Identification of a Novel Gene, *Vin-1*, in Murine Leukemia Virus-Induced T-Cell Leukemias by Provirus Insertional Mutagenesis

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The BL/VL3 radiation leukemia virus is a nondefective retrovirus which induces clonal or oligoclonal T-cell leukemia in mice. To study the role of provirus insertional mutagenesis in the development of these neoplasias, we searched for common provirus integration sites in BL/VL3 radiation leukemia virus-induced tumors. Using cellular sequences flanking a provirus cloned from one of these thymomas, we found that the viral genome was integrated into a common region, designated *Vin-1*, in a low percentage (5%) of these tumors. The proviruses found in this locus were integrated in the same orientation, close to a CpG-rich island, at proximity of a transcriptional unit encoding a 6-kb RNA. *Vin-1* RNA was detected in several organs of the adult mouse. *Vin-1* RNA levels were high in tumors having a provirus inserted within the *Vin-1* region but were also high in some other tumors whose *Vin-1* region was not found to be rearranged. *Vin-1* was found to be well conserved among mammalian species and was mapped to mouse chromosome 6, between *raf* and *K-ras-2*. *Vin-1* appears to be a novel gene which may be involved in tumor development.

The BL/VL3 radiation leukemia virus (RadLV) mixture is a highly leukemogenic, thymotropic murine leukemia virus (MuLV) (4, 14, 20, 22) initially recovered from a lymphoid cell line (BL/VL3) established from a thymoma (T-cell leukemia) of a C57BL/6 mouse (21). This thymoma was itself induced by a crude virus mixture initially derived from an X-ray-induced thymoma and passaged several times in mice (13, 14, 21). From BL/VL3 cells, we have previously reported the molecular cloning of several variants of ecotropic retroviruses responsible for the high leukemogenic potential of BL/VL3 RadLV stocks (30). As the parental BL/VL3 RadLVs, these cloned BL/VL3 RadLVs are thymotropic and rapidly induce T-cell leukemia when inoculated into susceptible mouse strains (30).

The mechanism by which these nondefective BL/VL3 RadLVs induce thymomas has not yet been investigated. However, for many such nondefective MuLVs, activation of cellular proto-oncogenes via provirus integration constitutes an important step in the tumorigenic process (for reviews, see references 18 and 26). As insertional mutagens, these proviruses, when inserted within or around transcribed regions, can activate gene expression, decrease transcription, or alter gene structure, thus conferring a growth advantage upon targeted cells. In rodents, *c-myc*, *Ha-ras*, *tpl-1/ets-1*, *lck*, *Mis-1/pvt-1/Mlvi-1*, *Gin-1*, *Pim-1*, and *Mlvi-4* genes as well *Dsi-1*, *Pim-2*, *Mlvi-2*, and *Mlvi-3* regions have all been identified as common MuLV provirus integration sites (26), and their rearrangements were found to correlate with the development of T-cell leukemia.

We have studied whether BL/VL3 RadLV induces T-cell lymphomas by insertion of its proviruses into particular chromosomal regions. To test this hypothesis, we looked for common provirus integration sites in thymomas induced by this retrovirus. Our analysis has identified a region of the mouse chromosome 6 which is rearranged at a low frequency in RadLV- and Moloney MuLV-induced-T-cell leukemias.

Moreover, this region is well conserved among mammalian species and harbors a gene encoding a 6-kb transcript.

MATERIALS AND METHODS

Animals and viruses. Mice were from our breeding colony. BL/VL3 (clone V-13) RadLV has been described previously (30). Newborn (<48-h-old) C57BL/Ka or SIM.R mice were inoculated intraperitoneally with 0.15 ml of filtered (pore size, 0.45 µm; Millipore Corp., Bedford, Mass.) tissue culture medium containing BL/VL3 (clone V-13) RadLV, a molecularly cloned derivative of BL/VL3 RadLV. Thymomas developed between 2 to 8 months postinoculation. Mice were sacrificed when they were moribund. Tumors induced in Fisher 344 and Lewis rats by Moloney MuLV have been described previously (19, 37).

DNA extraction and restriction endonuclease digestion. High-molecular-weight tumor DNAs were prepared from primary thymomas as previously described (27). They were digested with various restriction endonucleases (New England Biolabs, Inc., Beverly, Mass.; Boehringer Mannheim Biochemicals, Montreal, Quebec, Canada; P-L Pharmacia, Montreal, Quebec, Canada) under conditions recommended by the manufacturers as previously described (19, 24). Bacteriophage and plasmid DNAs were prepared essentially as described elsewhere (24).

Agarose gel electrophoresis and hybridization procedure. Digested DNA samples were electrophoresed on 1% agarose gels and transferred onto nitrocellulose or nylon membranes (Amersham Co., Montreal, Quebec, Canada) by the Southern technique (34) as described previously (19, 24, 27). Specific DNA fragments were detected by hybridization with ³²P-labeled cloned DNA fragments. These cloned DNA fragments were excised from vectors and separated by agarose gel electrophoresis. DNA fragments were isolated by electroelution onto NA45 affinity paper (Schleicher & Schuell), phenol-chloroform extracted, and precipitated with ethanol as described previously (27). Probes were labeled by the random priming method with the Klenow fragment of

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DNA polymerase I in the presence of hexamers (7). Nitrocellulose filters were hybridized in 50% formamide-3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-Denhardt solution at 42°C. Nylon membranes were hybridized in 4× SET (1× SET is 0.15 M NaCl, 30 mM Tris, and 2 mM EDTA, pH 7.4) with 0.1% sodium pyrophosphate, 0.2% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 0.5 mg of heparin per ml at 65°C for 16 h. After hybridization, all membranes were washed in 2× SSC for 15 min at room temperature, in 0.1× SSC-0.5% SDS for 1 h at 65°C, and three times for 2 min each in 0.1× SSC at room temperature. Membranes were air dried and exposed on RP-Royal X-Omat film (Eastman Kodak Co., Rochester, N.Y.) with a Cronex Lightning-Plus intensifying screen (DuPont Co., Wilmington, Del.) at -70°C.

Molecular cloning procedure. DNA from the BL/VL3 RadLV-induced tumor 133-1 (80 µg) was digested with *EcoRI* and centrifuged on a sucrose density gradient (10 to 40%) as previously described (19, 28, 36). Fractions of 0.5 ml were collected and ethanol precipitated. Aliquots of DNA from each fraction were separated by agarose gel electrophoresis, transferred onto a nitrocellulose membrane, and hybridized with a BL/VL3 RadLV U3 long terminal repeat (LTR)-specific probe (30). Fractions containing viral fragments from 5 to 20 kbp were pooled and ligated with *EcoRI*-cleaved Charon 4A arms (1 µg). DNA from another fraction, containing the 3.4-kbp fragment hybridizing to the U3 LTR probe, was ligated to *EcoRI*-cleaved lambda gt10 arms (1 µg). The ligated DNAs were packaged as described elsewhere (19, 24). Recombinant phages harboring RadLV sequences were identified with the ³²P-labeled BL/VL3 RadLV-U3 LTR-specific probe.

Phages were eluted, and their DNA was extracted on a double 5 and 40% glycerol cushion. Inserts were subcloned into plasmid vectors as described elsewhere (24, 28, 36).

Probes. The 265-bp BL/VL3 RadLV U3 LTR-specific probe was excised from the cloned BL/VL3 V13 RadLV genome (30) by *PstI*-*ScaI* digestion, treated with Klenow enzyme, ligated to *HindIII* linkers, and subcloned into the pEMBL8 vector. This probe specifically detects RadLV proviruses. The ecotropic MuLV-specific AKV-5 probe has previously been isolated from the AKV virus and corresponds to the amino-terminal region of the p15E protein (12). The Moloney U3 LTR probe consists of a 0.3-kbp *PstI*-*SacI* fragment from a mutated Moloney MuLV LTR (27, 28, 31).

RNA expression analysis. RNA was isolated from various tissues by the method of Chomczynski and Sacchi (2), separated on 1% formaldehyde-agarose gels, transferred onto nylon membranes, and hybridized as described above. Washings were performed for 15 min at room temperature in 2× SSC, for 1 h at 65°C in 0.5× SSC-0.5% SDS, and in 0.5× SSC three times for 2 min each at room temperature.

Somatic cell hybrids. Isolation of the mouse-hamster cell hybrids has been described previously (15-17). Mouse chromosomes were identified directly by Giemsa-trypsin banding followed by staining with Hoechst 33258 (Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J.). Hybrid cells were typed for the presence of specific mouse markers as described previously. In this study, 26 hybrids were selected from a larger panel of 76 hybrids for analysis.

Genetic crosses. AKR/J and C58/J mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. NFS/N mice were obtained from the Division of Natural Resources, National Institutes of Health, Bethesda, Md. Wild-derived *Mus musculus musculus* (Skive) were provided by M. Potter (National Cancer Institute) from his colony at Hazelton

Laboratories, Rockville, Md. (contract N01-CB-71085). *M. musculus musculus* (Skive) males were mated with C58/J, NFS/N, or AKR/J females, and the F₁ females were mated with *M. musculus musculus* males to produce first-backcross mice. For the second cross, NFS/N females were mated with *M. spretus* males (Hazelton Laboratories) and the F₁ females were mated with C57BL/6 males.

DNA was extracted from the livers of inbred mice, *M. musculus musculus*, and the backcross progeny. DNAs were digested with restriction enzymes, electrophoresed on 0.4% agarose gels, and transferred to nylon membranes (Hybond N+; Amersham) as described above. Hybridization and wash conditions were as described above. The genetic marker *K-ras-2* was typed by using a 618-bp segment of *v-Ki-ras*, the oncogene contained in Kirsten murine sarcoma virus. *raf* was typed by using a 290-bp segment of the *v-raf* oncogene of the 3611 murine sarcoma virus. Both probes were obtained from Oncor (Gaithersburg, Md.).

RESULTS

Cloning of virus-cell junction fragments of BL/VL3 RadLV-induced thymoma 133-1. The molecularly cloned BL/VL3 RadLV is a leukemogenic retrovirus which induces T-cell leukemia (thymoma) in a high percentage (80 to 100%) of inoculated C57BL/6 or SIM.R mice after a relatively short latent period (2 to 8 months) (30). To analyze the structure of the BL/VL3 proviruses in these tumors, BL/VL3 RadLV-induced T-cell lymphoma DNAs were analyzed by restriction endonucleases with a BL/VL3 RadLV U3 LTR and the AKV-5 ecotropic MuLV-specific probes.

EcoRI was most suitable for this analysis because it does not cleave the BL/VL3 RadLV genome. However, it is likely to cleave recombinant viral genomes (derived from BL/VL3 RadLV) such as mink cell focus-forming (MCF) viruses, which typically harbor an *EcoRI* site at 6.9 kbp (6). Therefore, this enzyme allows the detection of complete newly acquired ecotropic BL/VL3 RadLV proviruses flanked by cellular sequences and of a portion of recombinant BL/VL3 RadLV proviruses (cleaved by *EcoRI*), also flanked by cellular sequences.

All thymomas tested, induced by BL/VL3 RadLV either in C57BL/6 (9 of 9) or in SIM.R (22 of 22) mice, showed the presence of multiple newly acquired retroviral sequences ranging from 2 to 20 kbp and detected by hybridization with the BL/VL3 RadLV U3 LTR-specific probe (Fig. 1). The presence of discrete bands in all tumors indicated that these T-cell lymphomas were monoclonal or oligoclonal in origin. In addition, the presence of subgenomic-length fragments detected in all tested tumors with the AKV-5 (data not shown) or the BL/VL3 U3 LTR-specific probe suggested the presence of recombinant proviruses of the MCF type. The AKV-5 probe has previously been shown to detect MCF recombinant viruses in AKR and in Gross passage A MuLV-induced thymomas (12, 36). The BL/VL3 U3 LTR-specific probe is also likely to hybridize to some MCF viruses arising from the recombination of the inoculated ecotropic BL/VL3 RadLV with endogenous xenotropic sequences. In fact, one of the proviruses cloned from tumor 133-1 was found to have the structure of a typical MCF viral genome, as it harbors an *EcoRI* site at 6.9 kbp (see below).

To study the role of insertional mutagenesis by proviruses in the genesis of BL/VL3 RadLV-induced T-cell lymphomas, all of the BL/VL3 RadLV proviruses flanked by their adjacent cellular sequences were cloned from a single tumor. Tumor 133-1 (Fig. 1, lane 14) was chosen because it contains

FIG. 1. Southern blot analysis of proviruses present in representative BL/VL3 RadLV-induced T-cell lymphomas. DNAs (20 μ g) from various tumors were digested with *Eco*RI, electrophoresed on 1% agarose gels, transferred to nitrocellulose membranes, and hybridized with the BL/VL3 RadLV U3 LTR-specific probe. Lanes: 1 and 15, 32 P-labeled *Hind*III-digested λ DNA; 2, BL/VL3 cell line; 3 and 4, control liver DNAs from normal C57BL/6 and SIM.R mice; 5 to 14, BL/VL3 RadLV-induced T-cell lymphomas 108-1, 108-2, 132-5, 132-6, 132-7, 132-8, 136-1, 137-1, 139-1, and 133-1.

a low number (six) of easily detectable newly acquired proviral *Eco*RI fragments of 16, 12, 9.4, 7.7, 6, and 3.4 kbp. The 5- to 20-kbp and the 1- to 5-kbp size-selected *Eco*RI fragments from tumor 133-1 DNA were ligated to Charon 4A and lambda gt10 arms, respectively, and cloned. Recombi-

nant phages were identified with the 32 P-labeled BL/VL3 RadLV U3 LTR-specific probe.

All six detectable *Eco*RI fragments containing viral sequences (clones 4A-1, 4A-5, 4A-11, 4A-21, 4A-29, and gt10-5) were successfully cloned, as judged by comigration of *Eco*RI and *Pst*I-cleaved cloned proviral DNAs with proviruses of tumor 133-1 DNA also cleaved with *Eco*RI and *Pst*I (data not shown). These cloned proviruses were subcloned into plasmid vectors and analyzed by restriction endonuclease digestions and Southern blot with the BL/VL3 RadLV probe U3 LTR (data not shown). Two of these (clones 4A-1 and 4A-5) were found to represent complete BL/VL3 RadLV genomes, while four (clones 4A-11, 4A-21, 4A-29, and gt10-5) did not contain a complete RadLV genome. One clone (4A-21) was found to contain an *Eco*RI site at position 6.9 on the viral genome, a feature typical of MCF recombinant proviruses.

Cellular fragments adjacent to each provirus were subcloned, and those free of repetitive sequences were used as probes to screen other tumor DNAs to search for common proviral integration sites. Using one of these probes (PRO.6) derived from clone 4A-1 (Fig. 2A), we were able to identify rearrangements in DNAs of other T-cell leukemias (see below). This region, which represents a common proviral integration site, was designated *Vin-1* (VL3 integration site 1).

Molecular characterization of the *Vin-1* region. A crude restriction map of the *Vin-1* region was obtained by digestion of normal mouse cellular DNA with various restriction

FIG. 2. Restriction map of the mouse and rat *Vin-1* locus. (A) Schematic representation of the provirus inserted within the *Vin-1* locus and cloned from murine BL/VL3 RadLV-induced thymoma 133-1 (clone 4A-1). (B) Diagram of the normal mouse *Vin-1* allele with the locations of the RadLV proviruses found in this region. (C) Diagram of the normal rat *Vin-1* allele showing the location of the Moloney MuLV provirus found in this region. Symbols: arrows, transcriptional orientation of the proviruses; thick line, viral sequences; open boxes, retroviral LTRs. The dashed line around probes PRO.6 and PRO.7 indicates that exact positions of these murine probes have not been determined precisely within the given rat fragments. Restriction sites: B, *Bam*HI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; RV, *Eco*RV; S, *Sma*I; Sc, *Sac*I; ScII, *Sac*II.

FIG. 3. Southern blot analysis of MuLV-induced T-cell lymphomas with a probe from the *Vin-1* region. *KpnI*-digested DNAs (20 μ g) from various tumors were screened with *Vin-1*-specific probe PRO.6. (A) Murine BL/VL3 RadLV-induced T-cell lymphomas 1363-13, 133-1, 1362-4, 1362-5, 1362-7, 1362-9, 1362-11, 1362-12, 1363-11, and 1363-12 (lane 2 to 11). Lane 1, liver DNA from mouse 1363-13. (B) Rat Moloney MuLV-induced T-cell lymphomas. Lanes: 1, 1213-1; 2, 1213-2; 3, 1213-3; 4, 1213-4; 5, 1213-9; 6, 1213-10; 7, 1213-11; 8, 1213-12; 9, 1213-13.

endonucleases, alone or in combination, and hybridization with probe PRO.6 or PRO.7 (data not shown). A summary of these data is presented in Fig. 2B. This analysis has allowed us to select appropriate restriction endonucleases to screen for new rearrangements at both the left and right sides of the proviral integration site of tumor 133-1 as well as to map more precisely the integration sites of each provirus found within this region.

Detection of proviruses integrated within the *Vin-1* region of MuLV-induced T-cell leukemias. To determine the frequency at which *Vin-1* was occupied by proviruses in other BL/VL3 RadLV-induced thymomas, we first screened 39 of these *KpnI*-digested tumor DNAs (series 1363) by the Southern technique, using 32 P-labeled probe PRO.6. *KpnI* was chosen because of a relatively long region (~22 kbp) could be screened with the available probes and because it cleaves within the BL/VL3 RadLV LTR. In addition to the original tumor 133-1 (Fig. 3A, lane 3), one tumor (1363-13) (5%) showed a novel tumor-specific fragment in addition to the germ line fragment of the normal allele (lane 2). Using the same probe and the same restriction endonuclease, we also screened DNAs from thymomas induced by other MuLVs and found rearrangements within the *Vin-1* region in 1 (2%) of 48 Moloney MuLV-induced rat thymomas (series 1213) (Fig. 3B, lane 3).

Therefore, these results indicate that *Vin-1* is a common proviral integration site. Although the frequency of its occupancy does not appear high, it remains highly significant, considering the low probability of two independent proviral integration events occurring at random in the same 22-kbp genomic fragment (26).

Structural organization of the proviruses within the *Vin-1* region. Rearrangements of a given DNA region within the genome of a tumor cell can arise by different molecular mechanisms. To determine whether the rearrangements detected with *KpnI* within the *Vin-1* region indeed reflected the integration of a provirus or one of its recombinant MCF-type proviruses and, if so, to map each of them more precisely and to identify their transcriptional orientation, we used *Vin-1* (PRO.6 and PRO.7) and U3 LTR-specific probes to perform a restriction analysis on the tumor DNAs.

In tumor 1363-13 DNA, *KpnI* digestion revealed a novel 17-kbp fragment hybridizing with probes PRO.6 (Fig. 3A, lane 2) and PRO.7 (not shown), suggesting the presence of a novel provirus, thus introducing a *KpnI* site within this fragment. The same DNA digested with *BamHI* or *HindIII* and hybridized with probe PRO.7 showed novel fragments of 5.3 and 20 kbp, respectively, in addition to the normal 6.8- and 11.5-kbp alleles (Fig. 4A, lanes 1 and 2). On *EcoRI*-digested DNA, the probe detected only the 5.4-kbp germ line fragment (lane 4). In addition, probe PRO.6 did not detect rearrangements in *HindIII*-, *BamHI*-, or *EcoRI*-digested DNA (data not shown). These results are consistent with the presence of a BL/VL3 RadLV provirus integrated downstream of *Vin-1* probe PRO.7, between the *EcoRI* (at +0.7 kbp) and *BamHI* (at +6.3 kbp) sites. The length of the rearranged *HindIII* fragment (~20 kbp) suggests that this provirus is full length, since the BL/VL3 RadLV genome does not harbor any *HindIII* site.

Additional digestions with *KpnI*-*BamHI* or *PstI* revealed, with *Vin-1* probe PRO.7, novel 2.1-kbp (Fig. 4A, lane 6) and 1.1-kbp (lane 8) fragments, respectively, in addition to the 6.8-kbp (lane 5) and 1.2-kbp (lane 7) normal alleles. The sizes of these rearranged fragments allowed the determination of the provirus orientation and confirmed its localization (Fig. 4B). The presence of a novel *PstI* site 450 bp upstream from the novel *KpnI* site is consistent with the presence of a provirus (which harbors *PstI* and *KpnI* sites at 40 and 470 bp, respectively) being oriented away from *Vin-1* probe PRO.7. Moreover, the 1.1-kbp rearranged *PstI* fragment allowed the precise localization of the provirus at +1.2 kb. The rehybridization of the same filter with the BL/VL3 RadLV U3 LTR-specific probe did not bring additional confirmation on the orientation of this provirus, since too many newly acquired fragments hybridized with this probe in the region which would have been of interest (data not shown).

A similar analysis was performed to localize the Moloney MuLV provirus inserted within the *Vin-1* region in the rat tumor 1213-3. A crude restriction map of the rat *Vin-1* locus was first obtained from single and double digestions of normal rat DNA by using various restriction endonucleases and hybridized with mouse *Vin-1* probes PRO.6 and PRO.7, using the Southern blot technique (data not shown). The restriction map obtained is shown in Fig. 2C. Rat tumor DNA 1213-3 was digested with *BamHI*, *EcoRV*, and *HindIII*. With *BamHI*, hybridization with *Vin-1* probe PRO.6 revealed a rearranged fragment of 9.1 kbp in addition to the normal 11.5-kbp allele (Fig. 5A, lane 1), while *EcoRV* and *HindIII* digestions did not revealed rearrangements (lanes 2 and 3). With the same restriction endonuclease digestion, *Vin-1* probe PRO.7 detected novel rearranged 8.0-kbp

FIG. 4. Localization and orientation of the BL/VL3 RadLV provirus inserted within the *Vin-1* region. Normal mouse and tumor DNAs (20 μ g) were digested with restriction endonucleases, electrophoresed on 1% agarose gels, transferred onto nylon membranes by the Southern technique, and hybridized with a *Vin-1*-specific probe. (A) DNA from the BL/VL3 RadLV-induced thymoma 1363-13, digested with *Bam*HI (lane 1), *Hind*III (lane 2), *Eco*RI (lane 4), *Bam*HI-*Kpn*I (lane 6), and *Pst*I (lane 8); DNA from normal liver digested with *Hind*III (lane 3), *Bam*HI-*Kpn*I (lane 5), and *Pst*I (lane 7). Hybridization was with 32 P-labeled *Vin-1* probe PRO.7. 32 P-labeled *Hind*III-digested λ DNA was used as markers. G, germ line. (B) Corresponding partial restriction endonuclease map of the novel *Vin-1* DNA fragments in tumor 1363-13. Symbols: thick line, viral sequences; open boxes, LTR; closed boxes, *Vin-1* or U3 LTR probe; thin line, cellular *Vin-1* sequences. Restriction endonucleases: B, *Bam*HI; K, *Kpn*I; H, *Hind*III; P, *Pst*I; RI, *Eco*RI.

*Bam*HI, 6.6-kbp *Eco*RV, and 7.0-kbp *Hind*III fragments in addition to the normal 11.5-, 8.5-, and 4.0 kbp-alleles, respectively (lanes 5 to 7). In addition, with a *Bam*HI-*Kpn*I digestion, probe PRO.7 revealed a novel 6.2-kbp fragment in addition to the normal 11.5-kbp germ line fragment (lane 8). Together, these results are consistent with the presence of the provirus integrated at +5.4 kbp from the reference *Bam*HI site between *Vin-1*-specific probes PRO.6 and PRO.7, with a transcriptional orientation pointing toward *Vin-1* probe PRO.7 (Fig. 5B). The orientation of the provirus was confirmed by rehybridizing the *Eco*RV-digested DNA of tumor 1213-3 with a Moloney MuLV U3 LTR-specific probe. The novel 6.6-kbp *Eco*RV fragment, hybridizing with *Vin-1* probe PRO.7, comigrated with a fragment detected with the Moloney MuLV U3 LTR-specific probe (Fig. 5A, lanes 9 and 10). Moreover, as expected, the novel rearranged 6.2-kbp *Bam*HI-*Kpn*I fragment detected with probe PRO.7 (lane 8) was not detected with the Moloney MuLV U3 LTR-specific probe (data not shown), while the novel rearranged 6.0-kbp *Bam*HI-*Kpn*I fragment detected with probe PRO.6

(lane 11) was detected with the Moloney MuLV U3 LTR-specific probe (lane 12).

Interestingly, the three proviruses detected within the *Vin-1* region are all inserted in the same transcriptional orientation.

Expression of the *Vin-1* gene. Common provirus integration sites have often been found at the 5' or 3' end of transcriptional units (18, 26). Moreover, restriction endonuclease analysis of the *Vin-1* region has shown the presence of four *Sma*I sites and one *Sac*II site within a 4-kbp *Eco*RI-*Bam*HI fragment (Fig. 2B). The recognition sites for these enzymes are rich in GC, frequently clustered in islands, and often located in the vicinity of transcriptionally active regions (1, 9, 23, 29, 32). To determine whether the *Vin-1* locus harbors a transcriptional unit, Northern (RNA) analysis was performed on RNA extracted from various tissues of young adult mice, using 32 P-labeled probe PRO.6. This analysis revealed the presence of a 6-kb RNA in lung, thymus, brain, testis, spleen, liver, and kidney of young adult (6- to 8-week-old) mice (Fig. 6A). In kidney, an additional shorter and

FIG. 5. Localization and orientation of the Moloney MuLV provirus inserted within the *Vin-1* region. Normal rat or tumor DNAs (20 μ g) were processed as for Fig. 4. (A) DNA from rat Moloney MuLV-induced thymoma 1213-3 digested with BamHI (lanes 1 and 5), EcoRV (lanes 2, 6, 9, and 10), HindIII (lanes 3 and 7), and BamHI-KpnI (lanes 8, 11, and 12) and hybridized with *Vin-1*-specific probe PRO.6 (lanes 1 to 3 and 11) or PRO.7 (lanes 5 to 9) or with the Moloney MuLV U3 LTR-specific Moloney MuLV probe (lanes 10 and 12). Lane 9 is a reproduction of lane 6; lanes 9 to 12 represent two distinct filters first hybridized with *Vin-1* probes and then rehybridized with the U3 LTR probe. Lane 4, 32 P-labeled HindIII-digested λ DNA. G, germ line; arrows, rearranged fragments. (B) Corresponding partial restriction endonuclease map of the novel *Vin-1* DNA fragments in tumor 1213-3. Symbols: thick line, viral sequences; open boxes, LTR; closed boxes, *Vin-1* or U3 LTR probe; thin line, cellular *Vin-1* sequences. Restriction endonucleases: B, BamHI; K, KpnI; H, HindIII; P, PstI; RV, EcoRV.

more abundant RNA species was detected (Fig. 6A, lane 8). No RNA was detected in salivary gland of the same animal (lane 9).

In two thymomas (1213-3 and 133-1) which were found to have a provirus inserted in the *Vin-1* region, the levels of the 6-kb RNA species were high (Fig. 6B, lanes 1 and 11). Additional shorter transcripts were also detected in these two tumors. The levels of the 6-kb RNA species were also high in some, but not all, of the thymomas which did not show *Vin-1* rearrangement (lanes 5 and 7).

These results indicate that the *Vin-1* region harbors a gene which is transcriptionally active in some tissues of the adult mice. The transcription of the *Vin-1* gene appears to be affected by the integration of a provirus in its vicinity or possibly within it.

Conservation of the *Vin-1* gene among mammals. Common provirus integration sites often reflect the presence of proto-oncogenes or less frequently of antioncogenes (18, 26). These genes have been shown to be well conserved throughout evolution. To determine whether this holds true for the murine *Vin-1* gene, we searched for homologous sequences in other species. DNAs from mouse, rat, hamster, cat, cow, chicken, and human genomes were digested with *Pst*I and

hybridized at high stringency with 32 P-labeled probe PRO.6. In each of the DNAs tested except that from chicken, at least one specific DNA fragment was detected (Fig. 7), indicating a very good conservation of these sequences among mammals and suggesting that *Vin-1* gene may have an important function.

***Vin-1* maps to chromosome 6 and appears to be a novel gene.** *Vin-1* chromosome location was first assessed by using a series of hamster-mouse somatic cell hybrids, each containing a different subset of murine chromosomes (15–17). DNAs from these cell hybrids were cleaved with *Eco*RI and analyzed by the Southern technique with *Vin-1*-specific probe PRO.6. The murine fragment (5.5 kbp) could be distinguished from the homologous hamster fragment (4.1 kbp) by its length (data not shown). A total of 26 Chinese hamster-mouse hybrid cell DNAs were analyzed and scored as negative or positive for the presence of the 5.5-kbp mouse-specific fragment. A correlation between the presence or absence of each of the mouse chromosomes and the presence or absence of the *Vin-1*-hybridizing fragment has been calculated (data not shown). This analysis revealed that *Vin-1* maps to chromosome 6.

To position the *Vin-1* locus in the mouse linkage map,

FIG. 6. Northern blot analysis of expression of the *Vin-1* gene. (A) RNAs (20 μ g) from various organs of an 8-week-old CD-1 mouse were screened with *Vin-1*-specific probe PRO.6. Lanes: 1, lung; 2, thymus; 3, brain; 4, testis; 5, liver; 7, spleen; 8, kidney; 9, salivary gland. Lane 6 contains RNA from BALB/3T3 mouse fibroblasts used as positive controls. (B) RNAs (20 μ g) from Moloney MuLV-induced rat thymomas 1213-3, 1213-8, 1213-10, and 1213-14 (lanes 1 to 4), from BL/VL3 RadLV-induced mouse thymomas 1363-3, 1363-8, 1363-11, and 140-2 (lanes 5 to 8), and from thymomas 140-2, 140-4, 133-1, and 1362-3 (lanes 9 to 12). Migration of 28S and 18S rRNAs is shown. Hybridization was with *Vin-1* probe PRO.6. Exposure times were 7 days (lanes 1 to 4), 10 days (lanes 5 to 8), and 16 h (lanes 9 and 12). Autoradiograms of the same filters hybridized with the 32 P-labeled actin probe are shown at the bottom.

analysis of two genetic crosses was used. DNAs from parental mice were first typed by Southern blot analysis for restriction fragment length polymorphisms, using the *Vin-1* probe PRO.7. DNAs from parental NFS/N, C58/J, and C57BL/6 mice produced a 11.5-kbp *Vin-1*-reactive fragment, whereas *M. musculus musculus* and *M. spretus* produced fragments of 12.4 kb (Fig. 8). Southern blot analysis identified the 11.5-kbp NFS/N fragment in 54 of the 119 mice in the *M. musculus musculus* backcross and in 46 of the 93 mice of the *M. spretus* cross, consistent with single-gene segregation ratios. The pattern of *Vin-1* segregation was compared with that of other markers on chromosome 6 (Tables 1 and 2), and the *Vin-1* locus was mapped in the distal end of chromosome 6 near the oncogene loci *K-ras-2* and *raf* with gene order *raf-Vin-1-K-ras-2*. Three other genetic loci which could be involved in oncogenesis have been mapped in this region, *int-41* (a common provirus integration site), *Fgf-6* (fibroblast growth factor 6), and *Tnfr-2* (tumor necrosis factor receptor 2). The restriction endonuclease maps of *int-41* (8), *Fgf-6* (5), and *Tnfr-2* (10) are distinct from that of *Vin-1*. Moreover, the *Tnfr-2* RNA in normal spleen is 2.3 kb in length (10), while that of *Vin-1* in the same organ is 6 kb. These results indicate that *Vin-1* is distinct from either of these loci. Therefore, *Vin-1* appears to be a novel gene likely to be involved in leukemogenic transformation by MuLVs.

DISCUSSION

The BL/VL3 RadLV is a replication-competent leukemogenic retrovirus that does not harbor an oncogene. In this study, we have tested whether, like other MuLVs, it induces neoplasia partly through its ability to act as an insertional

FIG. 7. Interspecies conservation of the *Vin-1* locus. DNAs (20 μ g) from various species were digested with *Pst*I and analyzed by Southern blot with the *Vin-1*-specific probe PRO.6. Hybridization and washing were done at high stringency as described in Materials and Methods. Lanes: 1, mouse; 2, rat; 3, hamster; 4, cat; 5, cow; 6, human; 7, chicken.

FIG. 8. Southern blot hybridization of *Hind*III-digested liver DNA from NFS/N $\times$ *M. musculus musculus* backcrosses with the 32 P-labeled *Vin-1* probe PRO.7. Lanes: 1, NFS/N parental mouse; 2 to 7, individual *M. musculus musculus* backcrossed mice.

mutagen by activating cellular oncogenes or by inactivating antioncogenes. We found a common proviral integration site, designated *Vin-1*, in BL/VL3 RadLV-induced tumors. This locus was also occupied by a provirus in one additional Moloney MuLV-induced thymoma. Although the frequency of provirus occupancy of this locus was found to be low (2 to 5%), its identification is highly significant, since the probability that two such integration events occurred independently in two different tumors, in a 30-kbp fragment, is very low, around 10^{-10} for random integrations (26). This suggests that the presence of a provirus within the *Vin-1* locus provides some selective growth advantage to the targeted cells and that the *Vin-1* region participates to the malignant phenotype.

A low frequency of provirus occupancy has also been observed for many other loci (26). Several factors appear to influence the frequency of such provirus integration within a given locus. These include the virus (3, 26, 33, 36) or the mouse (3, 26, 33) strains used, the stage of malignancy at which the tissue is analyzed (25), and possibly the presence in the mouse genome of several distinct genes which can be activated independently by provirus insertion, inducing similar effects and possibly cooperating with each other. For instance, the activation of *c-myc* and *N-myc* by Moloney MuLV proviruses in *Pim-1* transgenic mice has been demonstrated to act in synergy to promote the development of T-cell tumors (35). Similarly, we found that the *c-myc* and

TABLE 2. Recombination between restriction fragments

Locus pair	No. of recombinants/total	% Recombination $\pm$ 1 SE ^a
<i>Raf</i> , <i>Vin-1</i>	10	4.7 $\pm$ 1.5
<i>Vin-1</i> , <i>K-ras-2</i>	12	5.7 $\pm$ 1.6

^a Percent recombination between restriction fragments and standard error was calculated according to Green (11) from the number of recombinants in a sample size of 212.

Pim-1 genes were rearranged in BL/VL3 RadLV-induced T-cell leukemias at frequencies of 20% (8 of 39) and 18% (7 of 39), respectively (data not shown). In one of the tumors showing *Pim-1* rearrangement, the *Vin-1* locus was also rearranged (tumor 1363-13). Thus, *Vin-1* and *Pim-1* may act cooperatively to transform T cells.

The *Vin-1* locus has been mapped on mouse chromosome 6 between the *raf* and *K-ras-2* genes and appears to be a unique gene (with a transcriptional unit encoding a 6-kb RNA) not previously described. This gene is likely to be involved in tumorigenesis. The *Vin-1* gene is relatively well conserved among mammalian species and is transcribed at high levels in several organs of the mouse.

Provirus within the *Vin-1* locus were found to be inserted within a 4-kbp fragment, all in the same transcriptional orientation, in a region presenting the characteristics of a CpG-rich island. CpG-rich islands are preferentially found in close proximity of promoter regions or at the 5' ends of various genes (1, 9, 23, 29); the presence of recognition sites for restriction enzymes which recognize infrequent GC-rich sequences has been found to be a good indication for the presence of a gene (23). The identification of one *Sac*II and of four *Sma*I recognition sites in the vicinity of the PRO.6 *Vin-1* fragment, which contains a *Vin-1* exon, suggests that this exon may be at the 5' end of the *Vin-1* gene.

In tumors which showed provirus integration within *Vin-1*, *Vin-1* RNA levels were found to be elevated, suggesting that this rearrangement may have activated *Vin-1* transcription. The mechanism by which such activation may occur is still unclear. Further analysis to localize the exons of the *Vin-1* gene with its upstream and downstream sequences will be required to clarify this issue. However, if indeed these proviruses are inserted at the 5' end of the gene (as the presence of the CpG-rich island suggests) in an opposite transcriptional orientation, they may activate gene expression through their LTR enhancers or by disrupting important upstream regulatory elements, or both, as previously reported for several other genes (18, 26).

Interestingly, *Vin-1* RNA levels were also high in some tumors which were not found to have proviruses integrated within the *Vin-1* locus. In these tumors, proviruses integrated at the other end of the gene in a region undetectable with the available *Vin-1* probes may contribute to the high *Vin-1* levels. Alternatively, other genetic events, such as mutations, insertions, deletions, or translocations of *Vin-1* sequences, may have spontaneously occurred in these tumors and may contribute to the high levels of *Vin-1* RNA. Finally, transactivation of the *Vin-1* gene may have occurred in these tumors. Further experiments on this set of tumors should yield useful information on the control of *Vin-1* gene expression.

Several provirus integration sites have previously been shown to encode novel proto-oncogenes (18, 26). The *Vin-1* gene, whose identification is reported here, represents an excellent candidate as a putative novel proto-oncogene.

TABLE 1. Segregation of the *Vin-1*-hybridizing restriction fragment with alleles of *K-ras-2* and *raf* in the progeny of backcrosses

Mice	Inheritance of the NFS/N allele			No. of mice	
	<i>raf</i>	<i>Vin-1</i>	<i>K-ras-2</i>	Cross 1 ^a	Cross 2 ^b
Nonrecombinants	+	+	+	50	39
	-	-	-	59	43
Single recombinants	+	+	-	2	3
	-	-	+	2	4
	+	-	-	3	0
	-	+	+	2	4
Double recombinants	+	-	+	1	0
	-	+	-	0	0

^a (NFS/N or C58/J $\times$ *M. musculus musculus*)F₁ $\times$ *M. musculus musculus*.

^b (NFS/N $\times$ *M. spretus*)F₁ $\times$ C57BL/6.

First, the integration of BL/VL3 RadLV or Moloney MuLV provirus in the *Vin-1* region of different T-cell lymphomas suggests that this molecular event provides a growth advantage to these cells and that this locus is involved in tumor formation. Second, the conservation of *Vin-1* among mammalian species underscores the importance of this gene. Third, the gene encoding the 6-kb *Vin-1* transcript, identified with a probe present only 4 kbp away from the initial integration site, appears to be activated by the provirus insertions. The molecular cloning of the *Vin-1* cDNA should provide the tools for analyzing the *Vin-1* gene structure, to test its function directly, and to examine its role in tumorigenesis.

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