Rauscher Murine Leukemia Virus: Molecular Cloning of Infectious Integrated Proviral DNA

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The integrated proviral genome of Rauscher murine leukemia virus was molecularly cloned in a bacteriophage Charon 4A vector after the proviral sequences were enriched by sequential RPC-5 column chromatography and sucrose gradient centrifugation. A recombinant DNA clone, λ-RV-1, possessing a 12-kilobase-pair EcoRI insert, was shown to contain the entire 8.8-kilobase-pair leukemia virus genome flanked by rat cellular sequences at the 5' and 3' ends. This DNA fragment was biologically active, inducing the release of virion-associated reverse transcriptase activity with as little as 10 ng of DNA insert. The virus induced XC plaque formation at high titers on NIH/3T3 and BALB/3T3 cells and demonstrated identity with the parental virus in radioimmunoassays for the highly type-specific gag gene-coded p12 protein. The molecularly cloned Rauscher murine leukemia virus should be useful in studying the molecular mechanisms involved in the transformation of specific lymphoid target cells by chronic mouse leukemia viruses.

Rauscher murine leukemia virus (R-MuLV) and Moloney murine leukemia virus (M-MuLV) belong to a class of replication-competent retroviruses which induce leukemias in newborn mice after prolonged periods of latency and replicate in tissue culture cells without causing transformation. Recently, it has been possible to show that different mouse leukemia viruses show a high degree of specificity in their target cells for transformation. R-MuLV and M-MuLV induce tumors of the B and T lymphoid cells, respectively (16). The advent of recombinant DNA and nucleotide sequencing techniques has made it possible to study retroviral genomes in a precise biochemical manner. The M-MuLV genome has been molecularly cloned as biologically active DNA (2). In studies aimed at defining at a molecular level the mechanisms responsible for the different target cell specificities of R-MuLV and M-MuLV, we reasoned that it would be important to obtain molecular clones of R-MuLV. In the present report, we describe the isolation, amplification by recombinant DNA techniques, and initial biological characterization of the integrated form of this virus.

Restriction analysis of unintegrated R-MuLV DNA. Molecular cloning of the R-MuLV genome required knowledge of which restriction enzymes cleaved within the viral genome. Linear unintegrated R-MuLV DNA isolated from the Hirt supernatant (11) of newly infected cells was used for this analysis. Restriction enzymes which did not cleave the 9.0-kilobase-pair (kbp)

viral DNA included EcoRI, SalI, and XhoI (Fig. 1). In contrast, HindIII, BamHI, and SstI all cut the genome at least twice. The lack of a suitable one-cut enzyme precluded cloning the full-length molecule in its unintegrated form. However, the availability of several enzymes which did not cleave the viral DNA made it feasible to attempt to clone the integrated form of the virus.

Molecular cloning of the integrated R-MuLV genome. Mouse cells, the permissive host for propagation of R-MuLV, contain multiple copies of endogenous mouse type C viruses. These sequences cross-react with those of R-MuLV, making it difficult to detect the exogenous viral genome. Thus, to clone the integrated infectious form of the viral genome we utilized NRK cells, whose endogenous viruses do not cross-react under conditions of stringent hybridization with those of R-MuLV (A. Habara, unpublished data). Because integration of type C viruses occurs at multiple sites within the host cell genome (1, 4, 22), we infected NRK cells at a multiplicity of infection of about 0.1 and transferred single cells within 24 h to microtiter plates. We reasoned that the onset of virus replication by single cells in microtiter would be likely to lead to interference with subsequent infection. By this approach, we hoped to obtain clonal cell lines in which virus integration occurred at a single or relatively few sites.

When high-molecular-weight cellular DNA obtained from a singly infected NRK clone was cleaved with EcoRI, we detected two fragments
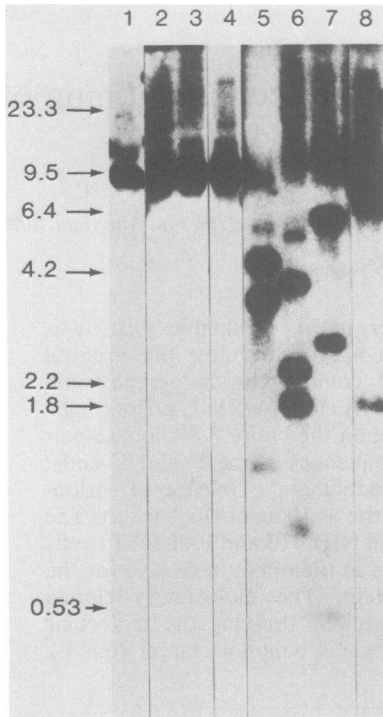
FIG. 1. Restriction endonuclease analysis of unintegrated R-MuLV DNA. Linear unintegrated R-MuLV DNA isolated from Hirt supernatants of R-MuLV-infected NIH/3T3 cells was digested with various restriction enzymes and electrophoresed through a 1.0% agarose gel, transferred to diazobenzyloxymethyl paper (28), and hybridized with ^{32}P-labeled R-MuLV cDNA probe. λ DNA cleaved with HindIII served as a molecular weight marker. Lanes: 1, untreated DNA; lanes 2 through 8, R-MuLV DNA after cleavage with EcoRI (lane 2), XhoI (lane 3), SalI (lane 4), HindIII (lane 5), BamHI (lane 6), SacI (lane 7), or PvuI (lane 8).

of 12 and 15 kbp by using R-MuLV cDNA as a probe. The EcoRI-digested DNA was enriched for these fragments by RPC-5 column chromatography (27) followed by sucrose density gradient fractionation. The enriched DNA fragments were ligated with the purified arms of phage Charon 4A and packaged in vitro (25). From pooled fractions containing the 12-kbp fragment, we obtained a single positive clone which reacted with ^{32}P-labeled cDNA by screening approximately 2 × 10^4 plaques (3). With the larger fragment, screening a similar number of plaques failed to reveal a positive clone.

Restriction endonuclease mapping of λ-RV-1 DNA. To identify the R-MuLV genome within λ-RV-1 DNA, we performed restriction endonuclease mapping (Fig. 2). This map was constructed by analyzing the total insert as well as

the PstI subclones of the molecule. Some enzymes, including EcoRI and XhoI, did not cleave within the molecule, whereas PvuI, PvuII, ClaI, and SalI cleaved the molecule once. The rest of the restriction enzymes tested had multiple cleavage sites. These enzymes included PstI, HindIII, XbaI, BamHI, BstEII, BglI, BglII, SacI, KpnI, SmaI, and AvaI.

To establish biochemically that our cloned molecule contained the R-MuLV proviral genome, we compared the restriction digests of the cloned molecule with those of unintegrated linear R-MuLV DNA isolated by the Hirt procedure (data not shown). The R-MuLV-specific fragments were identified by hybridization with R-MuLV-specific cDNA. When restriction enzymes which cleaved at multiple sites were used in this analysis, several internal fragments of the cloned DNA showed the same mobility as those generated with linear, unintegrated R-MuLV DNA. The restriction fragments from the cloned DNA that differed in mobility but hybridized with the R-MuLV cDNA probe were presumed to be located at the termini of the proviral DNA and to contain cellular flanking sequences as well.

Long terminal repeats (LTRs) have been shown to be a common structural feature of the proviral DNAs of avian and mammalian retroviruses (12, 19). An examination of the restriction map revealed the occurrence of a constellation of restriction sites, KpnI, SacI, SmaI, and AvaI, in two regions approximately 8 kbp apart and localized at positions 2.5 to 3.1 kbp and 10.7 to 11.3 kbp on the physical map of the molecule. It seemed likely that these two respective 0.6-kbp domains resided within the viral LTRs. AvaI and HindIII sites were present at either end but outside of these domains. These sites presumably occurred in flanking host cellular sequences, thus defining the outer limits of the two LTRs (Fig. 2).

Orientation of integrated R-MuLV DNA with respect to genomic viral RNA. The gene order of type C retroviruses is known to be 5'-gag pol env-3' (for a review, see reference 29). It is also known that R-MuLV and M-MuLV have a similar genomic structure and code for related structural components. Comparison of the restriction maps of each revealed certain common sites which helped us to orient R-MuLV sequences within the 12-kbp DNA fragment. Thus, a PvuI site, which occurred 0.9 kbp from the 5' terminus of M-MuLV DNA, and two PstI sites, localized 1.0 and 1.15 kbp from the 5' terminus, were found in an identical configuration within the 12.0-kbp R-MuLV DNA fragment, 3.35, 3.45, and 3.6 kbp, respectively, from one end. These results oriented this end as containing the 5' terminus of the R-MuLV genome. Similarly,
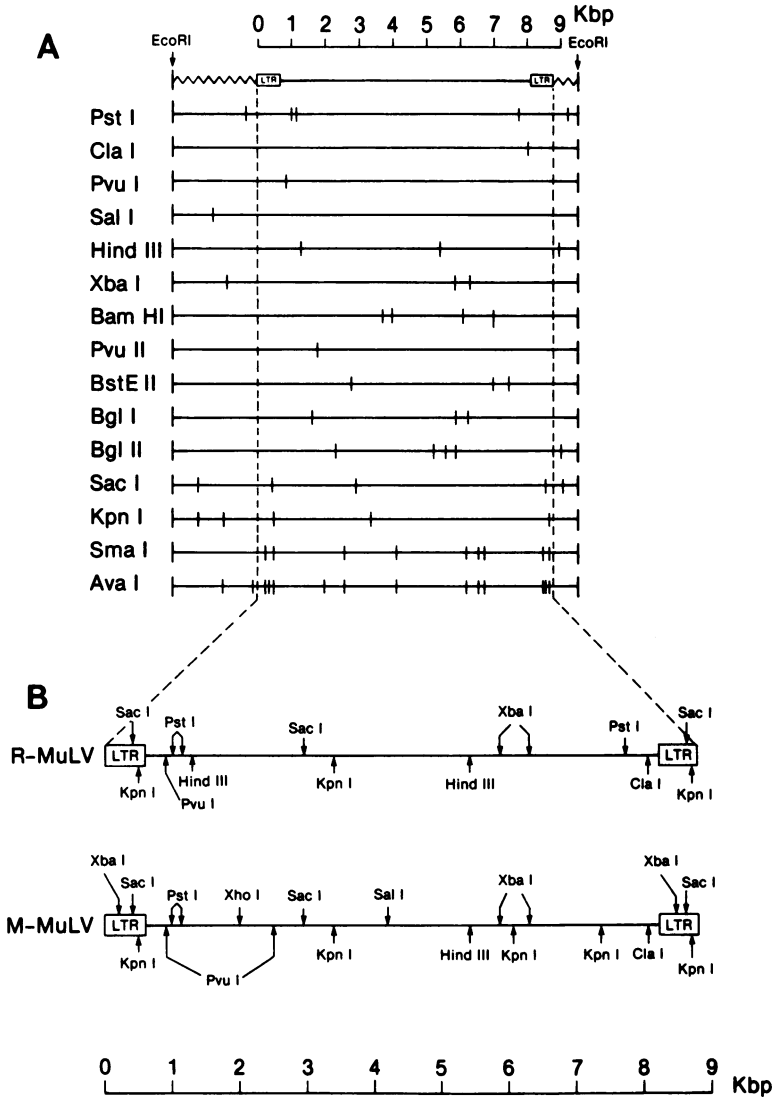
FIG. 2. (A) Restriction endonuclease map of R-MuLV proviral DNA derived from analysis of the 12-kbp λ-RV-1 DNA insert. ——, 8.8-kbp-long R-MuLV genome; open rectangle, LTRs. Flanking host cellular sequences are represented by wavy lines. (B) Comparison of common restriction endonuclease sites on the restriction maps of M-MuLV (7) and R-MuLV DNAs.

at its 3′ end the M-MuLV proviral genome contained a ClaI restriction site which occurred at a position 8.05 kbp from the 5′ terminus. A unique ClaI site was found in the cloned 12-kbp molecule in an identical configuration at a position 10.5 kbp from the 5′ end of the molecule. These results helped to confirm the orientation of this end as the 3′ terminus of the R-MuLV genome.

Biological activity of cloned R-MuLV DNA. To determine whether λ-RV-1 DNA was biologically active, we analyzed it by transfection on NIH/

3T3 cells. As little as 10 ng of DNA insert was able to induce the release of particle-associated reverse transcriptase activity (Table 1). There was no significant difference in infectivity whether or not the insert had been cleaved from its vector with EcoRI.

R-MuLV is an ecotropic virus which is infectious for NIH/Swiss and BALB/c mouse cells and induces XC plaque formation in both cell lines (18, 23). Thus, we examined the host range of a virus grown in cells transfected with the R-MuLV DNA clone. This virus, designated RV-1,

TABLE 1. Biological activity of λ-RV-1 DNA[a]

Recombinant DNA	EcoRI cleaved	Polymerase release (pmoles/ml) with DNA insert (ng) of:				
		1,000	100	10	1	0.0
λ-RV-1	No	>200	50	10, 50	<0.1	<0.1
	Yes	>200	100	15, 25	<0.1	<0.1

[a] NIH/3T3 cell cultures were transfected with serial dilutions of λ-RV-1 DNA as described previously (5, 8). At 4 weeks, tissue culture fluids were assayed for virion-associated reverse transcriptase activity as previously described (23). The results are expressed as picomoles of thymidine triphosphate incorporated per milliliter of tissue culture fluid.

induced XC plaque formation in both NIH/3T3 and BALB/3T3 cells at equivalent titers of 2 × 10^5 to 5 × 10^5 XC PFU/ml. In contrast, there was no detectable transmission of virus to cells from a variety of species susceptible to other host range variants of MuLV, including xenotropic, amphotropic, and dualtropic MuLVs (data not shown). These findings established that the tissue culture characteristics of the RV-1 virus were indistinguishable from those of R-MuLV.

Type-specific antigenic determinants have been readily demonstrated in the products coded for by the *gag*, *pol*, and *env* genes of mouse type C viruses (10, 24). To further characterize the
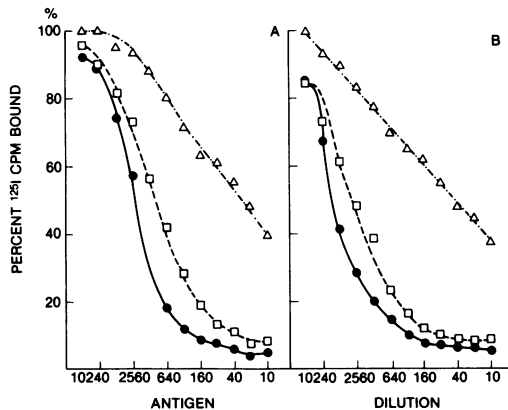

FIG. 3. Immunological typing of the p12 proteins of (A) RV-1 virus and (B) parental R-MuLV. Detergent-disrupted viruses were assayed at twofold serial dilutions for their ability to compete with ^{125}I-labeled p12 proteins for limiting antibody as described previously (10). The results are expressed as the percent of the total ^{125}I counts per minute in the antigen-antibody precipitate standardized to results in the absence of competing antigen. Reactivity in the anti-R-MuLV:^{125}I-labeled R-MuLV p12 type-specific immunoassay (●); anti-M-MuLV:^{125}I-labeled M-MuLV p12 type-specific immunoassay (△); and anti-BALB:virus-2:^{125}I-labeled R-MuLV p12 group-specific immunoassay (□).

virus, we performed typing radioimmunoassays for R-MuLV *gag* gene-coded p12. The RV-1 virus, like the parental R-MuLV, completely displaced labeled R-MuLV p12 in binding limiting anti-R-MuLV p12 serum (Fig. 3). In contrast, both viruses demonstrated only partial competition in a typing immunoassay for M-MuLV p12 (Fig. 3). Other viruses, including ecotropic AKR and M-MuLV and xenotropic BALB:virus-2 and NIH-MuLV, demonstrated only limited competition in the R-MuLV p12 assay (data not shown). These results established that the p12 of RV-1 virus is immunologically indistinguishable from that of R-MuLV.

Knowledge of the molecular structure of leukemia viruses has led to insights on how these viruses cause tumors. The viral LTR contains signals for RNA transcription and for RNA capping and adenylation (6, 13, 17, 20, 26). Several laboratories have recently reported that integration of avian leukosis viruses near the cellular analog of an avian retrovirus *onc* gene, *myc*, leads to its transcriptional activation (9, 14, 15). By this model, the random low-frequency integration of a retrovirus LTR near a cellular gene with transforming potential might lead to its activation as a transforming gene. Whether this model can be generalized to other systems or fully explains tumor induction by avian leukemia viruses awaits further analysis.

As one approach toward investigating the mechanisms by which leukemia viruses induce tumors, we compared the target cells for transformation by different mouse leukemia viruses. These studies revealed evidence for a rather high degree of specificity. Thus, R-MuLV transforms a specific subset of cells within the B lymphoid differentiation pathway, whereas M-MuLV transforms cells predominately with the T lymphoid cell series (16). As determined by heteroduplex analysis under stringent hybridization conditions, their genomes are colinear (S. G. Devare, unpublished data). Restriction mapping provides another means of measuring relationships between respective genes. Comparison of the maps of R-MuLV and M-MuLV indicated no long stretch of extremely well conserved sequences (Fig. 3). Nonetheless, two enzymes, *Pvu*I and *Cla*I, cleaved toward the 5' and 3' ends, respectively, of each viral genome at approximately the same positions (Fig. 3). Preliminary comparison of the nucleotide sequences at these sites revealed marked similarities as well (A. Habara, unpublished data). Thus, it may be possible to utilize these sites in the construction of in vitro recombinants in efforts to localize the regions of each viral genome that are responsible for its target cell specificity. At the same time, such studies may shed additional light on the mechanisms by

which these leukemia viruses transform lymphoid cells.

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