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Molecular Cloning of Unintegrated and a Portion of Integrated Moloney Murine Leukemia Viral DNA in Bacteriophage Lambda

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A covalently closed circular form of unintegrated viral DNA obtained from NIH 3T3 cells freshly infected with Moloney murine leukemia virus (M-MLV) and a portion of the endogenous M-MLV from the BALB/Mo mouse strain have been cloned in bacteriophage lambda. The unintegrated viral DNA was cleaved with restriction endonuclease *Hin*dIII and inserted into the single *Hin*dIII site of lambda phage Charon 21A. Similarly high-molecular-weight DNA from BALB/Mo mice was cleaved sequentially with restriction endonucleases *Eco*RI and *Hin*dIII and separated on the basis of size, and one of the two fractions which reacted with an M-MLV-specific complementary DNA was inserted into the *Hin*dIII site of Charon 21A. Recombinant clones containing M-MLV-reacting DNA were analyzed by restriction endonuclease mapping, heteroduplexing, and infectivity assays. The restriction endonuclease map of the insert derived from unintegrated viral DNA, λ·MLV-1, was comparable to published maps. Electron microscope analysis of the hybrid formed between λ·MLV-1 DNA and 35S genomic M-MLV RNA showed a duplex structure. The molecularly cloned λ·MLV-1 DNA contained only one copy of the long terminal repeat and was not infectious even after end-to-end ligation of the insert DNA. The insert DNA derived from endogenous M-MLV, λ·MLV$_{int}$-1, contained a DNA stretch measuring 5.4 kilobase pairs in length, corresponding to the 5′ part of the genomic viral RNA, and cellular mouse DNA sequences measuring 3.5 kilobase pairs in length. The viral part of the insert showed the typical restriction pattern of M-MLV DNA except that a single restriction site, *Pvu*II, in the 5′ long terminal repeat was missing. Reconstructed genomes containing the 5′ half derived from the integrated viral DNA and the 3′ half derived from the unintegrated viral DNA were able to induce XC plaques after transfection in uninfected mouse fibroblasts.

In the life cycle of RNA tumor viruses, the viral genetic material is converted into DNA, some of which integrates in the host chromosomal DNA (2, 31). Several forms of viral DNA, ranging from linear to covalently closed circles, have been identified in infected cells (33). However, a detailed knowledge of the structure of the intermediates leading to the integrated forms of viral DNA remains largely obscure. The study of the integrated murine leukemia viruses (MLVs) has been hampered by the large number of related endogenous viral sequences in uninfected cells. Use of recombinant DNA techniques, however, make it feasible to enrich and amplify DNA fragments of single-copy genes of eucaryotes. The BALB/Mo mouse strain (12) contains the Moloney MLV (M-MLV) as an endogenous virus, which has been localized in a single *Eco*RI fragment of 27 kilobases (kb) (27). This fragment is derived from the *Mov-1* locus of BALB/Mo mice (5). BALB/Mo mice are viremic from birth and develop lymphatic leukemia later in life (13), indicating the presence of a complete active M-MLV genome. In this study, we report the molecular cloning of (i) unintegrated circular forms of M-MLV DNA isolated from cells freshly infected with M-MLV (the DNA was cleaved with restriction endonuclease *Hin*dIII to generate the linear form) and (ii) a portion of the integrated form of M-MLV present in BALB/Mo mice (the 27-kb *Eco*RI cell DNA fragment was cleaved with restriction endonuclease *Hin*dIII to generate a fragment of about 8.9 kb which contains M-MLV sequences). Both the unintegrated circular M-MLV DNA cleaved with *Hin*dIII and the 8.9-kb *Hin*dIII fragment from the mouse DNA were cloned in the unique *Hin*dIII site of lambda phage Charon

21A DNA (3). The natures of the cloned DNAs were examined by restriction endonuclease mapping, R-loop formation, and infectivity assays.

MATERIALS AND METHODS

Preparation of unintegrated closed circular DNA. About 5×10^8 NIH 3T3 cells were infected with a 24-h tissue culture supernatant from M-MLV-producing clone 4A cells at a multiplicity of infection of 1. At 16 h postinfection, cells were harvested and a Hirt supernatant was prepared. After RNase treatment and *Eco*RI digestion, circular DNAs were separated from linear DNA by centrifugation in cesium chloride-ethidium bromide gradients (30). The material banding at the bottom one-third was collected, the ethidium bromide was removed by isopropanol extraction, and the DNA was dialyzed.

Preparation of mouse DNA fragments containing M-MLV sequences. High-molecular-weight mouse DNA was prepared from livers of 2-month-old BALB/Mo mice as previously described (15, 27). The DNA was digested to completion with *Eco*RI at 2 U/μg for 4 h, and the fragments were separated by zonal centrifugation in a BXXIX rotor of an International centrifuge. A 50-mg amount of DNA yielded about 1 mg of 18- to 35-kb DNA fragments. After cleaving with endonuclease *Hin*dIII, the fragments were separated on agarose gels and electroeluted, and the fractions hybridizing with an M-MLV-specific probe were used for cloning.

Cloning. DNA from lambda phage Charon 21A was digested with restriction endonuclease *Hin*dIII, followed by treatment with bacterial alkaline phosphatase to reduce self-ligation of the phage arms (5 U/μg of DNA in 50 mM Tris-hydrochloride, pH 9.0, for 30 min at 56°C). For cloning unintegrated M-MLV DNA, about 10 to 20 ng of *Hin*dIII-cut M-MLV DNA was mixed with 150 ng of *Hin*dIII-cut, phosphatase-treated Charon 21A vector DNA and precipitated with ethanol, and the pellet was incubated for 1 h at 42°C in 3 μl of DNA ligase buffer (66 mM Tris-hydrochloride [pH 7.5], 10 mM dithiothreitol, 7 mM $MgCl_2$, 80 μM ATP) to allow the DNA to dissolve; 0.4 U (1 μl) of T4 DNA ligase was added, and after incubation at room temperature for 2 h the DNA was packaged in vitro into infectious lambda phage particles with M-1 buffer by the protocol provided by Blattner and colleagues. The efficiency of packaging of Charon 21A DNA alone was 3×10^8 PFU/μg. After digestion with *Hin*dIII and treatment with bacterial alkaline phosphatase and ligation, the efficiency of packaging of phage DNA was $\pm 10^5$ PFU/μg; ligation of the insert DNA to the *Hin*dIII-cut bacterial alkaline phosphatase-treated Charon 21A DNA increased the efficiency of packaging by a factor of 5. Generally, the efficiency of packaging varied from 1×10^5 to 5×10^5 PFU/μg of substrate-vector DNA. For cloning integrated M-MLV DNA, the same protocol was used except that about 2 μg of an 8.9-kb *Hin*dIII fragment was ligated with 5 μg of phosphatase-treated Charon 21A arms.

Screening of recombinants. The recombinants were plated on 14-cm plates, and the DNA from the plaques was adsorbed onto nitrocellulose filters essentially by the technique of Benton and Davis (1). Plaques that showed hybridization to M-MLV complementary DNA (cDNA) probes (7, 27) were further subcloned until pure recombinant plaques were obtained. The recombinant phages were propagated in *Escherichia coli* DP50 *supF* in NZYDT broth as described in the protocols designed by Blattner et al. (3) and Leder et al. (17). All experiments were performed in a P2 laboratory facility, and certified EK2 or EK1CV vectors were used. The National Institutes of Health Guidelines were followed for the entire cloning procedure.

Restriction endonuclease analysis. The natures of the cloned DNAs were examined by restriction endonuclease mapping as described previously (31). The restriction endonuclease-digested DNA was fractionated on agarose gels ranging from 0.5 to 1.2% in a buffer containing 40 mM Tris base, 1 mM EDTA, and 5 mM sodium acetate, adjusted with acetic acid to pH 7.9 (20). The fragments were identified either by ethidium bromide staining (24) (present at 0.5 μg/ml in both the gels and electrophoresis buffer) or by the Southern blotting technique (26), using either a Moloney cDNA probe (7) or nick-translated DNA fragments (19). DNA fragments were nick translated with [α-^{32}P]dCTP (2,000 to 3,000 Ci/mmol) from Amersham Corp., *E. coli* polymerase I from Boehringer Mannheim Corp., and DNase I from Worthington Biochemicals Corp. After incubation at 15°C for 90 min, sodium dodecyl sulfate and EDTA were added to final concentrations of 0.5% and 10 mM, respectively. Proteinase K was added at 200 μg/ml, and the mixture was incubated for 30 min at 37°C and passed over a Sephadex G-50 column to remove unincorporated nucleotides.

Hybridization to nitrocellulose-bound DNA was performed as described previously (32). The sizes of the restriction fragments were computed from the standard molecular weight markers in the same gel (lambda DNA cleaved with *Eco*RI: 22.2, 7.3, 5.75, 4.9, and 3.8 kb; lambda DNA cleaved with *Hin*dIII: 24.2, 9.9, 6.7, 4.4, 2.2, and 1.95 kb; M13 replicative form digested with *Hpa*II: 1.6, 0.82, 0.65, 0.54, 0.47, 0.45, 0.38, 0.18, 0.16, and 0.13 kb).

Electron microscopy. Samples of DNA and RNA were dissolved in a solution containing 80% formamide, 0.4 M NaCl, 0.01 M PIPES [piperazine-*N*-*N*'-bis(2-ethanesulfonic acid)] (pH 6.3), and 0.001 M EDTA at 3 and 1 μg/ml, respectively. The DNA was heated separately at 68°C for 5 min and then mixed with RNA, followed by incubation at temperatures which slowly decreased from 55 to 51°C over a 4-h period. Molecules were prepared for electron microscopy, and contour lengths of the molecules were measured as described previously (4).

Infectivity assays. Infectivity of cloned M-MLV DNA was assayed by the XC plaque assay (22). DNA was transfected in NIH 3T3 mouse fibroblasts by the calcium phosphate method (9). Briefly, 4×10^5 cells were plated onto 35-mm plates the day before transfection. The DNA to be assayed was coprecipitated with calcium phosphate as described previously (16) and added directly to the recipient cells. After 5 h, the DNA was removed, and the cells were treated briefly with 20% dimethyl sulfoxide. After overnight recovery, cells were transferred to new plates at 10% confluency. After 5 days, the cells were UV irradiated and overlaid with XC cells. XC plaques were counted at day 9 after

fixing and staining the cells with hematoxylin.

RESULTS

Identification and characterization of the clone derived from unintegrated M-MLV DNA λ·MLV-1. (i) Physical mapping by restriction endonuclease. Figure 1A shows the restriction endonuclease map of in vitro-synthesized M-MLV DNA (8, 31). Since the recombinant clone was made by utilizing the *Hin*dIII site, Fig. 1B shows the physical map of λ·MLV-1 with the *Hin*dIII cleavage site at 0 map unit.

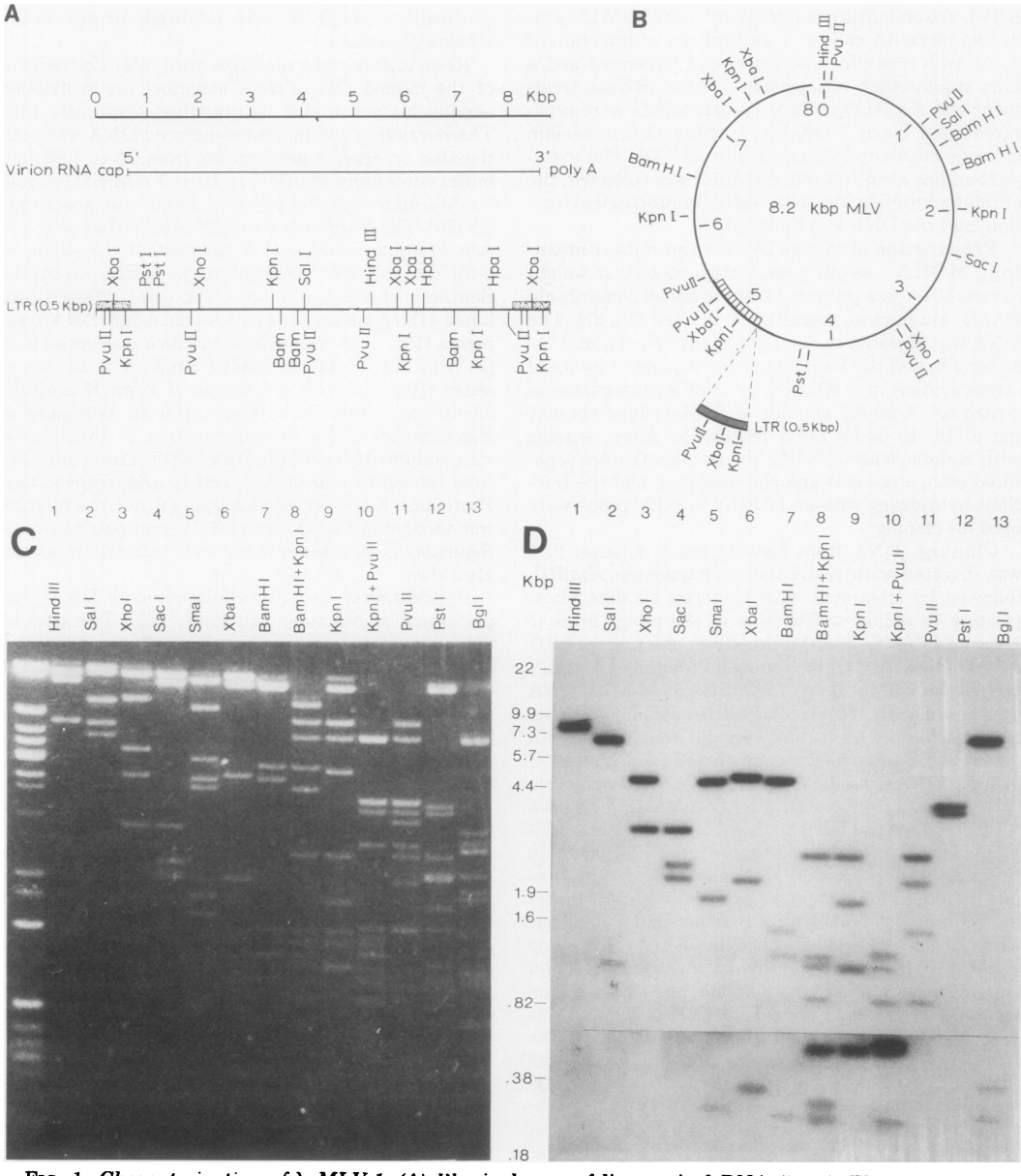

FIG. 1. *Characterization of λ·MLV-1. (A) Physical map of linear viral DNA (8, 31). The shaded areas indicate the location of the LTR. (B) Physical map of λ·MLV-1 with HindIII at 0 map unit. The shaded area is the location of the LTR from the 3' end of the genome. The dashed-line triangle is the expected position of the second LTR at the 5' end. (C) Ethidium bromide staining of restriction endonuclease-digested λ·MLV-1 DNA. (D) Physical map of the insert derived from λ·MLV-1 DNA. Lanes 1 to 13 are autoradiographs of restricted endonuclease-digested λ·MLV-1 DNAs, followed by Southern transfer and hybridization to total M-MLV cDNA. λ·MLV-1 was cleaved with HindIII, followed by digestion with the enzymes specified in the figure.*

A single recombinant clone, λ·MLV-1, which hybridized to M-MLV cDNA was obtained from a primary screening of about 5,000 plaques. Figure 1C, lane 1, shows the size of the insert in λ·MLV-1 to be 8.2 kb after cleavage with *Hin*dIII. Digestion with restriction endonucleases *Sal*I, *Xho*I, *Sac*I, *Sma*I, *Xba*I, *Bam*HI, *Kpn*I, *Pst*I, *Bgl*I, and *Pvu*II revealed a pattern which is in agreement with the map shown in Fig. 1A. However, only one of the two *Pvu*II sites shown in the long terminal repeat (LTR) of Fig. 1A could be detected (see below). The results also indicated that λ·MLV-1 contains only one copy of the LTR shown in Fig. 1A and B. For instance, digestion with *Hin*dIII and *Bam*HI should generate four fragments. The largest fragment should be about 5.4 kb if it contained two copies of the LTR. However, Fig. 1D, lane 7, shows that the largest fragment is only 4.8 kb. Digestion with λ·MLV-1 DNAs cleaved by *Pvu*II (lane 11) and *Sac*I (lane 4) did not show a 600-bp fragment, which would be expected if two copies of the LTR were present (Fig. 1B).

(ii) Heteroduplex formation. Figure 2A shows a heteroduplex formed between λ·MLV-1 and 35S M-MLV genomic RNA. Only a single loop structure of an average size of 8.0 ± 0.73 kb can be seen. An enlargement of the RNA-DNA hybrid loop of the heteroduplex is shown in Fig. 2B. The results indicate that λ·MLV-1 contains sequences homologous to those present in the M-MLV genomic RNA.

Identification and characterization of the clone derived from integrated M-MLV DNA, λ·MLV$_{int}$-1. A single recombinant clone which hybridized to M-MLV cDNA was ob-

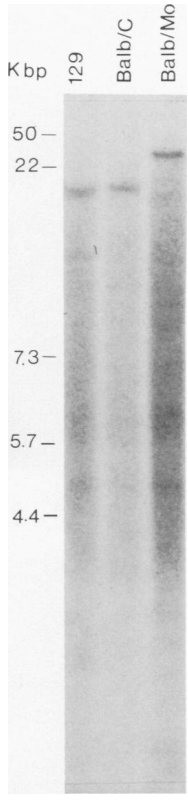

FIG. 3. *Identification of the cellular origin of λ·MLV$_{int}$-1. Autoradiographs of EcoRI-digested 129, BALB/c, and BALB/Mo mouse DNAs, followed by Southern blotting transfer and hybridization to the nick-translated XhoI to HpaI fragment derived from the cellular part of λ·MLV$_{int}$-1.*

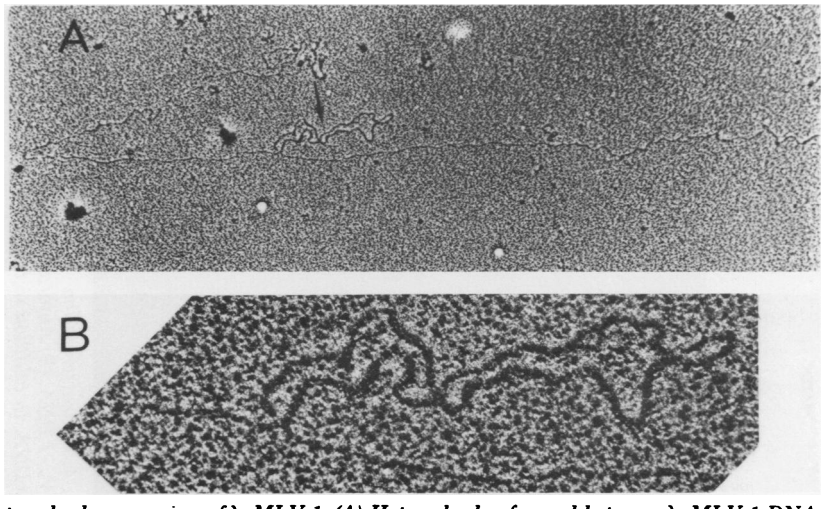

FIG. 2. *Heteroduplex mapping of λ·MLV-1. (A) Heteroduplex formed between λ·MLV-1 DNA and 35S M-MLV genomic RNA. The arrow points to the RNA-DNA hybrid. More than 50 molecules were identified. The average size of the hybrid molecules was 8.0 ± 0.73 kb. (B) Enlargement of the RNA-DNA hybrid shown in (A).*

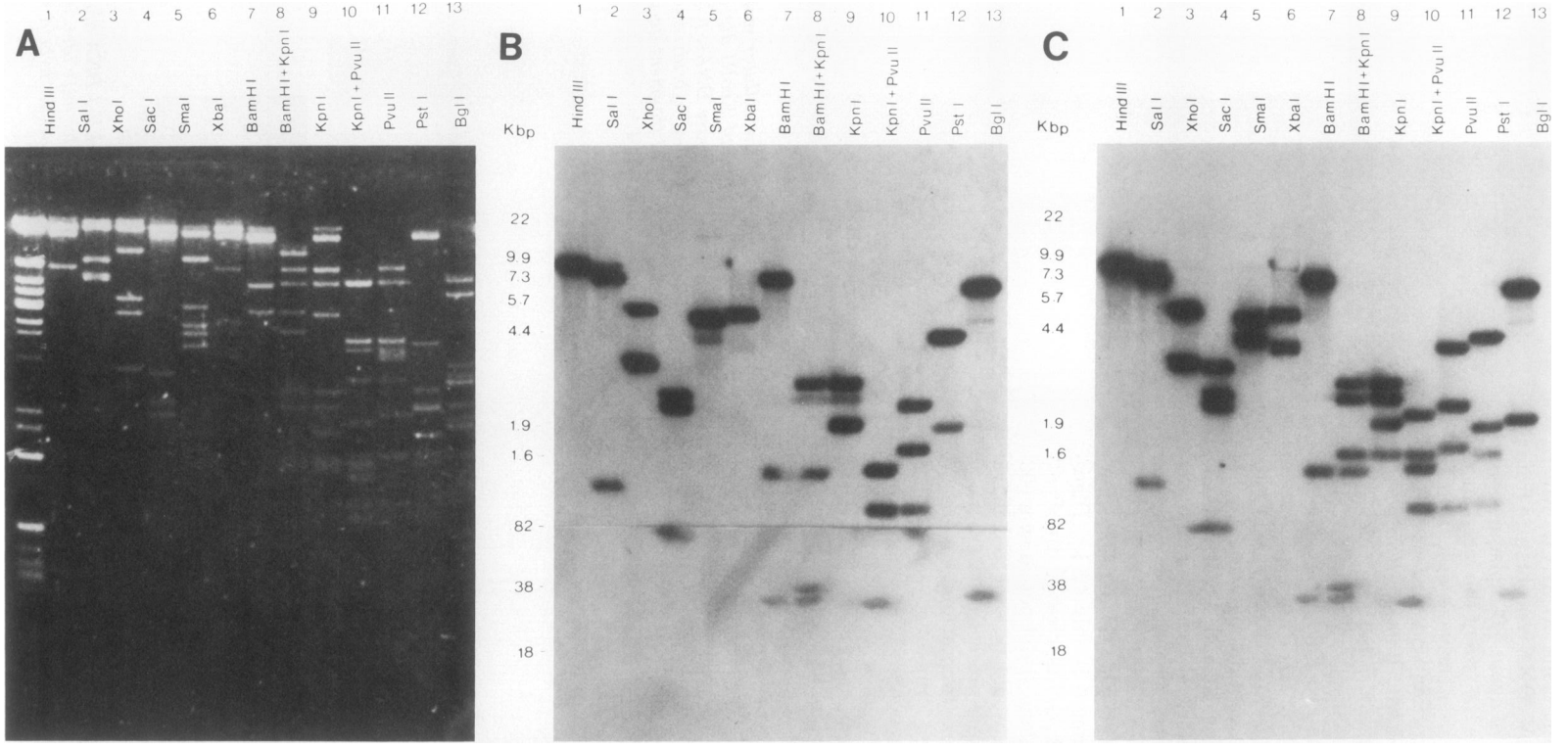

FIG. 4. *Characterization of λ·MLV$_{int}$-1 by restriction endonuclease mapping. (A) Ethidium bromide staining of restriction endonuclease-digested λ·MLV$_{int}$-1 DNA. (B) Autoradiographs of restriction endonuclease-digested λ·MLV$_{int}$-1 DNA, followed by Southern blotting transfer and hybridization to M-MLV cDNA. In addition to the enzymes listed in each lane, all samples were treated with HindIII to release the insert from the phage arms. (C) Same as (A), except that hybridization was performed with a plasmid clone containing the insert from λ·MLV$_{int}$-1. (D) Physical map of the insert derived from λ·MLV$_{int}$-1.*

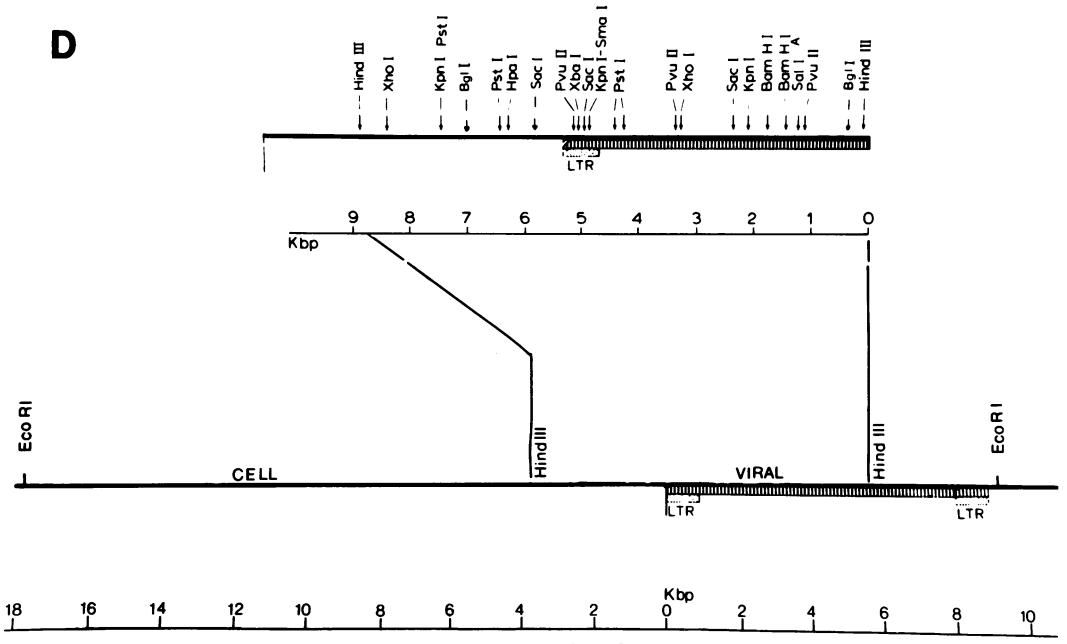

FIG. 4—*continued*

tained from a primary screening of approximately 12,000 plaques. The nature of the recombinant clone was identified as about 5.4 kb of viral and 3.5 kb of cellular sequences. The restriction map of the viral sequences agreed with that shown in Fig. 1A, except that only one *Pvu*II site was detected in the 5'-end LTR instead of the reported two sites (8). The restriction map of the cellular sequences agreed with the map determined for the 27-kb *Eco*RI fragment obtained from BALB/Mo mice (v.d. Putten et al., unpublished data). To ascertain that λ·MLV$_{int}$-1 was derived from the DNA corresponding to the *Mov-1* locus of the BALB/Mo mouse strain, we hybridized the *Xho*I to *Hpa*I cellular fragment of this clone (after labeling with [α-^{32}P]dCTP by nick translation) to Southern blots of *Eco*RI-digested DNA from the 129, BALB/c, and BALB/Mo mouse strains (Fig. 3). This probe recognized an 18-kb fragment in 129 and BALB/c DNAs and a 27-kb fragment in BALB/Mo mouse DNA, indicating that λ·MLV$_{int}$-1 is indeed derived from the *Mov-1* locus of BALB/Mo mice. Furthermore, it showed that integration of M-MLV has occurred without causing large deletions in the cellular DNA, as the size difference between the *Eco*RI fragments recognized in BALB/c and BALB/Mo mice corresponds with the genomic size of M-MLV. The λ·MLV$_{int}$-1 contains one copy of the LTR at its 5' end. Digestion with restriction endonucleases *Pvu*II and *Kpn*I showed the presence of a 280-bp fragment, expected from the LTR (Fig. 4B, lane 10). Thus, it appears that as do avian sarcoma viruses (11, 23) integrated M-MLV contains a copy of the LTR at its 5' end. Figure 4A to C shows the characterization of λ·MLV$_{int}$-1 by utilizing several restriction endonucleases and different probes; Fig. 4B shows an autoradiograph obtained with M-MLV cDNA as a probe, whereas in Fig. 4C, a plasmid probe containing the insert of λ·MLV$_{int}$-1 was used. Therefore, the fragments seen in Fig. 4C but absent from Fig. 4B contain exclusively mouse sequences, whereas fragments seen at a relative higher intensity in Fig. 4C as compared with Fig. 4B comprise both mouse and viral sequences. A detailed physical map of λ·MLV$_{int}$-1 is shown in Fig. 4D.

Infectivity. The λ·MLV-1 recombinant clone derived from unintegrated closed circular DNA was not infectious in any of the following forms: (i) λ·MLV-1 DNA, (ii) λ·MLV-1 DNA cleaved with *Hin*dIII, (iii) λ·MLV-1 DNA plus *Hin*dIII and the insert of 8.9 kb isolated on agarose gels (Fig. 5, lane A), and (iv) isolated insert ligated end-to-end by T4 DNA ligase (Fig. 5, lane B). None of these forms of DNA were infectious as assayed by the XC plaque assay (27). The λ·MLV$_{int}$-1 lacked the 3' half of the viral genomic information and thus remained uninfectious. However, when λ·MLV$_{int}$-1 was supplemented with viral DNA representing the 3' half of the genome, it was found to be infectious. Figure 6 shows a diagrammatic sketch of how the infectious DNA molecule was constructed. The λ·

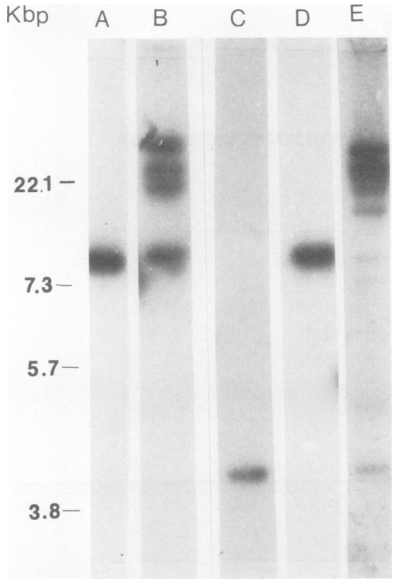

FIG. 5. *Size analysis of insert DNAs used for transfection. The lanes shown are autoradiographs of unligated and ligated fragments after agarose gel electrophoresis, followed by Southern blotting transfer and hybridization to M-MLV-1 cDNA. (A) Isolated insert from λ·MLV-1 DNA; (B) same insert as visualized in (A) but after ligation; (C) isolated HindIII to PstI fragment derived from λ·MLV-1 DNA; (D) isolated insert derived from λ·MLV$_{int}$-1 DNA; and (E) HindIII to PstI fragment (C) plus the isolated insert shown in (D) after ligation.*

MLV-1 DNA was cleaved with restriction endonucleases *Pst*I and *Sal*I. The *Hin*dIII to *Pst*I fragment of 4.1-kb size contained the 3′-half viral genomic sequence and about 0.51-kb sequences from the 5′ end of the genomic RNA (from *Pst*I to *Kpn*I as in Fig. 1A). The 4.1-kb *Hin*dIII to *Pst*I fragment (Fig. 5, lane C) treated with bacterial alkaline phosphatase and the 8.9-kb λ·MLV$_{int}$-1 insert (Fig. 5, lane D) were ligated with T4 DNA ligase (Fig. 5, lane E). In another experiment, we digested the λ·MLV-1 DNA with *Hin*dIII and *Xho*I (Fig. 1A and 6). The 5.1-kb *Hin*dIII to *Xho*I fragment was ligated to the 8.9-kb insert of λ·MLV$_{int}$-1. Table 1 shows the specific infectivity of the in vitro-constructed infectious DNA molecules. Unligated DNA fragments were not infectious. Since M-MLV is NB tropic, we wanted to determine the tropism of the virus released by cells infected with in vitro-constructed DNA molecules. Table 2 shows that, like the parental virus, the virus released from cells infected with reconstructed DNA was NB tropic.

DISCUSSION

An unintegrated circular form and a portion of an integrated form of M-MLV DNA were cloned in lambda phage Charon 21A. Since M-MLV DNA has a single *Hin*dIII site (8, 31), unintegrated closed circular M-MLV DNA was linearized by cleaving with restriction endonuclease *Hin*dIII and ligated into the unique *Hin*dIII site of Charon 21A phage DNA (3). An integrated form of M-MLV DNA was obtained from the BALB/Mo mouse strain (12), which contains M-MLV as an endogenous virus. This endogenous virus is present in a single Mendelian locus (*Mov-1*) which has been mapped genetically on chromosome no. 6 (5), and M-MLV is expressed in hematopoietic tissues from early life on (12, 13). The expression of the endogenous virus leads to the development of a lymphatic leukemia later in life (13), accompanied by the amplification of M-MLV sequences in the tumor tissues. Restriction endonuclease cleavage of BALB/Mo mouse DNA from nontarget tissues such as brain and liver, followed by hybridization with M-MLV-specific probes, has indicated that the genetically transmitted M-MLV genome can be detected in a single 27-kb *Eco*RI fragment (27). This *Eco*RI fragment was isolated and cleaved with *Hin*dIII, generating two M-MLV-containing fragments of 8.9 and 3.9 kb. The 8.9-kb *Hin*dIII fragment was cloned into the *Hin*dIII site of Charon 21A. The relative inefficiency of the Charon 21A cloning vehicle in accommodating DNA fragments larger than 6 to 7 kb in a unique restriction site may be responsible for the identification of a single M-MLV-reacting plaque obtained from either unintegrated or integrated M-MLV DNA. The physical map of the λ·MLV-1 was comparable to the published maps (8, 31) except that only a single 600-bp LTR was present and one *Pvu*II site was missing. The exact positions of the various restriction sites (8) on the viral genome have been mapped more precisely. λ·MLV-1 lacks one of the two *Pvu*II sites in the LTR (Fig. 1A). The nucleotide sequences of the 5′ LTR from λ·MLV$_{int}$-1 (26b) and of the 3′ LTR from a cloned M-MLV cDNA transcript (26a) have been determined. In both cases only one *Pvu*II site could be detected in the LTR (Fig. 1A). A comparison of the LTR sequences from M-MLV with that of murine sarcoma virus (R. Dhar, W. C. McClements, L. Enquist, and G. F. van de Woude, Proc. Natl. Acad. Sci. U.S.A., in press) reveals a direct duplication of 73 nucleotides in the latter case. The direct duplication contains the additional *Pvu*II site. The absence of the direct repeat of 73 bp from the LTR of λ·MLV$_{int}$-1 or λ·MLV-1 does not appear to be essential for infectivity, as the in vitro recombinant DNA is able to produce infectious virus (Table 1). It has been reported that molecularly cloned retroviral

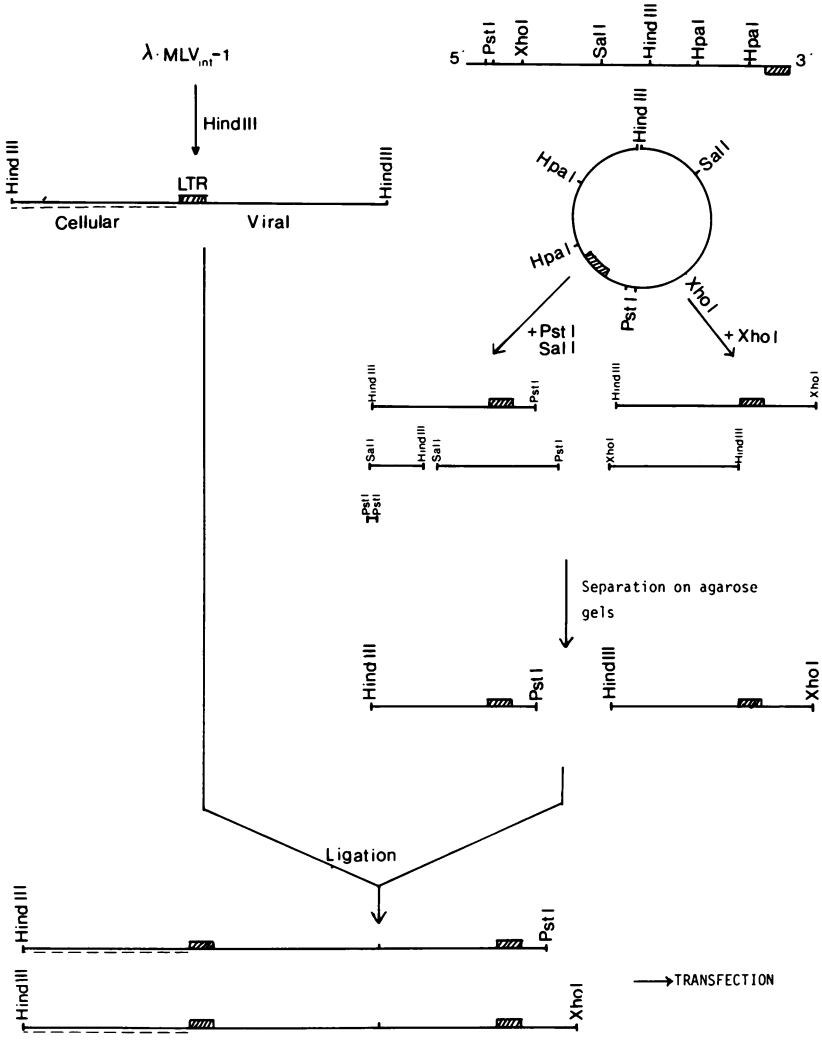

FIG. 6. *Flow sheet showing the in vitro construction of infectious DNA molecules.*

TABLE 1. *Specific activity of in vitro-constructed infectious DNA molecules*

Nature of DNA	Amt of DNA used (μg)	No. of XC plaques (avg of two plates)	XC PFU/μg of DNA
λ·MLV$_{int}$-1 (8.9 kb)	0.24	0	
λ·MLV$_{int}$-1 (ligated)	0.21	0	
*Hin*dIII to *Pst*I (4.1 kb)	0.18	0	
*Hin*dIII to *Pst*I (ligated)	0.18	0	
*Hin*dIII to *Xho*I (5.1 kb)	0.50	0	
*Hin*dIII to *Xho*I (ligated)	0.60	0	
λ·MLV$_{int}$-1 (8.9 kb) plus *Hin*dIII to *Pst*I (4.1 kb)	0.02	2	100
	0.06	7	116
	0.18	26	144
λ·MLV$_{int}$-1 (8.9 kb) plus *Hin*dIII to *Xho*I (5.1 kb)	1.5	300	150

DNAs can lose one or two LTRs, presumably by homologous recombination (10, 14, 28). Similarly, it is possible that λ·MLV-1 has lost one LTR during molecular cloning.

The insert DNA was not infectious when transfected in any of the following forms: (i) λ·M-MLV DNA, (ii) λ·M-MLV DNA cleaved with *Hin*dIII, (iii) λ·M-MLV DNA cleaved with *Hin*dIII and the insert isolated on agarose gels, and (iv) isolated insert, end-to-end ligated by T4 DNA ligase (Fig. 5, lane B). This result suggests either that a mutation has occurred somewhere in the 5′ two-thirds of the viral genome or that the presence of two LTRs is essential for infection. However, when λ·MLV-1 was annealed to M-MLV genomic RNA and analyzed by S1 mapping, only a single band of an average size of 8.5 kb could be detected on alkaline agarose gels (I. M. Verma, unpublished data). Thus, it appears

TABLE 2. *Tropism of the virus released from cells transfected with in vitro-constructed infectious DNA*[a]

Virus	XC plaque-forming titers (PFU/ml)		
	NIH 3T3 (*Fv-1*^{n})	BALB/3T3 (*Fv-1*^{b})	F 2408
In vitro recombinant	3.1×10^5	0.8×10^5	2.9×10^2
M-MLV	5.8×10^5	1.8×10^6	10^3

[a] After two transfers of NIH 3T3 cells transfected with in vitro-reconstructed DNA molecules (0.18 μg of DNA, Table 1), 24-h tissue culture fluid was assayed on NIH 3T3 cells (N-tropic), BALB/3T3 cells (B-tropic), and F 2408 (rat) cells. For a comparison, 24-h tissue culture fluid from mouse fibroblasts chronically infected with M-MLV was also assayed in parallel.

that λ·MLV-1 has no major deletion. A similar phenomenon is observed with cloned Moloney murine sarcoma virus DNA; only insert DNAs containing two copies of the LTR gave rise to focus formation (30). In contrast, Oliff et al. (21) have recently shown that molecularly cloned Friend MLV containing one copy of the LTR is able to infect cells and produce Friend MLV. The reason for this discrepancy remains obscure for the moment. The clone containing the insert derived from the integrated M-MLV DNA has been characterized by restriction endonuclease mapping (Fig. 4). The viral portion of the insert was comparable by restriction endonuclease mapping from in vitro- and in vivo-synthesized M-MLV viral DNAs (8, 31), except for the absence of one *Pvu*II site from the 5′ LTR. The restriction fragments characteristic for the *Mov-1* integration site (fragments containing both viral and cellular sequences) could also be generated from this clone, with the exception of some fragments which were generated by enzymes sensitive for DNA methylation. Furthermore, a ^{32}P-labeled fragment (*Xho*I to *Hpa*I) derived from the cellular part of this clone recognized specifically a 27-kb *Eco*RI fragment in DNA from BALB/Mo mice, whereas an 18-kb fragment was detected in DNA from the BALB/c and 129 mice, which were the parental strains of the BALB/Mo mouse strain (12). The observed difference in size of 9 kb between BALB/Mo and its parental strain (Fig. 3) is in agreement with the insertion of a single M-MLV genome and suggests that no major deletions have occurred in the cellular DNA during the integration process. Analysis with a variety of restriction enzymes did not allow us to determine whether the germ line integration of M-MLV took place into the BALB/c or 129 genome, since the same restriction fragments were generated from both strains. A more detailed analysis will be necessary to clarify this point.

To study the biological activity of λ·MLV$_{int}$-1 DNA, the insert was ligated to the 4.1-kb *Hin*dIII to *Pst*I fragment or the 5.1-kb *Hin*dIII to *Xho*I fragment from the λ·MLV-1 cloned DNA. These fragments contained the 3′-end genomic sequences missing from λ·MLV$_{int}$-1 DNA. These reconstructed genomes (Fig. 6) were infectious, indicating that no defect has been introduced in the mouse-derived clone during the cloning procedure. The same conclusion can be drawn for the 3′ part of λ·MLV-1 DNA. The specific infectivity of the reconstructed molecules was similar to that observed for in vitro-synthesized genome-length DNA (16) but much lower than that reported for unintegrated M-MLV (25) or integrated, molecularly cloned AKR MLV DNA (18). It is, however, difficult to get an exact estimate as the ligated molecules of the right length were not individually isolated. It is interesting to note that a molecular clone derived from an integrated viral genome, which is not expressed in the tissue from which the DNA was isolated (mouse liver), is infectious. Therefore, we will focus our attention on the regulatory functions, specified by the cellular sequences present in this clone, as well as on the extent of base modification present in the integrated M-MLV, which is expected to be related to the expression of the M-MLV genome specified by the *Mov-1* locus (6).

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