNA fragments were prepared essentially as described by Hayward (16). The molecular weights of the viral poly(A)⁺ fragments and the number of nucleotides per RNA were calculated as described previously (31). (A) Lanes 1 through 5 show autoradiographs after the hybridization of HindIII fragments with the [32 P]RNA probe in the absence of poly(A)⁺ RNA (lane 1) and in the presence of 2.1-kb poly(A)⁺ RNA (lane 2), 3.3-kb poly(A)⁺ RNA (lane 3), 4.8-kb poly(A)⁺ RNA (lane 4), and 30S viral poly(A)⁺ RNA (lane 5). 32 P-labeled λ HindIII fragments were used as molecular weight

genome RNA, assuming that a 0.6-kbp terminal repeat was present at the left and right ends of both linear DNA molecules (12). Considering the extraordinary specificity of restriction enzymes for recognition sequences and the occurrence of these sequences at about once every 4,000 base pairs, the sequences of those segments are likely to be almost identical, allowing for the insertion or deletion of short segments. The segment 1.3 to 3.7 kbp from the right end is reported to be derived from a rat endogenous 30S RNA genome (5), 20 to 40 copies of which are present per haploid genome in rat cells (23; Tsuchida, unpublished data). The function and organization of these sequences in rat chromosomes and whether these sequences are identical in all copies are questions that remain to be resolved.

The XbaI sites of Ha-MSV DNA at both the right end and the left end were not found in Ki-MSV DNA. Moreover, SacI sites at both ends were not found on either end of cloned Ki-MSV DNA (Tsuchida, unpublished data). These results are at variance with the results obtained in heteroduplex studies, but this may be explained by considering that a slight mismatching of base pairs cannot be detected by heteroduplex analysis (7). Also, the MLV sequences of Ki-MSV and Ha-MSV are derived from different sources (15, 18), and the T₁ oligonucleotide maps for the first 1,000 nucleotides in the 3' ends of these viral RNAs (5) do not coincide (29).

The region(s) of nonhomology between the Ki-MSV and the Ha-MSV genomes observed in heteroduplex studies may reside somewhere be-

markers. (B) Areas corresponding to the 3.3- and 2.0-kbp Ki-MSV HindIII fragments in each strip were cut out, and radioactivities were measured with a liquid scintillation counter. The ordinate represents the percentage of radioactivity remaining in a fragment after competition with unlabeled poly(A)⁺ RNA fragments of different sizes. The abscissa represents the length of the poly(A)⁺ fragment used to compete with the [32 P]RNA probe. Symbols: $\circ$, competition of 3.3-kbp HindIII fragment with poly(A)⁺ RNA; $\bullet$, competition of 2.1-kbp HindIII fragment with poly(A)⁺ RNA.

tween 0.9 kbp from the left end and 3.7 kb from the right end, as no restriction sites examined thus far coincide here. Further analysis of the restriction sites within this region will be most interesting, as Wei et al. (41) have recently reported that the region between 0.4 and 1.5 kb from the 5' end of the Ha-MSV RNA genome but not the first 3.0 kb from the 3' end contains sequences essential for transformation.

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