Mouse mammary tumor virus (MMTV) is a latently oncogenic B-type retrovirus that primarily causes murine mammary cancers but also induces T-cell lymphomas at a lower frequency (6, 7, 29). Like other slowly oncogenic retroviruses, MMTV exerts its tumorigenic effects by the activation of cellular proto-oncogenes in the vicinity of integrated proviruses. Using the “proviral tagging” approach, nine common sites of integration, namely, Wnt1, Fgf3, Notch4, Wnt3, Cyp19, Fgf4, Fgf8, Int6, and Wnt10b, have been identified in MMTV-induced mammary tumors (22, 24, 25, 35). Like mammary tumors, MMTV-induced T-cell tumors have acquired new copies of MMTV proviruses in chromosomal DNA in addition to endogenous MMTVs found in the germ line (7, 29). These acquired proviruses have a large deletion (350 to 500 bp) of negative regulatory elements (14) in the long terminal repeat (LTR) U3 region compared to mammotropic MMTVs, and often these deletions are accompanied by duplications of the sequences flanking the deletion (5, 23, 28, 38).

Type B leukemogenic virus (TBLV) is a replication-competent thymotropic retrovirus whose genome is >98% identical to that of milk-borne MMTV (3, 6). Unlike MMTV, which induces mammary tumors after a long latent period (6 to 9 months), TBLV induces a high incidence of T-cell lymphomas in mice after a very short latent period (42 to 55 days) (4). Like changes in other MMTVs that induce lymphomas, the alterations in the U3 region of the TBLV LTR include a deletion of 440 nucleotides and a 122-nucleotide substitution, consisting of sequences flanking the region (5). When linked to a c-myc transgene, the TBLV LTR has been shown to induce CD4+ CD8– murine T-cell lymphomas, similar to those produced by TBLV injection (31). These results suggest that overexpression of c-myc RNA from the TBLV LTR is sufficient to initiate T-cell disease in transgenic mice. Additional experiments indicate that infection of TBLV LTR-myc-transgenic mice with MuLV accelerates leukemogenesis. Thus, activation of c-myc leads to initiation of lymphomas, but other genes are required for progression (10).

Currently, only one “common” region of proviral integration (Tblvi1) has been identified in 20% of 55 primary TBLV-induced tumors; this site maps to the mouse X chromosome and activates one or more hitherto-unidentified genes (30). To identify additional common integration sites, 30 TBLV-induced primary tumors were generated by intrathymic inoculation of newborn mice with concentrated virus as described previously (27). Tumors arose after 2 to 3 months in approximately 40 to 50% of the mice, within the thymus, spleen, and lymph nodes and often in more than one of these tissues. Southern blot analysis of tumor cells revealed proto-oncogene rearrangements (see above).

The c-myc locus is rearranged in TBLV-induced primary tumors. Proviral insertions near the c-myc proto-oncogene are relatively common among lymphoid tumors induced by retroviral infection. Therefore, independently derived TBLV-induced tumors were screened for virally induced rearrangements near c-myc. Of the 30 tumors screened, 2 (7%), namely, T16 and T17, showed novel bands hybridizing to the pSVcmyc1 probe (19) by Southern blotting (Fig. 1A). PCR and Southern blotting analysis indicated that the proviral integrations in the T16 and T17 tumors are ca. 0.5 and 1.9 kb, respectively, downstream and in the same transcriptional orientation as the c-myc gene (Fig. 1B). To determine if the tumors induced by TBLV were clonal, DNA was extracted from the T16 and T17 tumors after intraperitoneal passage in weanling BALB/c mice. As detected by Southern blotting, both the T16 and T17 tumors showed a change in the proviral integration pattern during passage, and the T16 integration pattern simplified during passage (Fig. 2A). These results suggested that the original tumor was a heterogeneous population, although a dominant cell type could be detected as assessed by T-cell receptor β-chain rearrangements (see above). To determine if tumor cell growth selects for TBLV integrations near c-myc, Southern blotting was used to monitor viral integration sites after passage of the T16 and T17 tumors.
FIG. 1. Analysis of proviral insertions near the c-myc locus in TBLV-induced T-cell tumors. (A) Southern blot analysis of TBLV-induced tumors. Tumors were induced by intrathymic inoculation of concentrated TBLV from the 485-10 cell line (6) into BALB/c or C57BL/6 mice. Tumor DNAs (15 μg) were digested with EcoRI, BamHI, EcoRV, or XbaI prior to Southern blotting and hybridization with the pSVcmyc1 probe (19); this probe contains genomic DNA spanning c-myc exons 2 and 3. DNA extractions, Southern blotting, and hybridizations were performed as described previously (27). Genomic tumor cell DNA derived from different organs within the same animal showed identical patterns of additional TBLV integrations, indicating a common origin (data not shown). No rearrangements were detected in DNA from other TBLV-induced tumors spanning approximately 48 kb of the c-myc locus (25 kb upstream and 23 kb downstream of the locus) (data not shown). The positions of molecular size markers are shown. (B) Diagram of TBLV insertions in the c-myc locus. The c-myc gene is transcribed (5' to 3') from left to right, in the same transcriptional orientation as the proviruses in the T16 and T17 tumors. The location of TBLV insertions was confirmed using one primer from the c-myc gene [c-myc 695(+) 5' GGA CTG TAA GCT TCA GCC ATA 3'] and another from the TBLV LTR [TBLV LTR 546(-) 5' TTG GGA ACC GCA CCT GTT CTT 3']. PCR was performed using the Expand long-template PCR system (Boehringer Mannheim). Sequencing was performed as described previously (36). The positions of some restriction enzyme sites are shown: RV, EcoRV; B, BamHI; X, XbaI; H, HindIII; RI, EcoRI. The two coding exons are shown as white boxes, and the 5' noncoding exon is shown by a shaded box. The location of the pSVcmyc1 probe, used for Southern hybridizations, is shown.
in vivo (Fig. 2B and C). As expected, data obtained after one or two tumor passages in mice showed retention of the TBLV provirus near c-myc in the primary tumor. In addition, we observed a second TBLV integration close to c-myc after two passages of the T17 tumor in mice (Fig. 2B, lane 7). Thus, it appears that TBLV insertions near c-myc are selected during tumor passage in vivo.

**Activation of c-myc expression in TBLV-induced tumors.** To determine if c-myc was overexpressed in TBLV-induced tumors, we performed Northern analysis on total RNA extracted from a number of TBLV-induced tumors. The T16 and T17 tumors showed a three- to fivefold overexpression of c-myc RNA relative to normal thymus RNA after normalization for RNA loading on the gel transfers by hybridization to a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (9) (Fig. 3A and B). No novel c-myc transcripts were detected. With the exception of the T7 and T9 tumors, similar results were obtained with other TBLV-induced tumors (2- to 5.6-fold c-myc overexpression). Three T-cell lymphomas that were not induced by TBLV (ERLD, C6XL, and ASLI) had c-myc levels that were 0.9 to 2.1 times the level in normal C3H mouse thymus (Fig. 3C). Thus, the level of c-myc expression was elevated in most TBLV-induced tumors tested, although many tumors had no TBLV integration near c-myc detectable by Southern blotting (data not shown).

**Cloning and chromosomal mapping of the Pad7 locus.** Because of the low number of TBLV-induced tumors with detectable c-myc integration, we attempted to identify additional common TBLV integration sites. After screening of a partial lambda phage library from the T8 tumor (containing three TBLV insertions), we obtained an integrated TBLV provirus and cellular flanking sequences. The locus identified by this probe was designated Pad7. Southern analysis on other tumor DNAs using three different restriction enzymes suggested that the Pad7 locus is not a common integration site in TBLV-induced tumors.

To determine the chromosomal map position of the Pad7 locus and its possible colocalization with known cellular oncogenes, we tested the progeny of two sets of multilocus crosses: (NFS/N × C58/J × Mus musculus musculus) × M. m. musculus (18) and (NFS/N × Mus spretus) × M. spretus or C58/J (1) by Southern blotting and hybridization with the Pad7 probe. No recombination was observed between Myc and Pad7 in 198 mice, indicating that, at the upper limit of the 95% confidence level, Myc and Pad7 are within 1.5 centimorgans of each other (Fig. 4).

DNAs digested with 13 rare-cutting enzymes were analyzed by pulsed-field gel electrophoresis (15) to identify fragments containing Pad7, Myc, and Pvt1. The Pvt1 locus also is a common integration site for murine leukemia viruses on mouse chromosome 15 and is located approximately 270 kb from c-myc (17, 34). No physical linkage could be established between Pad7 and either Myc or Pvt1 (data not shown).

As in leukemias induced by other retroviruses, c-myc appears to be activated two- to sixfold by an enhancer insertion mechanism in most TBLV-induced T-cell lymphomas, and this conclusion is supported by our failure to detect new c-myc transcripts that are initiated within the TBLV LTR. It has been proposed that murine leukemia virus proviruses integrated in the Pvt1 or Mlvi4 locus activate c-myc expression by long-range (up to 300 kb) cis effects (20, 21, 33). Similar long-range effects on c-myc expression also have been observed in mouse or human tumors carrying chromosomal translocations that juxtapose the c-myc gene to other cellular gene enhancers (12, 13, 16, 26, 39). Thus, one explanation for c-myc overexpression is that the TBLV proviral enhancer exerts its effect over a very long distance (>300 kb). Long-range effects of TBLV integrations could be due to proviral insertions that affect a locus control region; such regions may coordinate control of multiple genes, including those near Pad7.
FIG. 3. Elevated c-myc expression in TBLV-induced T-cell lymphomas. Total RNA (20 μg) was analyzed using 1.0% agarose gels containing 2.2 M formaldehyde as described previously (27). The positions of the 18S and 28S rRNAs are shown. RNA was extracted as described previously (37). C3H mouse normal thymus RNA was used in lanes 1 and 3, whereas RNA extracted after one passage of the T8 tumor was used in lane 2. RNAs extracted from TBLV-induced tumors after one or two intraperitoneal passages (P1 or P2) in mice are shown in lanes 6 to 8. RNAs from the T17 tumor were obtained from two separately injected mice. Lanes 1 and 2 were derived from a different gel than lanes 3 to 8. (A) Northern blot hybridized to the pSVcmyc1 probe (19). (B) Northern blot hybridized to a GAPDH probe. The blots used for panel A were stripped prior to hybridization with the GAPDH probe. (C) Levels of c-myc RNA in TBLV-induced T-cell lymphomas. Total RNAs from normal thymus or T-cell tumors were analyzed on Northern blots and hybridized to c-myc and GAPDH probes. Levels of c-myc RNA after normalization to GAPDH levels relative to normal C3H mouse thymus RNA (assigned a value of 1) are shown. In some cases, intact RNA was not available from primary tumors. The T-cell lines ERLD, C6XL, and ASL-1 (2, 7, 27) were derived from spontaneous or X-ray-induced tumors appearing in animals that were not infected with TBLV. RNA from passage 1 of the T17 tumor was prepared from two different animals.
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