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Low-Frequency Loss of Heterozygosity in Moloney Murine Leukemia Virus-Induced Tumors in BRAKF1/J Mice

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To identify potential involvement of tumor suppressor gene inactivation during leukemogenesis by Moloney murine leukemia virus (M-MuLV), a genome-wide scan for loss of heterozygosity (LOH) in tumor DNAs was made. To assess LOH, it is best to study mice that are heterozygous at many loci across the genome. Accordingly, we generated a collection of 52 M-MULV-induced tumor DNAs from C57BR/cdJ × AKR/J F_1 (BRAKF1) hybrid mice. By using direct hybridization with oligonucleotides specific for three different classes of endogenous MuLV-related proviruses, 48 markers on 16 of 19 autosomes were simultaneously examined for allelic loss. No allelic losses were detected, with the exception of a common loss of markers on chromosome 4 in two tumors. The three autosomes that lacked informative endogenous proviral markers were also analyzed for LOH by PCR with simple-sequence length polymorphisms (SSLPs); one additional tumor showed LOH on chromosome 15. Further screening with chromosome 4 SSLPs identified one additional tumor with LOH on chromosome 4. Therefore, in total, the average fractional allelic loss was quite low (0.002), but the LOH frequency of 6% on chromosome 4 was highly statistically significant ($P < 0.0005$). Detailed SSLP mapping of the three tumors with LOH on chromosome 4 localized the region of common LOH to the distal 45 centimorgans, a region syntenic with human chromosomes 1 and 9. Candidate tumor suppressor genes, *Mts1* ($p16^{INK4a}$) and *Mts2* ($p15^{INK4b}$), have been mapped to this region, but by Southern blot analysis, no homozygous deletions were detected in either gene. One of three tumors with LOH on chromosome 4 also showed a proviral insertion near the c-*myc* proto-oncogene. These results suggested that tumor suppressor inactivation is generally infrequent in M-MuLV-induced tumors but that a subset of these tumors may have lost a tumor suppressor gene on chromosome 4.

Moloney murine leukemia virus (M-MuLV) has been extensively studied as a leukemogenic agent. When inoculated into neonatal NIH Swiss or BALB/c mice, it induces T lymphomas with 100% incidence and with a mean latency of 16 to 17 weeks (20). Leukemogenesis by M-MuLV and other nonacute viruses is a multistep process. The best understood mechanism involved in nonacute retroviral carcinogenesis is long terminal repeat (LTR) activation of proto-oncogenes (11, 20, 36). In mice, T lymphomas induced by M-MuLV predominantly show LTR activations of the proto-oncogenes c-*myc*, *pim-1*, and *pvt-1*. In addition to proto-oncogene activation, M-MuLV requires other steps for leukemogenesis (21).

Recently, the contributions of tumor suppressor gene mutations during the genesis of a variety of human and rodent tumor types have been documented. Tumor suppressor genes contribute to a malignant phenotype through their inactivation or loss (50, 92). To date, tumor suppressor gene mutations in M-MuLV-induced leukemias have not been extensively examined. However, for the Friend spleen focus-forming virus (SFFV) acute transforming virus, one of the secondary changes observed in Friend SSFV-induced erythroleukemia cells is deletion or inactivation of the p53 tumor suppressor gene (16, 60). Also, in myeloid leukemias of BXH-2 mice, proviral integration at the *Evi-2* locus and disruption of normal Nf1 expression have been identified (53). Furthermore, in some M-MuLV-induced tumors, proviral insertions near known proto-oncogenes have not been detected (21). Thus, it seems possible that tumor suppressor gene alterations occur in leukemias induced by M-MuLVs.

For human cancers, loss-of-heterozygosity (LOH) studies have become important in identifying and cloning putative tumor suppressor genes (10, 23, 48, 51, 56, 70, 87, 95). Recently, LOH has been used to search the entire mouse genome for loci involved in several kinds of tumors (15, 39, 57, 96).

In this report, we describe the result of a genome-wide LOH analysis of M-MuLV-induced tumors in C57BR/cdJ × AKR/J F_1 (BRAKF1) mice. Although the overall genome-wide rate of LOH was quite low, chromosome 4 showed a significant rate of allelic loss. The patterns of partial allelic loss in two tumors were used to map the common region of LOH on chromosome 4 to the distal two-thirds of chromosome 4. Several candidate tumor suppressor genes that map to this region were tested for homozygous deletions, but none were observed. In addition, tumors were examined for proviral insertions adjacent to c-*myc*, *pim-1*, and *pvt-1*.

MATERIALS AND METHODS

Mice. C57BR/cdJ females and AKR/J males were purchased from the Jackson Laboratory (Bar Harbor, Maine). BRAKF1 mice were derived by crossing female C57BR/cdJ mice with male AKR/J mice.

Virus and inoculation of BRAKF1 mice. Viral stocks were clarified cell culture supernatants derived from NIH 3T3 fibroblasts productively infected with wild-type M-MuLV; portions of viral stocks were individually frozen at −70°C and were thawed and used only once (22). Virus infectivity titrations were performed by the UV-XC syncytial assay (69). BRAKF1 mice were inoculated intraperitoneally with 0.15 ml of virus stock (2×10^4 XC PFU) within 48 h of birth.

Tumor purification and DNA isolation. Moribund M-MuLV-infected BRAKF1 mice were sacrificed, and thymic tumor tissue was removed and rinsed gently with phosphate-buffered saline. Tumor cells were recovered by mincing the tumor in phosphate-buffered saline and passing the homogenate through a

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94-mm no. 150 mesh screen (Bellco Glass, Inc., Vineland, N.J.); the tumor cells passed through the screen, while the thymic stromal tissue was retained. DNA was extracted from the tumor cell suspensions by a modification of standard protocols (81) and suspended in 10 mM Tris–0.1 mM EDTA (pH 8). Uninfected AKR/J and C57BR/cdJ parental mice and uninfected BRAKF1 mice used for controls were sacrificed at 16 to 20 weeks of age, and thymus and spleen tissues were processed as described above.

Analysis of endogenous provirus loci in tumor DNAs from BRAKF1 mice. Analysis of endogenous proviral markers (see Fig. 1) was performed by agarose gel electrophoresis and hybridization in situ with the oligonucleotides JS-4, JS-5, and JS-6/10, which detect modified polytropic, polytropic, and xenotropic endogenous proviruses, respectively (78). The chromosomal locations of markers on the linkage map of the mouse genome have been described previously (28, 30). Detailed protocols for the detection of *Pmv*, *Mpmv*, and *Xmv* loci have been described previously by Coffin and collaborators (29, 30, 78, 79) and were only slightly modified. The dried agarose gel hybridization process was modified from that of Wallace and Miyada (91). Briefly, high-molecular-weight tumor or control DNA (18 to 22 μg) was digested overnight with either 100 U of *Eco*RI or 100 U of *Pvu*II (Gibco/BRL, Grand Island, N.Y.). Ammonium acetate (6 M) was added after digestion to bring the final ammonium acetate concentration to 2.5 M. Digested DNA was precipitated with 2 to 2.5 volumes of ethanol and resuspended in 25 μl of electrophoresis sample buffer (10 mM Tris [pH 8.0], 0.02 M EDTA, 5% Ficoll, 0.05% bromophenol blue, 0.05% xylene cyanol). Samples were separated by electrophoresis in 0.8% agarose–1× Tris-borate-EDTA gels (24 by 20 cm) by electrophoresis at 75 to 90 V for 18 to 20 h or until a 2.0-kb marker band (from lambda phage DNA cut by *Hin*dIII) was about 1 cm from the end of the gel. The gels were stained for 15 to 30 min with ethidium bromide (0.5 μg/ml), photographed, and processed as follows. Gels were cut in half lengthwise for ease of handling. They were then denatured in 0.5 M NaOH–1.5 M NaCl for 35 to 45 min and neutralized in 1.0 M Tris (pH 8.0)–1.5 M NaCl for 35 to 45 min. The gels were dried on Whatman 3MM paper in a slab gel vacuum dryer for 1 h at room temperature until they were flat and then for 40 to 75 min at 60°C until they were translucent and had a rubber-like consistency. The dried gels were removed from the paper by wetting in 5× SSPE (5× SSPE is 0.75 M NaCl, 0.05 M $NaH_2PO_4 \cdot H_2O$, and 0.005 M EDTA, pH 7.4) and stored frozen at −20°C (for up to 1 month), wetted and sealed in 5× SSPE. ^{32}P 5′ end labeling of oligonucleotide probes JS-4, JS-5, and JS-6/10 has been described previously (78). Gels were hybridized with 25 ml of 5× SSPE–0.1% sodium dodecyl sulfate–10 μg of denatured salmon sperm DNA per ml plus 1.0×10^6 to 2.0×10^6 cpm of labeled oligonucleotide (JS-4, JS-5, or JS6/10) per ml in a sealed plastic bag for 20 to 28 h at 62°C. Hybridized gels were then washed gently with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% sodium dodecyl sulfate (four times with 500 ml at room temperature for 15 min and then two times with 500 ml at 62°C for 30 min), air dried, and exposed to Kodak XAR-5 film at −70°C for 7 to 21 days with intensifying screens. Autoradiograms were examined visually, and losses of endogenous proviral markers were scored.

PCR analysis of SSLPs. Tumor DNAs were also analyzed for allele loss by PCR amplification of a number of loci that contained polymorphic simple-sequence repeats. Primers for all simple-sequence length polymorphisms (SSLPs) employed in this study have been described previously (reference 14 and the Massachusetts Institute of Technology [MIT] Genome Database [URL: http://www.genome.wi.mit.edu]) and were purchased from Research Genetics (Huntsville, Ala.). PCR assays for markers developed at MIT were carried out as described in protocols available from Research Genetics with some modifications. Allele sizes (in base pairs) for the SSLP markers have been reported for AKR/J mice but not for C57BR/cdJ mice. However, SSLP allele sizes have been reported for C57BL/6J mice, a strain related to the C57BR/cdJ strain (43). The sizes reported for C57BL/6J mice were therefore used to select presumptive polymorphic SSLP markers, and AKR/J and C57BR/cdJ parental DNAs were included in all PCR-SSLP analyses as controls. The PCR mixtures contained 100 to 200 ng of genomic DNA (or no template DNA for negative controls), 200 mM (each) dCTP, dATP, dTTP, and dGTP (Pharmacia), 0.3 μM each primer, 1.25 U of *Taq* DNA polymerase (Perkin-Elmer Cetus), 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, and 0.001% gelatin (Sigma). A reaction volume of 50 ml was overlaid with 50 ml of sterile mineral oil and subjected to 30 cycles of amplification in a DNA Thermal Cycler (Perkin-Elmer Cetus). After an initial denaturation for 3 min at 97°C, each cycle consisted of 15 s of denaturation at 94°C, 2 min of reannealing at 55°C, and 2 min of extension at 72°C. Amplified target DNAs were resolved in 3 to 4% MetaPhor agarose gels (FMC BioProducts) and stained with ethidium bromide. Allelic losses were scored visually from Polaroid photographs of the stained gels. The SSLP products for the two alleles in BRAKF1 hybrids were not always of equal intensity, but the relative intensities were extremely reproducible. We selected those SSLP markers that gave relatively equal intensities for the two alleles. LOH was detected by comparing tumor DNAs with at least two DNAs isolated from nontumor tissues of an uninfected BRAKF1 animal.

Southern blot analysis. Seven micrograms of high-molecular-weight DNA was digested with *Hpa*I (T-cell receptor beta [TCR-beta]), *Kpn*I (c-*myc*), *Eco*RI (*pim-1* and *pvt-1*), *Bam*HI (*Mts1* [$p16^{INK4a}$]), or *Sph*I (*Mts2* [$p15^{INK4b}$]) (Gibco/BRL), separated in a 0.8% agarose–1× Tris-borate-EDTA gel, and transferred to GeneScreen Plus (Dupont NEN, Boston, Mass.). Transfer, hybridization, and washing were performed according to the manufacturer's specifications. Random-primer-labeled probes were prepared by the standard protocol (82).

DNA fragments used as radioactive probes for Southern blot analyses included the following: the 600-bp *Eco*RI fragment from 86T5 (38), a cDNA clone for the TCR-beta locus; fragments for c-*myc*, *pim-1*, and *pvt-1* as described previously (22); and 426- and 385-bp fragments corresponding to exon 2 of mouse *Mts1* ($p16^{INK4a}$) and *Mts2* ($p15^{INK4b}$), respectively. Exon 2 fragments were generated by PCR with normal mouse DNA under conditions recommended by Perkin-Elmer Cetus, isolated from agarose gels, and used for random-primer probes. Primer sequences for *Mts1* ($p16^{INK4a}$) were 5′ TCCCTCTACTTTTTC TTCTGAC 3′ and 5′ TTGGGTTGCTTCTTCTTTTTC 3′ (94). Primer sequences for *Mts2* ($p15^{INK4b}$) were 5′ CCCCCACCCCACTAGAAAC 3′ and 5′ TAGATGGGGCTGGGGAGAA 3′ (94).

RESULTS

One hallmark of tumor suppressor gene loss in tumors is LOH. To rapidly screen for LOH in M-MuLV-induced tumors, we used a system developed by Frankel et al. (30) that utilizes endogenous nonecotropic MuLV proviruses as genetic markers. Endogenous MuLV proviruses are useful markers since (i) 30 to 60 copies are present per typical inbred mouse strain, and they are broadly distributed throughout the genome; (ii) they are inherited in a Mendelian fashion, and their positions on the mouse chromosomes have been mapped; (iii) there are many polymorphisms between inbred strains (i.e., some strains carry an endogenous provirus at a particular chromosomal locus, while others do not); and (iv) oligonucleotide probes are available to unambiguously detect them. A major advantage of the system is that multiple proviruses on different chromosomes can be detected simultaneously by restriction endonuclease digestion, gel electrophoresis, and hybridization.

To assess LOH, it was desirable to study mice that were polymorphic at many endogenous proviral loci across the genome. Accordingly, we used F_1 progeny from a cross between C57BR/cdJ females and AKR/J males, since the endogenous proviral patterns of these two inbred strains are very different (28). Figure 1 shows a linkage map of the mouse genome and the positions of the endogenous nonecotropic MLV proviruses in the C57BR × AKR F_1 (BRAKF1) mice. There was at least one polymorphic proviral marker on each of the 19 BRAKF1 mouse autosomes. Furthermore, at least one polymorphic proviral marker was donated by each parent for 10 of the 19 autosomes.

The endogenous nonecotropic MLV proviruses can be divided into three classes, i.e., modified polytropic, polytropic, and xenotropic, based on differences in their envelope genes (77). Each class can be detected by hybridization with oligonucleotide probes specific for that class, as developed by Stoye and Coffin (78). To identify particular proviruses, cellular DNA is digested with either *Eco*RI or *Pvu*II to generate viral-nonviral junction fragments; each junction fragment is diagnostic for a particular endogenous provirus. The proviral junction fragments are detected by direct hybridization of the dried gels with the radioactive class-specific oligonucleotide probes. JS-4 is used to detect proviruses of the modified polytropic class, JS-5 is used for the polytropic class, and JS-6/10 is used for the xenotropic class (78). Frankel et al. (30) have mapped essentially all of the endogenous proviruses visualizable in this manner to specific chromosomal locations (see below).

Figure 2 illustrates the detection of the modified polytropic proviruses in C57BR, AKR, and BRAKF1 mice. DNAs were digested with either *Eco*RI or *Pvu*II and then analyzed by agarose gel electrophoresis and hybridization with the JS-4 *Mpmv*-specific oligonucleotide probe. The modified polytropic proviral patterns as well as the polytropic and xenotropic patterns (not shown) of the C57BR and AKR parents were quite different, and, as expected, the F_1 mice had a pattern consisting

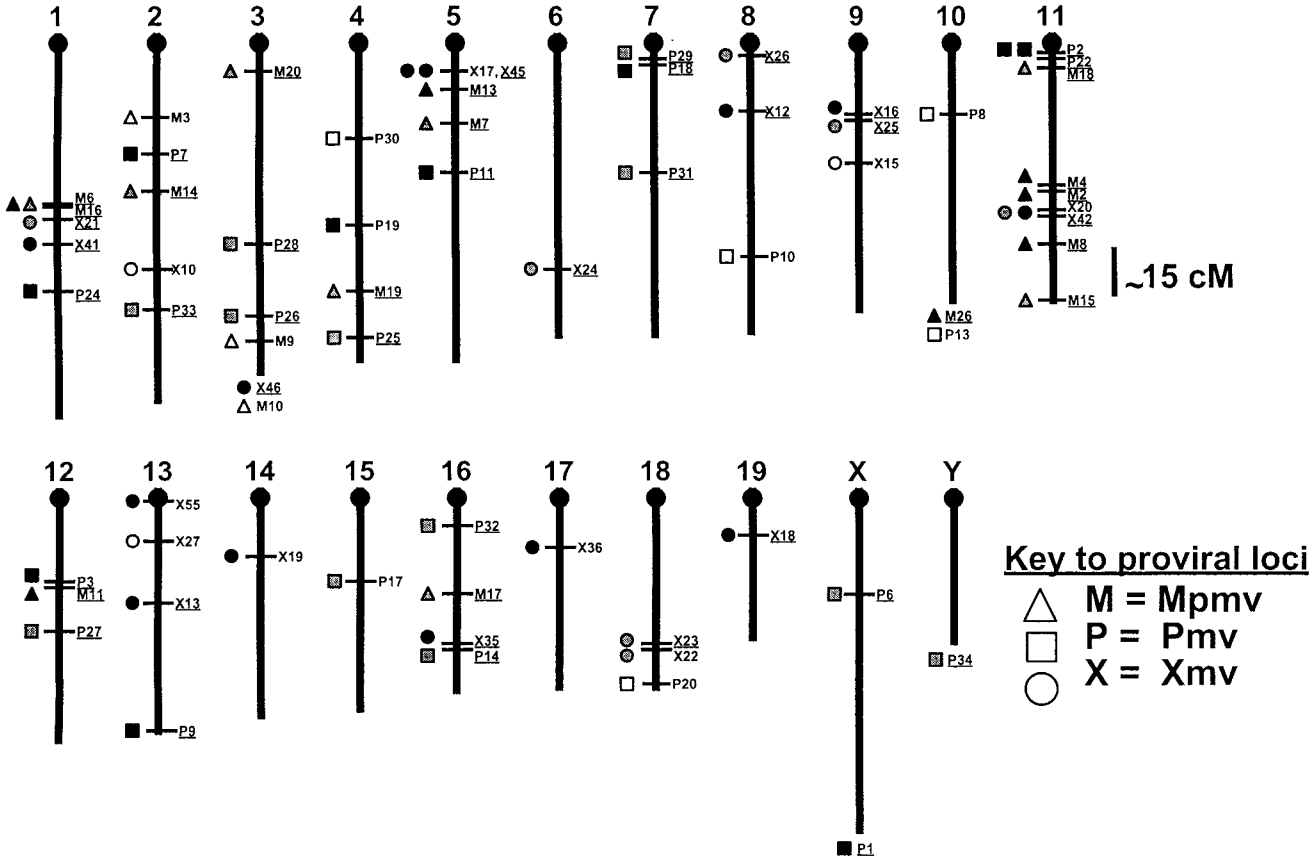


FIG. 1. Linkage map of endogenous nonecotropic MuLV proviruses in BRAKF1 mice. Map locations of 68 endogenous proviruses are shown. Positions of markers on the mouse genome linkage map are based on data from Frankel et al. (28, 30) and the Mouse Genome Database (URL: http://www.informatics.jax.org). Proviruses on chromosomes with unknown positions relative to mapped loci are shown at the bottom of their assigned chromosome. Filled symbols indicate proviruses present only in C57BR/cdJ (female) mice, and shaded symbols indicate proviruses present only in AKR/J (male) mice; thus, filled and shaded symbols indicate proviral loci that would be heterozygous (polymorphic) in BRAKF1 mice. Open symbols represent proviral markers contained by both parental strains; these would be homozygous in BRAKF1 mice. Underlined markers represent proviruses that were confidently detected with class-specific oligonucleotide probes and used to assess LOH in M-MuLV-induced tumor DNAs.

of the sum of the parental patterns (Fig. 1). Therefore, most proviral bands in the F_1 mice represented heterozygous proviruses that were therefore polymorphic (informative) markers. In tumors from BRAKF1 mice, loss (or significant reduction in intensity) of an endogenous proviral band indicated an LOH event. Occasionally, two or more proviruses had *Eco*RI or *Pvu*II junction fragments that were difficult to separate by electrophoresis; the alternate restriction enzyme digest generally allowed them to be distinguished (see below). Of the 68 polymorphic proviruses in the BRAKF1 background shown in Fig. 1, we could resolve and identify 48. On 16 of 19 autosomes we could identify at least one informative proviral marker donated by either parent, and on 10 of 19 autosomes we could identify informative markers donated by each parent. The locations of the 48 markers we used to assess LOH are indicated in Fig. 1.

For this study, we used tumor DNAs from BRAKF1 mice infected with M-MuLV. M-MuLV causes T-cell lymphoma with a latency of 3 to 4 months in randomly bred NIH Swiss mice (13). One- to 3-day-old neonatal BRAKF1 mice were inoculated intraperitoneally with 2×10^4 PFU of M-MuLV. These animals developed disease with essentially the same time course as observed in NIH Swiss mice (50% mortality in 17 weeks). Gross pathology at necropsy revealed markedly enlarged thymuses and frequent spleen and lymph node enlargement. The tumors resembled those that appear in NIH Swiss mice. Thymic masses were dissected from sacrificed animals, and DNA was extracted from tumor cell suspensions for further analyses.

BRAKF1 mice are *Fv-1*^{nn} (since both AKR/J and C57BR/cdJ mice are *Fv-1*^{nn}) and are therefore susceptible to both NB-tropic and N-tropic MuLVs (85). BRAKF1 mice also presumably carry one copy of endogenous Akv MuLV donated from the AKR/J male parent on chromosome 2. In theory, BRAKF1 mice were susceptible to both M-MuLV-induced and spontaneous Akv MuLV-induced leukemogenesis. Thymic tumors derived from M-MuLV-infected BRAKF1 mice were most likely induced by M-MuLV as opposed to activated endogenous Akv MuLV for several reasons. First, M-MuLV-infected BRAKF1 mice developed disease with a time course consistent with M-MuLV (average latency, 16 to 17 weeks) and not Akv MuLV (average latency, >25 weeks). Second, all uninfected BRAKF1 animals survived at least 30 weeks without signs of distress or disease, whereas all M-MuLV-infected animals became moribund within 26 weeks. Third, infection of mice as newborns with 2×10^4 PFU of M-MuLV would probably interfere with the spread of spontaneously activated endogenous Akv MuLV.

In LOH studies of tumor DNA, it was important to analyze DNA from relatively pure tumor cells; non-tumor-cell DNA

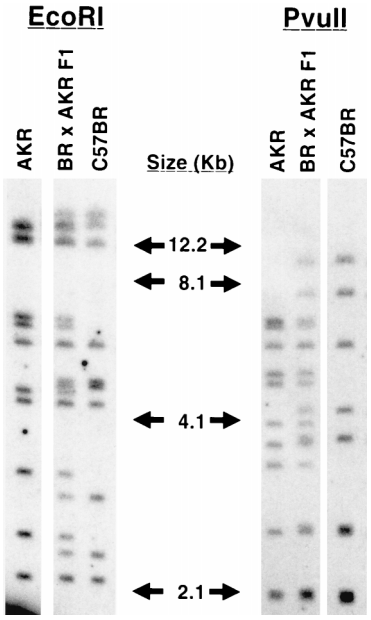


FIG. 2. Modified polytropic proviral content of AKR/cdJ, BRAKF1, and C57BR/J mice. Shown are autoradiograms of *Eco*RI- and *Pvu*II-digested genomic DNAs from C57BR/J female, AKR/J male, and BRAKF1 uninfected mice fractionated in agarose gels that were dried and directly hybridized to a radioactive JS-4 probe. Arrows show positions of *Hin*dIII-digested lambda DNA molecular size standards.

could mask allelic deletions. Thymic masses derived from M-MuLV-infected mice generally contain a very large majority of tumor cells as seen by flow cytometry (reference 5 and unpublished results). Furthermore, TCR-beta gene rearrangement can be used as a marker for T-lymphoid cells (26); it also can be used to screen clonal tumor cell populations for the presence of contaminating non-tumor cells (or tumors arising from separate clones). Contamination would result in decreased hybridization intensity of specifically rearranged TCR-beta-specific bands in Southern blot analysis. Fourteen M-MuLV-induced BRAKF1 tumors were analyzed for rearrangements at the TCR-beta chain locus. Southern blot analysis for TCR-beta rearrangement (5) showed that all 14 tumors had rearranged TCR-beta loci (data not shown), and for most tumors germ line-sized TCR-beta-specific restriction fragments were absent.

As an initial genome-wide screen for LOH in M-MuLV induced leukemia, DNAs from 52 thymic tumors were analyzed for endogenous proviral contents. Figure 3 shows representative results from this analysis. Modified polytropic (Fig. 3A and B), polytropic (Fig. 3C and D), and xenotropic (Fig. 3E and F) proviral patterns in tumor DNAs were visualized by using both *Eco*RI and *Pvu*II digests followed by gel hybridization with class-specific oligonucleotide probes (JS-4, JS-5, and JS-6/10). Losses of proviral bands in tumors were detected by comparing patterns in tumor DNAs with patterns in DNAs from uninoculated BRAKF1 animals. The lack of (or significant reduction in) intensity of a proviral band in a tumor DNA was considered an LOH event.

However, not all differences between the proviral patterns of tumor DNAs and DNAs from uninoculated animals reflected LOH events. In particular, differences due to sex also were evident. As expected, depending on whether the tumor was from a male or a female BRAKF1 animal, it lacked either *Pmv6* or *Pmv34*. For example, Fig. 3D shows that tumors 47, 48, and 49 (from male mice) lacked the polytropic proviral marker *Pmv6*. This marker is located on the X chromosome of the AKR male parent, and therefore it would not be present in BRAKF1 males. The lack of marker *Pmv6* in tumors 47, 48, and 49 is also shown in Fig. 3C. Similarly, Fig. 3C shows that tumors 46, 50, 51, 52, and 53 (from female mice) and the uninoculated BRAKF1 female mouse lacked the polytropic proviral marker *Pmv34*. *Pmv34* is located on the Y chromosome of the AKR male parent, so it would not be present in BRAKF1 females.

Figure 3A and B display modified polytropic proviral patterns in tumors as *Eco*RI and *Pvu*II junction fragments, respectively. The loss of the proviral marker *Mpmv19* in tumor 50 is shown in Fig. 3B. This marker is located on chromosome 4 in the BRAKF1 mice, and it was donated by the AKR/J parent. The loss of *Mpmv19* from tumor 50 was not evident in Fig. 3A because the *Mpmv19 Eco*RI junction fragment comigrated with another junction fragment that did not show LOH. Figure 3C and D display polytropic proviral patterns in tumors as *Eco*RI and *Pvu*II junction fragments, respectively, and both indicate that a second LOH represented by polytropic proviral marker *Pmv25* was present in tumor 50 DNA. Like *Mpmv19*, *Pmv25* is located on chromosome 4 in BRAKF1 mice, and it is also donated by the AKR/J male parent. Figure 3E and F display xenotropic proviral patterns in tumor DNAs. No losses of xenotropic proviral markers were detected in our screen of M-MuLV-induced lymphomas. The analysis shown in Fig. 3 provided firm evidence for LOH on chromosome 4 in tumor 50. This tumor lost two proviral markers that are both located on chromosome 4 and donated by the AKR/J male parent.

Figure 3C also shows that additional novel *Eco*RI junction fragments were present in most of the tumor DNA samples when the JS-5 (polytropic) probe was used. These proviral bands likely reflected infectious, replication-competent, recombinant mink cell focus-forming (MCF) viruses that appear during M-MuLV-induced tumorigenesis (35). In M-MuLV-infected animals, recombination in the envelope gene occurs between the infecting M-MuLV and an endogenous polytropic provirus. The resulting MCF recombinant virus contains primarily M-MuLV-derived sequences as well as a portion of the envelope gene derived from the polytropic virus. MCF proviruses are typically found integrated in M-MuLV-induced tumors, and MCF viruses may play roles in both early and late stages of leukemogenesis (6, 9, 11, 17, 19, 55, 72, 88). Since the region of *env* recombination includes the JS-5 oligonucleotide probe sequences, MCF recombinant proviruses hybridize with the polytropic class-specific probe. MCF virus-specific bands were evident as *Eco*RI but not *Pvu*II junction fragments in Fig. 3C and D. This was expected, since there should be only one internal *Eco*RI site in an M-MCF recombinant provirus, located in the polytropic *env* gene 5′ of JS-5, leading to novel viral-host cell junction fragments larger than 2 kb. The presence of discrete junction fragments was consistent with the mono/oligoclonal nature of M-MuLV-induced T lymphomas. In contrast, *Pvu*II sites are located internally and in the M-MuLV LTR, so that all MCF proviruses give a restriction fragment of 1.3 kb that hybridizes with JS-5 regardless of the insertion site; this fragment migrated out of the agarose gel shown in Fig. 3C. It was interesting that no evidence for MCF virus recombinants was observed when the modified polytropic probe JS-4 was used (Fig. 3A). MCF virus recombinants whose *env* sequences originate from an *Mpmv* endogenous virus have been reported (18, 80). However, while MCF virus recombinants generated by other MuLV strains (e.g., Friend MuLV) frequently contain *Mpmv*-derived *env* sequences, most M-MuLV-derived MCF viruses contain *Pmv*-derived *env* (18). Thus, the lack of detection of MCF proviruses in Fig. 3A was

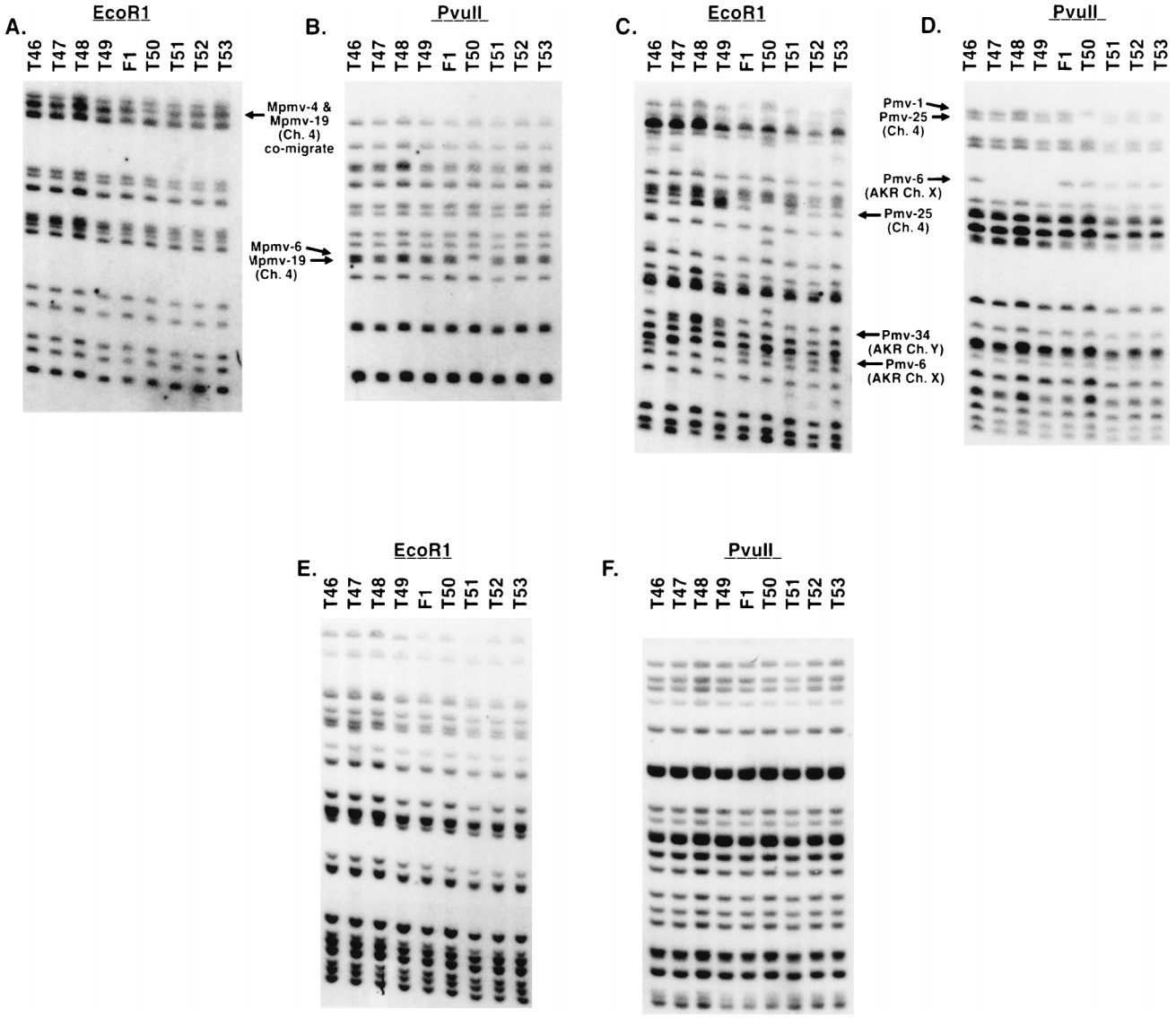


FIG. 3. LOH on chromosome 4 in M-MuLV-induced BRAKF1 tumors. Shown is a comparison of the endogenous modified polytropic (A and B), polytropic (C and D), and xenotropic (E and F) proviral contents of wild-type BRAKF1 and M-MuLV-induced BRAKF1 tumor DNAs. Forty-eight polymorphic (informative) endogenous proviral markers on 16 autosomes and the X chromosome could be identified. Autoradiograms of *Eco*RI- and *Pvu*II-digested tumor DNAs that were fractionated in agarose gels, dried, and directly hybridized to JS-5 (A and B), JS-4 (C and D), and JS-6/10 (E and F) are shown. F1, BRAKF1 control DNA from an uninfected BRAKF1 female mouse. The eight different tumor samples in each panel are denoted with the animal number preceded by T. Arrows in each panel indicate endogenous proviral markers described in the text. Ch., chromosome.

supportive of the notion that formation of MCF virus recombinants by M-MuLV generally involves only endogenous viruses of the polytropic class.

In the initial screen, a second tumor with the same pattern of LOH as tumor 50 was identified. Like tumor 50, tumor 19 lost proviral marker *Pmv25*. In addition, it also lost *Mpmv19*, as indicated in Fig. 4. These were the only two tumors with LOH identified in the initial screen.

Table 1 summarizes the results of the initial screen for LOH in M-MuLV-induced tumors by using the endogenous proviral markers. In total, DNAs were genotyped with 45 polymorphic endogenous proviral markers distributed on 16 of 19 mouse autosomes (and 2 endogenous proviral markers on the X chromosome). Although LOH can occur at random in tumor cells, the overall frequency of chromosomes showing LOH was low (0.2%), with most chromosomes showing no allelic loss (Table 1). As mentioned above, of 52 tumors examined, the only LOH events detected were in 2 tumors (tumor 19 and tumor 50). Tumors 19 and 50 each lacked endogenous proviruses *Pmv25* and *Mpmv19*, located on mouse chromosome 4. Moreover, both of these endogenous proviruses were derived from the AKR/J parent in BRAKF1 mice. Although the genome-wide rate of LOH was low, chromosome 4 showed a much higher rate of 3.8% in the initial survey. This rate was highly significantly different from the background genome-wide rate ($P < 0.0005$). Since LOH was relatively common at *Pmv19* and *Mpmv25* and since previous studies have implicated mouse chromosome 4 as containing a tumor suppressor gene(s) (34, 40), this supported the possibility that chromosome 4 contains a gene or genes in which inactivation is important for M-MuLV-induced tumorigenesis.

Endogenous viral *env* probes were useful in rapid genome

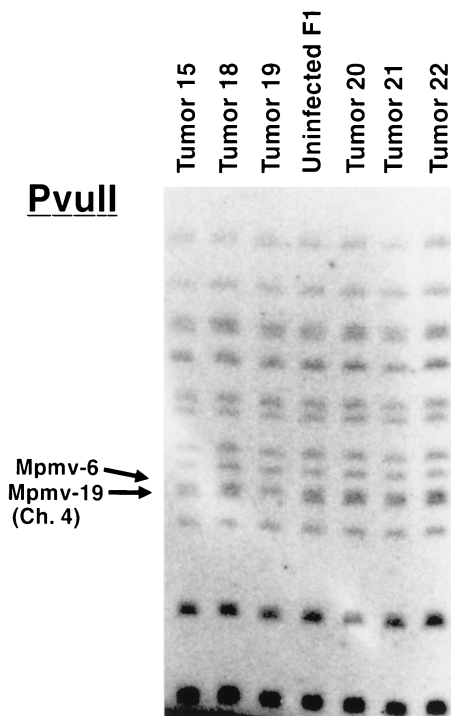


FIG. 4. LOH on chromosome 4 in tumor 19. Shown is a comparison of the endogenous modified polytropic content of wild-type BRAKF1 and eight additional M-MuLV-induced BRAKF1 tumor DNAs. An autoradiogram of *Pvu*II-digested genomic DNA fractionated in an agarose gel that was dried and directly hybridized to radioactive JS-5 probe as described in the legend to Fig. 3 is shown. The lower arrow indicates *Mpmv19*, a marker that is located on chromosome 4 (Ch. 4) in BRAKF1 mice and is donated by the AKR/J male parent. Tumor 19 lacked *Mpmv19*. The upper arrow indicates *Mpmv6*, a marker located on chromosome 1.

scanning for allelic losses, since they detected loci on multiple chromosomes simultaneously. However, approximately half of all allele loss events would be missed by this approach, because for any given endogenous proviral locus, only loss of the parental chromosome containing the integrated provirus could be visualized; loss of the parental chromosome lacking that endogenous provirus would not be detected. Furthermore, in BRAKF1 mice, loss of the C57BR-derived chromosome 4 could not be detected by using this technique, since neither endogenous provirus was on this chromosome. Thus, the actual frequency of LOH on chromosome 4 in the M-MuLV-induced tumors was expected to be approximately twice the rate observed in the initial screen. Another limitation of the initial screen was that informative endogenous proviral markers could not be resolved on chromosomes 14, 15, and 17.

Fortunately, a genetic map of the mouse, consisting of thousands of PCR-amplifiable SSLPs, has been developed (14). Therefore, PCR amplification of SSLP markers was used to screen the tumor set for additional LOH events on chromosome 4 and also for LOH on chromosomes 14, 15, and 17. Figure 5 illustrates PCR-SSLP analysis of M-MuLV tumor DNAs for LOH. The set of 52 tumors was examined for LOH at one SSLP-based locus each on chromosomes 4 (*D4Mit27*), 14 (*D14Mit115*), 15 (*D15Mit186*), and 17 (*D17Mit66*). The approximate positions of these SSLPs on the linkage maps of their respective chromosomes are indicated (Fig. 5) and have been described previously (reference 14 and the MIT Genome Database). SSLP markers were visualized by separation of PCR products by gel electrophoresis followed by ethidium bromide staining. Both parental alleles at each SSLP locus could be identified, and lack of (or significant reduction in) intensity of an SSLP-specific band in a tumor DNA was considered an LOH event. LOH events were confirmed by repeating the PCR-SSLP analysis on tumor DNA samples at least three times. No LOH events were detected on chromosomes 14 and 17, but one LOH event was detected on chromosome 15 in one tumor (tumor 2) (Fig. 5). In addition, a third tumor (tumor 73) was identified that lost the SSLP marker on chromosome 4. Additional SSLP markers from chromosome 4 were also lost in this tumor (see below). As predicted, tumor 73 lost SSLP markers from the C57BR parent but not from the AKR parent; this LOH would not have been detected in the initial screen with endogenous proviruses. SSLPs were also used to test for loss of material from chromosome 4 in tumors 19 and 50; as expected from the initial screen with endogenous proviruses, the chromosome 4 SSLP characteristic of the AKR parent was lost in both of these tumors.

Since the predominant and statistically significant LOH in M-MuLV-induced tumors on chromosome 4 was based on the lower-resolution genome-wide screen, LOH on this chromosome was examined in finer detail. To determine the common regions of loss on chromosome 4 in tumors 19, 50, and 73 and to thus infer the potential position of the putative tumor suppressor gene(s), a dense collection of 14 SSLPs distributed on chromosome 4 was examined. Figure 6 shows PCR-SSLP analysis of five SSLPs on chromosome 4 to illustrate identification of the LOH breakpoints. Of the three tumors, tumor 73 showed losses of all C57BR-specific chromosome 4 SSLP alleles, suggesting loss of the entire chromosome. However, some but not all chromosome 4 SSLP markers were lost in the

TABLE 1. Initial screen for marker loss in 52 tumors by using endogenous proviral markers

Chromosome[a]	Markers per chromosome[b]	% (no.) of tumors with marker loss[c]
1	5	0.0 (0)
2	3	0.0 (0)
3	3	0.0 (0)
4	2	3.8 (2)[d]
5	4	0.0 (0)
6	1	0.0 (0)
7	3	0.0 (0)
8	2	0.0 (0)
9	2	0.0 (0)
10	1	0.0 (0)
11	8	0.0 (0)
12	3	0.0 (0)
13	2	0.0 (0)
14	0	NA[e]
15	0	NA
16	4	0.0 (0)
17	0	NA
18	1	0.0 (0)
19	1	0.0 (0)
X	2	0.0 (0)

[a] Chromosome numbers in boldface type represent those for which polymorphic (informative) endogenous proviral markers originating from both parents (C57BR/cdJ and AKR) could be identified.

[b] Number of polymorphic (informative) endogenous proviral markers analyzed for LOH on each chromosome.

[c] Percentage and number of tumors with LOH at any endogenous proviral marker along each chromosome. A total of 52 tumors was examined in each case.

[d] The difference from the background rate (average FAL) of 0.2% was significant ($P < 0.0005$).

[e] NA, not available since there were no polymorphic endogenous proviral markers.

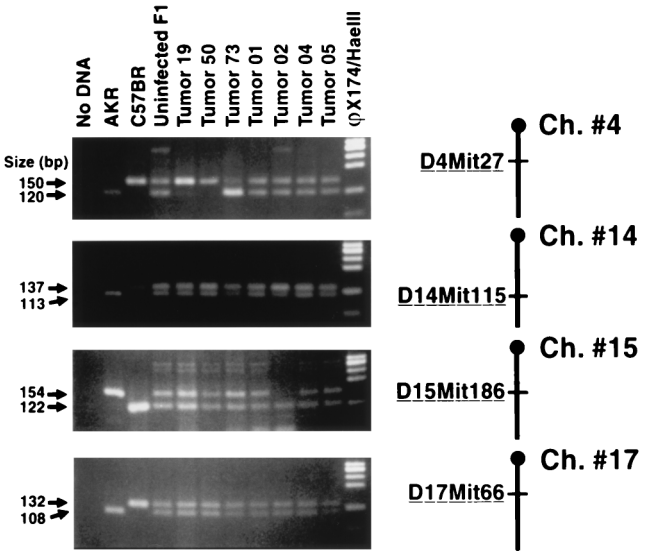


FIG. 5. LOH analysis of M-MuLV-induced tumor DNA samples with SSLP markers. Shown are examples of genotyping of the loci *D4Mit27*, *D14Mit115*, *D15Mit186*, and *D17Mit66* on DNAs from seven tumors, with parental (AKR/J and C57BR/cdJ) DNA, wild-type BRAKF1 DNA, and no-DNA samples as controls. The approximate chromosomal location of each SSLP locus is indicated to the right. PCR products were fractionated in agarose gels, stained with ethidium bromide, and photographed. *Hae*III-digested ϕX174 DNA molecular size standards are shown in the right lane. Sizes of SSLP-specific alleles for each parent of each locus are indicated to the left of arrows that denote their positions. Tumors 19 and 50 showed loss of the AKR allele and LOH and tumor 73 showed loss of the C57BR allele of *D4Mit27*. The low level of the 150-bp (C57BR/cdJ) fragment in tumor 73 DNA might have resulted from PCR amplification of contaminating normal cells in the tumor sample. Tumor 2 showed loss of the AKR allele of *D15Mit186*. These allelic losses were consistently reproducible. All tumors have retained both parental alleles at *D14Mit115* and *D17Mit66*. Although some tumors show possible allelic imbalances (e.g., *D14Mit115* in tumor 73 and *D17Mit66* in tumor 5), they were not evident in additional assays of the same DNA samples. Ch., chromosome.

other two tumors. For example, tumor 19 retained both parental alleles (C57BR/cdJ and AKR/J) for *D4Mit2*, *D4Mit108*, and *D4Mit271*, but it lacked the AKR alleles for *D4Mit27* and *D4Mit13*. Tumor 50 retained both parental alleles for *D4Mit2* and *D4Mit108*, but it lacked the AKR alleles for *D4Mit271*, *D4Mit27*, and *D4Mit13*. Thus the common region of LOH on chromosome 4 for these tumors was approximately the distal 45 centimorgans (cM) (Fig. 6). We also tested whether other M-MuLV-induced tumors in the collection had smaller regions of LOH or deletions on chromosome 4 that might have escaped detection by the endogenous provirus markers. The entire tumor set was rescreened with five additional SSLPs (*D4Mit82*, *D4Mit62*, *D4Mit69*, *D4Mit12*, and *D4Mit13*) located within the 45-cM region of common LOH (Fig. 7). However, no additional tumors with LOH at these loci were identified.

Ultimately, study of a larger collection of tumors should make it possible to define more precisely the common region of LOH and thus predict the location of a putative tumor suppressor gene(s). In advance of this, the potential involvement of potential known tumor suppressors could be inferred from the likely positions of the human tumor suppressor homologs based on synteny conservation between mouse and human chromosomes. The human homologs of genes within the common region of LOH (approximately the distal 45 cM of mouse chromosome 4) have been mapped to human chromosomes 9 and 1. Of these regions, 9p21 was particularly notable because it is a hot spot for LOH in a wide variety of human tumor types, including melanomas and leukemias (47, 63). The human 9p21 region includes the interferon gene cluster and the putative tumor suppressor genes *Mts1* ($p16^{INK4a}$) and *Mts2* ($p15^{INK4b}$). High frequencies of homozygous deletion of these loci have been reported for a variety of human cancer cell lines and primary tumors (47, 63, 67). These genes, several other mouse genes that map to the region of common LOH, and the assigned human syntenies are indicated in Fig. 7. It seemed possible that the LOH events in the M-MuLV-induced tumors with LOH on chromosome 4 may have reflected loss of the *Mts1* ($p16^{INK4a}$) and/or *Mts2* ($p15^{INK4b}$) genes. Therefore, the three tumors with allelic losses on chromosome 4 were tested for homozygous deletions in these two genes by Southern blot hybridization. For comparison, four tumors without apparent allelic loss on chromosome 4 were analyzed in parallel. The cDNA sequences of mouse *Mts1* ($p16^{INK4a}$) and *Mts2* ($p15^{INK4b}$) genes have been cloned and reported (68), so radioactive hybridization probes were prepared for exon 2 of *Mts1* ($p16^{INK4a}$) and exon 2 of *Mts2* ($p15^{INK4b}$) by PCR amplification from BRAKF1 mouse DNA. The Southern blot hybridizations indicated, however, that homozygous deletions were not detected in either *Mts1* ($p16^{INK4a}$) or *Mts2* ($p15^{INK4b}$) in any of the seven tumors examined. Bands representing *Mts1* ($p16^{INK4a}$) were present in parental DNAs (AKR and C57BR), in uninfected BRAKF1 DNA, and in each tumor DNA examined (not shown). All lanes were loaded with the same amount of total DNA, and the intensities of the hybridizations in the lanes representing tumors 19, 50, and 73 were approximately one-half of those of the other DNAs. This was expected since these tumors presumably lacked one copy of the *Mts1*

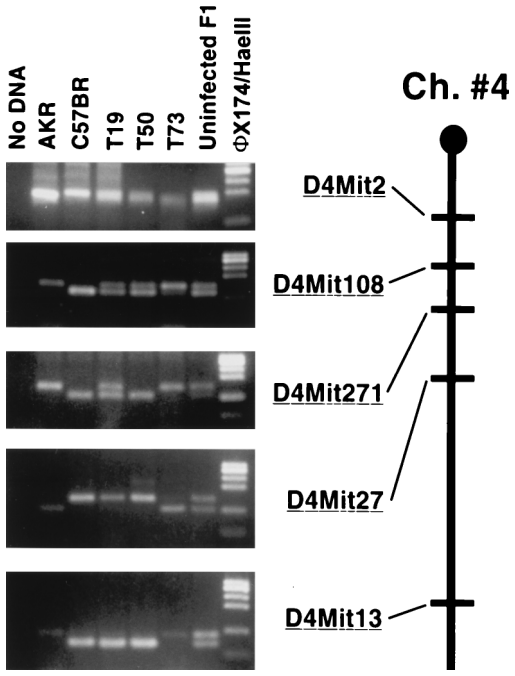


FIG. 6. PCR-SSLP analysis of chromosome 4 markers to define the common region of LOH in tumors 19, 50, and 73. To the left are scanned images of photographs of ethidium bromide-stained agarose gels of SSLP markers *D4Mit2*, *D4Mit108*, *D4Mit271*, *D4Mit27*, and *D4Mit13* that were PCR amplified from AKR/J and C57BR/cdJ parental, uninfected BRAKF1, and tumor 19, 50, and 73 DNAs. No DNA, PCRs that lacked template genomic DNA. To the right are indicated the approximate locations of markers on the linkage map of mouse chromosome (Ch.) 4. Tumor 19 DNA lacked the AKR alleles of *D4Mit27* and *D4Mit13*. Tumor 50 DNA lacked the AKR alleles of *D4Mit271*, *D4Mit27*, and *D4Mit13*. Tumor 73 DNA lacked the C57BR alleles of all five SSLP markers shown.

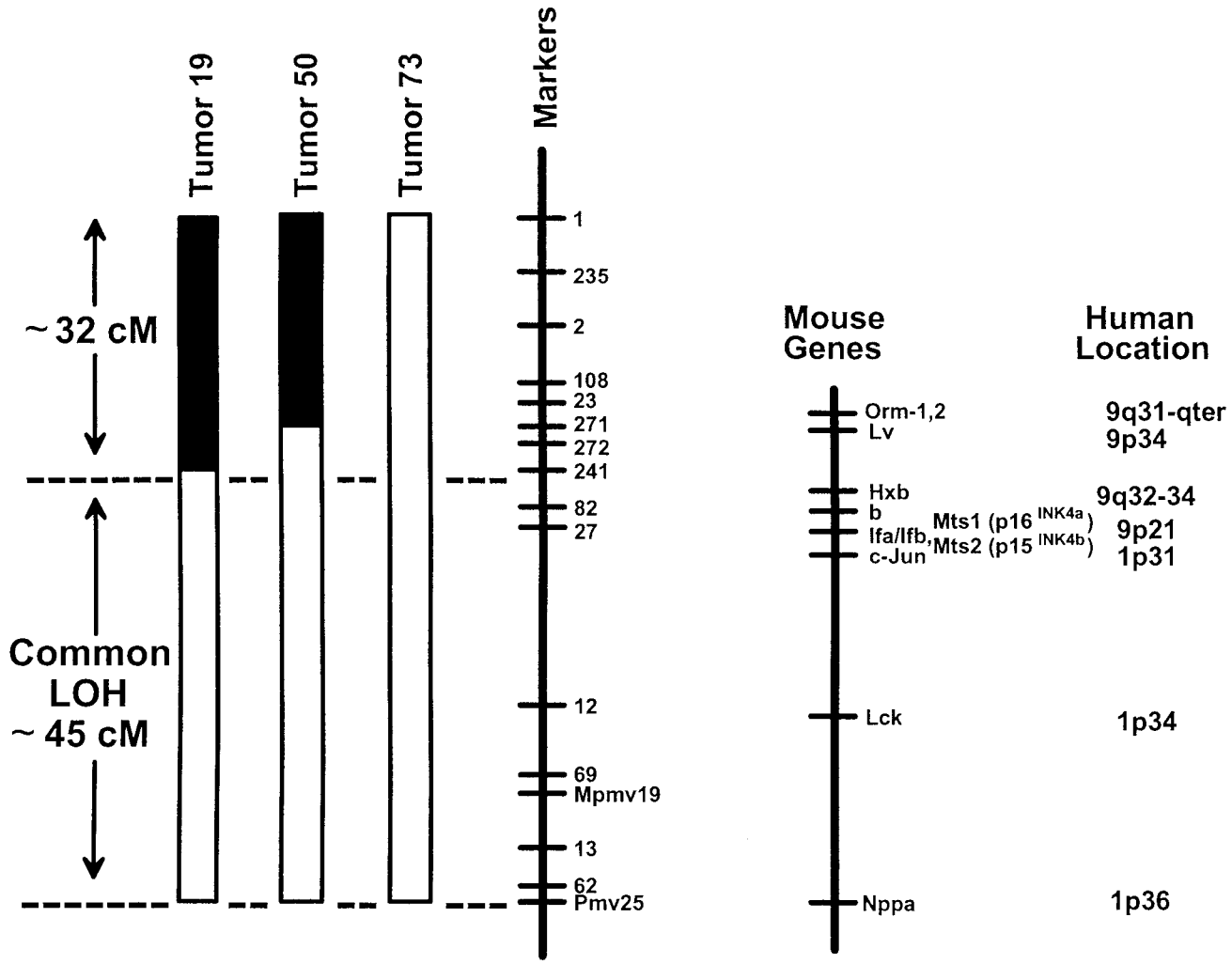


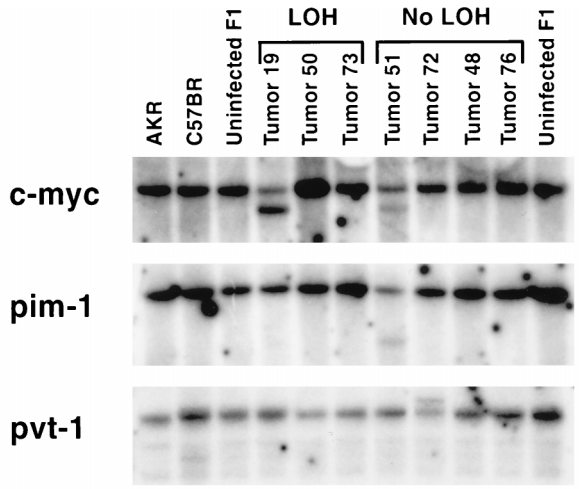
FIG. 7. Mapping the common region of LOH on chromosome 4. Chromosomes 4 from tumors showing LOH are shown on the left. A map of mouse chromosome 4 showing endogenous provirus and SSLP markers used for LOH analysis is shown in the center. The names of the SSLP markers are abbreviated to omit the prefix *D4Mit*. A genetic map showing the locations of known mouse genes on chromosome 4 is shown on the right. On the far right are the approximate cytogenetic locations of human homologs where these are known. For chromosome 4 in each of the tumors, filled areas represent regions where both parental SSLP alleles were retained, while open areas correspond to regions where markers from one of the parental chromosomes were missing. The smallest region of LOH overlap, corresponding to approximately the distal 45 cM of chromosome 4, is delineated by a dashed horizontal line.

($p16^{INK4a}$) gene. The same reduction in signal intensity in tumors 19, 50, and 73 was observed when *Mts2* ($p15^{INK4b}$) was examined by Southern blot analysis. Thus, the tumors with LOH on chromosome 4 did not appear to contain homozygous deletions of either *Mts1* ($p16^{INK4a}$) or *Mts2* ($p15^{INK4b}$). However, only large-scale deletions would be observed by this method, and it was possible that smaller inactivating deletions or point mutations that escaped detection were present.

One additional question was whether the putative inactivation of a tumor suppressor on chromosome 4 could substitute for activation of cellular proto-oncogenes in M-MuLV-induced tumors. The common mechanism of tumorigenesis in tumors induced by MuLVs is insertional activation of cellular proto-oncogenes (20, 21). In mice, T-lymphoblastic lymphomas induced by wild-type M-MuLV typically show proviral insertions adjacent to at least one of three cellular loci, i.e., c-*myc*, *pim-1*, or *pvt-1* (11, 33, 72, 75). It seemed possible that in M-MuLV-induced tumors with LOH on chromosome 4, tumor suppressor gene inactivation substitutes for proto-oncogene activation. Therefore, we tested whether the tumors that showed LOH on chromosome 4 lacked proviral insertions adjacent to c-*myc*, *pim-1*, and *pvt-1*. Tumor DNAs were tested by Southern blot hybridization with radioactive hybridization probes for each of these three loci as described previously (22). As indicated in Fig. 8, one of three tumors with allelic losses on chromosome 4 had a proviral integration near c-*myc*. In comparison, two of four tumors without detectable allelic loss on chromosome 4 had proviral integration near c-*myc*, *pim-1*, and/or *pvt-1*. Thus, LOH on chromosome 4 did not appear to obviate the need for proto-oncogene activation in at least some cases.

DISCUSSION

In this work, we studied a collection of 52 M-MuLV-induced T lymphomas for inactivation of tumor suppressor genes by searching for LOH. The primary screen was carried out by performing a rapid genome-wide survey for loss of endogenous proviral markers. Subsequent screening was carried out by PCR amplification of SSLP markers for chromosomes that did



Summary

1 of 3 tumors with Ch. 4 LOH show insertions
2 of 4 tumors without LOH show insertions

FIG. 8. Southern blot analysis of proviral insertions near c-*myc*, *pim-1*, and *pvt-1* in selected M-MuLV-induced tumor DNAs. Tumor DNAs were digested with *Kpn*I (c-*myc*) or *Eco*RI (*pim-1* and *pvt-1*), separated by agarose gel electrophoresis, and transferred to GeneScreen Plus. Membranes were hybridized with a c-*myc*, *pim-1*, or *pvt-1* probe, respectively. In the scanned autoradiograms shown, lanes representing AKR and C57BR parental and uninfected BRAKF1 DNAs indicate the positions of germ line bands. Novel bands in tumor DNAs indicated rearrangement of the gene locus, presumably due to proviral (M-MuLV or M-MCF) insertions. As shown, comparison of three tumors that showed chromosome 4 LOH events (tumors 19, 50, and 73) and four tumors that did not show chromosome 4 LOH (tumors 51, 72, 48, and 76) indicated that tumors 19 and 51 were rearranged at c-*myc*, tumor 51 was rearranged at *pim-1*, and tumor 72 was rearranged at *pvt-1*.

not contain informative endogenous proviral markers. The results indicated that the overall frequencies of endogenous proviral and SSLP marker loss were low in comparison to those found in allelotype studies of human tumors. Despite the low overall frequency of LOH observed, statistically significant ($P < 0.0005$) loss of chromosome 4 was observed. This suggests that a subset of M-MuLV-induced T-lymphomas may involve loss of a tumor suppressor gene located on mouse chromosome 4.

In LOH studies, a useful measure is the fractional allelic loss (FAL). The FAL in a tumor is defined as the number of chromosomal arms in which allelic loss is observed divided by the number of chromosomal arms for which allelic markers are informative (90). Thus, FAL values can be used to compare the extents of LOH in tumors within a collection, average FAL values can be used to compare the genomic stabilities of different tumor types, and FAL values are important in assessing the significance of specific LOHs in a tumor collection. Average FAL values for human tumors of different types have been reported to range from 0.125 (for bladder cancer [49]) to 0.32 (for osteosarcoma [95]) (73, 87, 90); i.e., in bladder cancer, 12.5% of evaluable chromosome arms show allelic loss. The average FAL for childhood acute lymphocytic leukemia (ALL) has been reported to be 0.123 (84). The average FAL in the M-MuLV-induced lymphomas studied here was much lower, 0.002 (four allelic losses observed for 1,716 evaluable chromosome arms). This indicated a high genetic stability of these tumors and that genetic events leading to M-MuLV-induced leukemia are only rarely accompanied by LOH. It also suggested that the rate of tumor suppressor gene inactivation in M-MuLV-induced T lymphomas is low in comparison to that in most human tumors.

Reports of recent LOH studies with other murine tumor systems have also described lower average FAL values than are found in human tumors. FAL values for different chemically induced tumors have ranged from 0.0004 (for urethane-induced hepatocellular carcinoma [57]) to 0.054 (for methylene chloride-induced lung carcinoma [39]). In insulinomas arising in genetically predisposed transgenic mice, the average FAL was 0.045 (15). The average FAL values for 2′,3′-dideoxycytidine- and 1,3-butadiene-induced T lymphomas in B6C3F1 mice were 0.044 and 0.042, respectively (96). Thus, the average FAL observed for the M-MuLV-induced T lymphomas was consistent with the generally lower FAL values of rodent tumors; the FAL of 0.002 determined in our study was the second lowest reported.

The low FAL rate observed in this study could result from several possibilities. One technical explanation could be that higher frequencies of LOH occurred but the endogenous proviral and SSLP markers did not detect them. However, this seems unlikely, since the number and distribution of these markers were comparable to those for restriction fragment length polymorphism-, SSLP-, and motif-primed PCR-based markers used in allelotype studies of human and murine tumors in which higher frequencies of LOH were observed (15, 39, 49, 73, 84, 87, 90, 95, 96). Moreover, our studies did identify LOH on chromosomes 4 and 15, and rescreening of the entire tumor collection with a dense set of SSLP markers distributed across chromosome 4 did not identify additional tumors with LOH beyond the original three.

A second technical possibility could be that additional LOHs occurred in M-MuLV-induced tumors but escaped detection due to contaminating normal DNA or because the tumors were oligoclonal in nature. Contamination with normal DNA was probably not a problem, since cells extruded from thymic masses of M-MuLV-induced T lymphomas (the method used for DNA extraction) are virtually pure tumor cell populations, as measured by flow cytometry or Southern blot hybridization for TCR-beta gene rearrangements (5). In a previous study of mouse tumors that used SSLP-PCR analysis, LOH could be reliably detected in the presence of as much as 30% contaminating normal-cell DNA (15). In our case, the initial screening for endogenous proviral markers was probably even less sensitive to contaminating DNA than PCR-based analyses, since it entailed direct in-gel hybridization without PCR amplification. Oligoclonality of the tumors was a greater potential problem, since oligoclonality of M-MuLV-induced T lymphomas has been previously documented (12). However, where oligoclonality has been observed, the tumors have generally consisted of a major clonal population and one or two minor populations. Thus, for these analyses, oligoclonal tumors may be considered the major tumor clone, with contaminating minor clones. Genetic changes detected in an oligoclonal tumor would reflect those of the majority clone, while changes in the minor clones would likely escape detection. We have found it possible to reliably detect a 50% reduction in band intensity in the hybridizations for endogenous proviral markers. Thus, it should have been possible to detect LOH in a majority clone in an oligoclonal tumor, as long as it comprised 50% or more of the tumor mass.

A third explanation for the rare LOH in M-MuLV-induced tumors could be that tumor suppressor genes might be inactivated by mechanisms that do not involve LOH, such as point mutations on both chromosomes, small deletions, translocations, or proviral insertion into the tumor suppressor gene.

Indeed, in erythroleukemia induced by the Friend virus complex (a mixture of Friend MuLV and oncogene-containing Friend SFFV), one of the oncogenic steps involves inactivation of one of the alleles of the p53 tumor suppressor gene by proviral insertion (2, 3). The remaining allele is inactivated by either point mutation or deletion (44). Thus, inactivation of tumor suppressor genes by proviral insertion in M-MuLV-induced T lymphomas is also possible.

The fourth and most likely possibility is that the very low FAL in M-MuLV-induced T lymphomas reflected infrequent inactivation of tumor suppressor genes in these tumors. As described in the introduction, the predominant changes that have been characterized in retrovirus-induced leukemias are insertional activations of proto-oncogenes (21). Indeed, sequential proto-oncogene activations may occur in M-MuLV-induced tumors (86). In addition, other virus-induced and spontaneous changes have been described for M-MuLV-induced leukemias (reviewed by Fan [21]). One possible reason for the low frequency of tumor suppressor inactivation in the M-MuLV-induced T lymphomas (and the low frequency in rodent tumors in general) is that these tumors arose relatively quickly (3 to 5 months after virus infection). Some studies with humans have indicated increased FAL with tumor progression (10, 61), and tumors in humans may take much longer to fully progress and be diagnosed than those in rodents.

Given the very low average FAL in these tumors, the finding that 3 of 52 tumors showed LOH for chromosome 4 was very noteworthy. In quantitative terms, 6% of the T lymphomas showed LOH for markers on chromosome 4, compared to the overall average rate of loss of 0.2% (FAL of 0.002) for all chromosomes. The LOH on chromosome 4 was statistically significant, with a P value of <0.0005. This suggests that these three tumors sustained inactivation of a tumor suppressor gene located on mouse chromosome 4. More-detailed analysis with a dense collection of SSLP markers for mouse chromosome 4 mapped the common region of LOH to the distal two-thirds (45 cM) of the chromosome.

Identification of the putative tumor suppressor gene involved in the M-MuLV-induced lymphomas would be very interesting. One approach has been to test known tumor suppressor genes that map to the distal portion of mouse chromosome 4 for deletion. This region of mouse chromosome 4 is syntenic with human chromosomes 1 and 9, including 9p. Frequent deletions of human chromosome 9p21–22 have been reported for numerous cancer types, including malignant melanoma (27), glioma (42, 66), lung cancer (58, 59, 65), head and neck cancer (89), nasopharyngeal cancer (41), familial melanoma (7), and ALL (37, 64, 67, 89). A putative tumor suppressor gene, *Mts1* ($p16^{INK4a}$) (an inhibitor of cyclin-dependent kinase CDK4), was mapped to human chromosome 9p21 by virtue of its homozygous deletion in many tumor cell lines (47, 63). It is noteworthy that homozygous deletions of *Mts1* ($p16^{INK4a}$) are extremely frequent in human T-ALLs (77%) (37) and are also found in precursor B-ALLs (15%) (82). As described in Results, we generated molecular probes for the mouse *Mts1* ($p16^{INK4a}$) gene and the related adjacent *Mts2* ($p15^{INK4b}$) gene and used them to test for homozygous deletions in the tumors with LOH on chromosome 4. However, these tumors did not show homozygous deletion of either gene. Thus, these genes did not appear to be the inactivated tumor suppressors, although inactivation of the remaining allele by point mutations, small deletions, or loss of protein expression (e.g., DNA methylation [32, 46]) would not have been detected.

Studies of human ALL and chemically induced mouse lung tumors have suggested the existence of an additional tumor suppressor gene adjacent to the *Mts2* ($p15^{INK4b}$) and *Mts1* ($p16^{INK4a}$) genes (40, 83, 94). In addition, LOH studies and somatic-cell fusion studies with human and mouse tumors have also suggested that a tumor suppressor gene may also reside on human chromosome 1 and mouse chromosome 4 (31, 34, 45, 71, 76, 96), although the precise location(s) has yet to be determined. When these are identified and cloned, it will be interesting to test them in the M-MuLV-induced T lymphomas.

It is also possible that the M-MuLV-induced T lymphomas with LOH on chromosome 4 had deletions of a novel tumor suppressor gene. Ultimately, it would be desirable to map and positionally clone such a gene. However, given the low frequency of LOH (6%), this would be very difficult. Based on experiments and calculations with another murine tumor system (15), we estimate that it would be necessary to survey over 3,000 M-MuLV-induced T lymphomas to obtain enough tumors with LOH at chromosome 4 to allow us to localize the region of common LOH to 1 cM (the maximum allowable for positional cloning).

It was interesting that the M-MuLV-induced lymphomas did not show evidence for loss of the p53 tumor suppressor gene (*Trp53*). Mouse *Trp53* is located on chromosome 11, and no LOH was observed for this chromosome, even though eight informative endogenous proviral markers spanning the entire chromosome were examined. Inactivation of p53 was plausible, given the fact that it is the most common mutated tumor suppressor gene in human tumors (50, 52, 74). Moreover, as mentioned above, erythroleukemias induced by the Friend MuLV complex show insertional inactivation of p53. Likewise, inactivation of the p53 gene has been described for non-T/non-B lymphomas induced by Cas-Br-E MuLV (4). On the other hand, a recent study of M-MuLV infection in $p53^{-/-}$ or $p53^{+/-}$ mice revealed weak interactions between M-MuLV leukemogenesis and p53 loss (1). Moreover, there was no evidence for insertional mutagenesis of p53 by M-MuLV in tumors from wild-type or $p53^{+/-}$ mice.

The results also showed that one M-MuLV-induced tumor showed LOH of chromosome 15. It is possible that this reflected loss of a tumor suppressor gene. Alternatively, this might have simply reflected the background rate of chromosomal instability. A third possibility is that the chromosome LOH on chromosome 15 might have reflected activation of the c-*myc* proto-oncogene, which is within 5 cM of the *D15Mit186* SSLP marker (Mouse Genome Database [URL: http://www.informatics.jax.org/]). Other studies of M-MuLV-induced lymphomas have reported trisomy of chromosome 15 (93); in addition, M-MuLV-induced tumors with this trisomy that also contain a proviral activation of c-*myc* have duplicated the chromosome with the c-*myc* activation (12). Thus, trisomy of chromosome 15 might be related to overexpression of c-*myc*. At the same time, it has been reported that in mouse tumors overexpressing c-*myc*, the unamplified allele was frequently lost (62). The loss might represent a secondary change to compensate for deleterious effects of chromosome trisomy. Consistent with this, some other tumors in the collection studied here showed allelic imbalance of the two *D15Mit186* alleles as judged by the intensities of the amplified SSLP bands (not shown). However, these tumors did not show proviral insertions at c-*myc* (not shown).

Given the specific LOH on chromosome 4 in a subset of the tumors, it seemed possible that loss of the putative tumor suppressor gene substituted for proto-oncogene activation in these tumors. Thus, these tumors were screened for activation of the proto-oncogenes that are known to be activated in M-MuLV-induced tumors, i.e., c-*myc*, *pim-1*, and *pvt-1*. If the

putative tumor suppressor gene substituted for proto-oncogene activation, then these tumors might lack activations. However, the frequency of proto-oncogene activations for the three tumors with LOH on chromosome 4 was not appreciably different from the frequency observed for four tumors that lacked LOH (one of three versus two of four, respectively). Thus, the putative tumor suppressor inactivation did not appear to substitute for proto-oncogene activations.

The use of endogenous proviral markers to screen for LOH was especially useful for performing initial rapid screening of large numbers of tumor DNA samples. Forty-five endogenous proviruses on 16 of 19 autosomes as well as both sex chromosomes could be screened by a total of six gel hybridizations (digestion with two restriction enzymes and hybridization with three oligonucleotide probes). This afforded substantial savings in labor and also in the amount of tumor DNA used. On the other hand, weaknesses in this technique included the fact that informative proviral markers were not present on three chromosomes in BRAKF1 mice, necessitating secondary screening with SSLP markers for those chromosomes. In addition, for six autosomes in these mice (including chromosome 4), informative endogenous proviral markers were present on only one parental chromosome. In principle, this would have simply reduced the observed frequency of LOH for these chromosomes by 50%. However, it is possible that specific LOHs were missed because of this. For instance, if chromosomal imprinting (8, 15, 39, 54) resulted in preferential loss of the maternal or paternal allele for one of these chromosomes, LOH related to the tumor suppressor gene might have been missed. (All of the BRAKF1 mice used here were the progeny of female C57Br and male AKR mice.) However, this was not a problem at least for the specific LOH on chromosome 4: of the three tumors showing this loss, two lost alleles from the AKR parent and one lost alleles from the C57Br parent.

It seemed possible that LOH might be observed more frequently in tumors induced by acute retroviruses that carry oncogenes. Therefore, we induced fibrosarcomas in BRAKF1 mice by infection with two M-MuLV-based viruses expressing the v-*src* oncogene of avian sarcoma virus [M-MuLV(src) and M-MuLV(+src)] (24, 25). However, when 28 tumors induced by these viruses were screened, only one tumor with an LOH (on chromosome 19) was found.

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