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Provirus Integration at the 3′ Region of N-*myc* in Cell Lines Established from Thymic Lymphomas Spontaneously Formed in AKR Mice and a [(BALB/c × B6)F_1→AKR] Bone Marrow Chimera

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Among 18 thymic leukemia cell lines which have been established from spontaneous thymic lymphomas in AKR mice as well as in bone marrow chimeras which were constructed by transplanting allogeneic bone marrow cells into irradiated AKR mice, three proviral integration sites were identified; near c-*myc*, N-*myc* and *pim*-1 loci. No integration site specific for chimeric leukemia cell lines was found. In three thymic leukemia cell lines which contained rearranged N-*myc* genes, insertions of long terminal repeats (LTRs) of murine leukemia viruses were detected at 18 or 20 bp downstream of the translational termination codon. These results demonstrate that the 3′ region of the N-*myc* gene is one of the integration targets for murine leukemia viruses in spontaneous thymic lymphomas. In these three cell lines, N-*myc* mRNA was stably transcribed and transcription of c-*myc* mRNA was down-regulated. The integrated murine leukemia viruses in AKR thymic leukemia were most likely AKV, though the DNA sequence of the LTR inserted in the genome of a leukemic cell line from [(BALB/c × B6)F_1→AKR], CAK20, was different from LTRs of murine leukemia viruses so far reported.

Key words: Thymic lymphoma — Bone marrow chimera — Murine leukemia virus — N-*myc*

In AKR mice, thymic lymphomas develop spontaneously by proviral insertion of ecotropic murine leukemia viruses (MuLV) into the genome of thymic lymphocytes. The retrovirus infects thymic lymphocytes through thymus epithelium.[1-4] In bone marrow chimeras MuLV infection occurs through thymus epithelium to the transplanted thymic lymphocytes, thus inducing thymic lymphomas.[5] However, the mechanisms of lymphomagenesis in this special situation have remained obscure.

By infecting Moloney and mink cell focus-forming (MCF) MuLV into new-born mice of AKR, C57BL/10 and BALB/c strains, two genes have been implicated in the onset of thymic lymphoma; c-*myc*, which regulates cell proliferation,[6-8] and *pim*-1, which has structural homology to tyrosine kinase genes.[9-11] More recently, frequent integration of Moloney MuLV in the 3′ flanking region of N-*myc* was reported.[12] Therefore, *pim*-1 may act synergistically with either c-*myc*[9] or N-*myc*[12] in the leukemogenesis of murine T cells. This assumption was substantiated by the finding that thymic lymphomas produced by neonatal injection with Moloney MuLV into *pim*-1 transgenic mice showed integration of Moloney MuLV provirus exclusively near either c-*myc* or N-*myc* locus.[13]

We report here that AKV-like proviruses were integrated 18 bp downstream of the translational stop codon of N-*myc* in spontaneous AKR thymic leukemia cell lines. In one thymic leukemia cell line which was established from a lymphoma in a bone marrow chimera, [(BALB/c×B6)F_1→AKR], the integration site was 20 bp downstream of the stop codon of the N-*myc* gene. In the latter, the DNA sequence of the long terminal repeat (LTR) inserted was not the same as that of any retroviral LTR so far reported. The sites where these retroviruses were integrated were close to the site which has been reported for Moloney MuLV.[12] Thus, the third exon of N-*myc* may be a preferential insertion site for not only LTR of Moloney MuLV but also LTR of various endogenous murine leukemia viruses.

MATERIALS AND METHODS

Cell lines CAK1.3, CAK4.4, CAK30, CAK31 were thymic leukemia cell lines from bone marrow chimeras constructed by transplanting BALB/c *nu/nu* bone marrow cells into 8.5 Gy-irradiated AKR mice. Methods of induction and the properties of CAK1.3 and CAK4.4 were reported in detail.[1] CAK21 and CAK24 were

thymic leukemia cell lines from bone marrow chimeras constructed similarly by transplanting BALB/c +/+ bone marrow cells into AKR mice. CAK20 was a thymic leukemia cell line from a bone marrow chimera constructed similarly by injecting (BALB/c×B6)F_1 bone marrow cells into an AKR mouse. CAK32 was a thymic leukemia cell line from a bone marrow chimera constructed similarly by injecting C3H bone marrow cells into an AKR mouse. CAK21 and CAK28 were thymic leukemia cell lines from chimeric mice which had been constructed in the following way: first, BALB/c bone marrow cells were injected into irradiated AKR mice, then the thymuses were removed and transplanted into BALB/c mice. NAK13, NAK19, NAK34, NAK36, NAK37, NAK38, NAK39 and L176 were cell lines derived from spontaneous thymic lymphomas in AKR mice. NOD1 was a cell line derived from a lymphoma that had spontaneously occurred in an NOD mouse.

Cell surface markers Various monoclonal antibodies (mAbs) were used for staining cell surface antigens. Anti-CD3 (145-2C11),[14] anti-TCR V_β 8 (F23.1)[15] and anti-B220 (RA3-6B2)[16] were provided by Drs. J. A. Bluestone, N. J. Bevan, and R. L. Coffin, respectively. All these mAbs were affinity-purified from culture supernatants or ascites. Anti-CD3 was biotinylated, and anti-TCR V_β 8 and anti-B220 were conjugated with fluorescein isothiocyanate (FITC) by standard methods. Biotinylated anti-Thy-1.2 (30-H12), phycoerythrin (PE)-conjugated anti-CD4 (G1.5) and FITC-conjugated anti-CD8 (53-6.3) were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA). Anti-Thy-1.2 (2D3) mAb was produced at this institution, and its specificity was determined by the reactivity with T-cells from Thy-1.1-bearing and Thy-1 congenic mice (S. Kobayashi, unpublished data). Two-color flow cytometry was performed in a FACScan (Beckton Dickinson). Cells were incubated with a mixture of biotin-anti-Thy1.2 and FITC-anti-B220 or a mixture of biotin-anti-CD3 and FITC-anti-TCR V_β 8 followed by PE-streptoavidin (Biomeda Corp., Foster City, CA). Cells were also stained with a mixture of PE-anti-CD4 and FITC-anti-CD4. For staining Thy1.1 by indirect immunofluorescence, cells were reacted with anti-Thy1.1 culture supernatant followed by FITC rabbit anti-mouse immunoglobulin (Zymed Laboratories, Inc., South San Francisco, CA). Dead cells were gated out on the basis of propidium iodide uptake and cell size. The data from 10,000 cells were accumulated on list mode by using a FACScan program, processed, and displayed as two-dimensional contour plots of the green vs. red logarithmic fluorescences.

Southern blot hybridization analyses Southern blot hybridization was carried out as follows. After electrophoresis of restriction enzyme-digested high-molecular DNA in a 0.8% agarose gel, the DNA was transferred to nitrocellulose membranes with 10×SSC (1×SSC; 150 m*M* sodium chloride, 10 m*M* sodium citrate). Hybridization was carried out using nick-translated probes at 42°C in a solution containing 50% formamide, 6×SSPE (1×SSPE; 150 m*M* sodium chloride, 10 m*M* sodium phosphate, and 1 m*M* EDTA, pH 6.5), 5× Denhardt's solution (50× Denhardt's solution; 1% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS) and 100 μg/ml single-stranded *E. coli* DNA. The membranes were washed twice with 2×SSC and 0.1% SDS at room temperature and finally with 0.1×SSC and 0.1% SDS at 60°C. DNA probes used were as follows: an approximately 7.7 kb *Eco*RI fragment of mouse N-*myc*[17] which was cloned by Taya *et al.*,[18] a 1.8 kb *Eco*RI/*Bam*HI fragment corresponding to the 5′ flanking region and a half of the first exon of N-*myc*, a 0.8 kb *Hinc*II/*Eco*RI fragment corresponding to the non-coding region of the third exon of N-*myc*, an approximately 6 kb *Bam*HI/*Bam*HI fragment of mouse c-*myc* which contained the 2nd and the 3rd exons,[19] a 1 kb *Hae*II/*Xba*I fragment of v-*myb*,[20] a 1 kb *Ava*I/*Eco*RI fragment of cDNA corresponding to the constant region and 3′ untranslated region of mouse TCR_γ chain,[21] a 1 kb *Pst*I/*Pst*I fragment of v-*fos*,[22] a 1.4 kb *Bgl*II/*Bgl*II fragment of v-*abl*,[23] a 0.8 kb *Pst*I/*Pst*I fragment corresponding to mouse TCR_β chain,[24] a 0.9 kb *Bam*HI/*Bam*HI fragment of murine *pim*-1,[10] a 1.7 kb *Eco*RI/*Eco*RI fragment of murine *lck* cDNA,[25] a 0.5 kb *Bgl*II/*Bgl*II fragment in the 3rd exon of mouse L-*myc*,[26] and a 0.5 kb *Pst*I/*Pst*I fragment of rat B-*myc*.[27]

Analysis of mRNA Total RNA was extracted by the method of Chomczynski and Sacchi[28] with slight modifications. The RNA (20 μg) was fractionated in a 1% agarose gel containing 2.2 *M* formaldehyde[29] and transferred to a nitrocellulose membrane with 20×SSC. Hybridization conditions were the same as for the Southern blot hybridization except that 250 μg/ml of single-stranded *E. coli* DNA was used. For superinduction of c-*myc* and N-*myc* mRNA by cycloheximide (CHX) treatment, 1×10^6 cells/ml were cultured for 16 h in RPMI1640 containing 0.5% fetal calf serum (FCS) and then cultured in RPMI1640 containing 10% FCS with or without 10 μg/ml CHX for 2.5 h. Then total RNA was extracted as described above.

Polymerase chain reaction (PCR) PCR[30] was carried out using the 5′GGCGAGGCAGCAGCAGTTGC3′ sequence (spanning from 6,255 to 6,274 of mouse N-*myc*[17]) as a 5′ primer and either an antisense 20-base sequence in MuLV U3 (5′ATGCCTTGCAAAATG-GCGTT3′) or a sense 20-base sequence in MuLV U5 (5′-CTCGCTGTTCCTTGGGAGGG3′) as the 3′ primer. These LTR-related oligodeoxynucleotides were chosen as a consensus sequence of Moloney MuLV, AKV and

MCF viruses.[7, 31–33] A twenty-five cycle amplification was carried out using annealing and polymerization temperatures of 55 and 72°C, respectively. The amplified DNA fragments were analyzed by 2% agarose gel electrophoresis.

DNA sequence The amplified DNA fragments containing N-*myc* and LTR junction were cut out, eluted from the gel and sequenced by the dideoxy chain termination method[34] with a modification involving the use of Taq DNA polymerase as recommended by the manufacturer's protocol (Taq-Track; Promega Corporation, Madison, MI). Sequences were determined using the two primers used for PCR amplification and an additional internal N-*myc* primer (5'ACGCTCGGACTTGCTAAACG3'; spanning from 6,293 to 6,312 of N-*myc*).

RESULTS

Establishment and surface phenotypes of thymic leukemia cell lines Table I summarizes the surface phenotypes of the cell lines used in this study. Expressions of *lck* mRNA and Thyl antigen were used as criteria for T-cells. T-cell lines derived from [BALB/c→AKR] bone marrow chimeras expressed Thyl.2 antigen, indicating their origin from bone marrow donors. Surface expression of CD4 and CD8 was studied to determine differentiation stages of cultured T-lymphoma cell lines. These cell lines were classified as $CD4^+/CD8^+$, $CD4^-/CD8^-$, $CD4^+/CD8^-$ and $CD4^-/CD8^+$. CAK21 was a mixture of various phenotypes, but its clonality was not studied. A portion of $CD4^-/CD8^-$ cell lines expressed TCR_γ (assayed by Northern analysis) and TCR_β (assayed by examining surface expression of V_β 8 antigen and by Northern analysis). The presence of B220 on the cell surface of two cell lines suggested that this group of cell lines was deviated from immature thymocytes found in the thymus. Cell lines of $CD4^+/CD8^+$ phenotype were TCR_γ-negative and mostly TCR_β-positive. All five $CD4^+/CD8^-$ cell lines expressed TCR_β but not TCR_γ. This finding suggested that these cell lines might have originated from relatively mature cells in the thymus. However, immediately after establishment of the cell lines, almost all thymic leukemia cell lines were either $CD4^+/CD8^+$ or $CD4^-/CD4^-$. During passage for over 2 years, 3 out of 6 double-positive cell lines (CAK1.3, CAK32 and NOD1) became $CD4^+/CD8^-$. The phenotype of CAK1.3 reported here (Table I) was apparently shifted from that previously reported.[1] Consistent presence of rearranged c-*myc* in CAK1.3 supported the occurrence of phenotypic change and excluded the possi-

Table I. Surface Phenotypes, TCR mRNA Expression and DNA Rearrangements of Leukemia Cell Lines

	Surface marker						mRNA		DNA rearrangement		
	Thyl	CD3	CD4	CD8	$V_\beta 8$	B220	TCR_γ	TCR_β	*pim*-1	c-*myc*	N-*myc*
CAK4.4	2	−	−	−	−	±	+		+	−	−
CAK20	2	+	−	−	−	−	−		−	−	+
NAK19	1	−/+	−	−	+/−	−	−		−	−	−
NAK34	1	+	−(+)	−	−	−	+	+	+	−	−
NAK36	1	+	−	−	+	±	−	+	−	−	+
NAK38	1	+	−(+)	−	+	−	+	+	−	−	−
CAK28	2	+	+	+	+	−	−	+	−	+	−
CAK31	2	+	+	+	−	−	−		−	−	−
CAK32	2	−	+	+(−)	−	−	−		−	−	−
NAK13	1	+/−	+	+	−	−	−	+	−	−	−
NAK39	1	−(+)	+	+	−	−	−		+	+	−
CAK1.3	2	+	+	−	+	−	−	+	−	+	−
CAK22	2	+/−	+	−	+/−	−	−	+	−	−	−
NAK37	1	+	+(−)	−	+	−	−	+	−	−	−
L176	1	+	+−	−	+	−	−		+	−	+
NOD1	2	+	+	−	−	−	−		−	−	−
CAK30	2	+	−(+)	+	−	−	+	+	−	−	−
CAK21	2	+	+/−	+/−	+	−	−		−	−	−

+/− and −/+ represent continuous differences in expression within clones. ± represents the presence of two populations with similar numbers. +(−) and −(+) represent the presence of two populations, but the number of that in parenthesis is relatively low. The level of TCR_β mRNA was generally low, so that in some cell lines the absence of the message could not be confirmed.

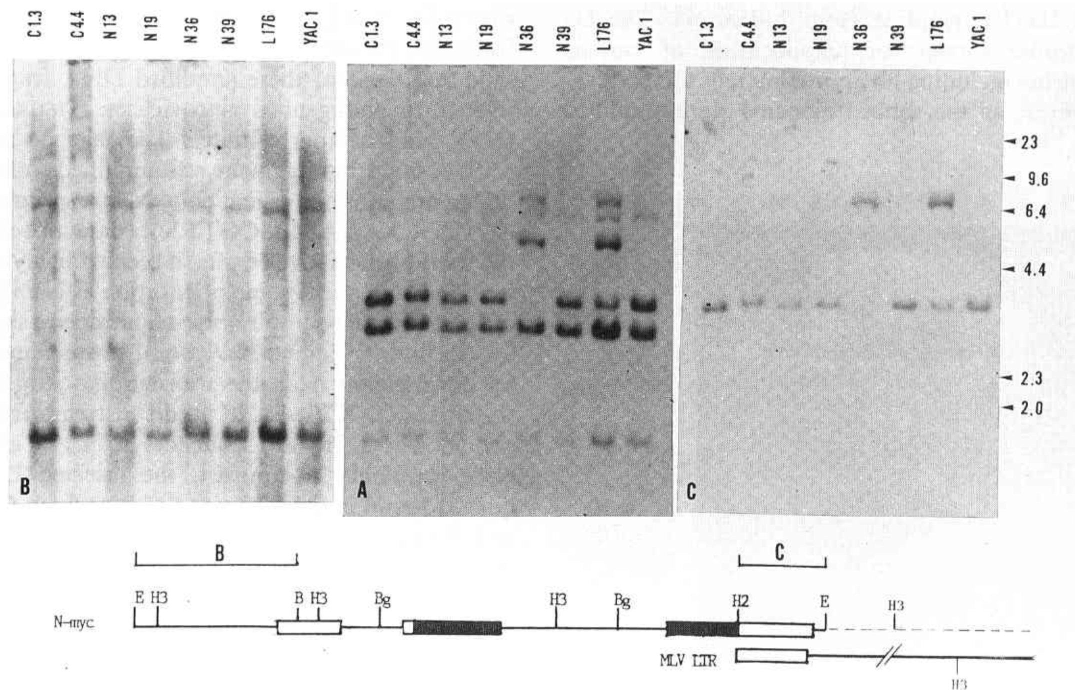

Fig. 1. Rearrangement of N-*myc*. DNA was digested with *Hin*dIII and transferred to a nitrocellulose membrane. A. Hybridized with 7.7 kb *Eco*RI fragment of mouse N-*myc* (probe A). B. The same membrane was rehybridized with 5′ *Eco*RI/*Bam*HI fragment (probe B). C. The same membrane was rehybridized with 3′ *Hin*cII/*Eco*RI fragment (probe C). C and N indicate CAK and NAK, respectively. The numbers on the right-hand side indicate the lengths of the size markers in kb.

bility of contamination with different clones during passages (data not shown). These results suggest that the established thymic leukemia cell lines may represent a random collection of subpopulations of thymic lymphocytes found in adult thymuses. No consistent relationship was found among origin of cells, surface phenotypes, TCR expressions and the oncogenes which were activated by retroviral insertion.

Insertion of retrovirus near *pim*-1, c-*myc* and N-*myc* genes This investigation was started in order to search for retroviral integration near cellular oncogenes having homologies to c-*myc*, since no attempt to do this had been made at that time. Southern hybridization analysis indicated that three cell lines (CAK1.3, NAK39 and CAK28) out of 18 cell lines studied had acquired LTR insertion near the c-*myc* gene. In these three cell lines, in contrast to the preferential insertion sites of injected MCF virus at the 5′ region of the first exon of *c-myc*,[8] retrovirus was inserted at the 3′ flanking region of c-*myc* (data not shown). We found three cell lines which had MuLV insertion near the N-*myc* locus. Fig. 1A shows a Southern hybridization analysis of selected cell lines probed with 7.7 kb genomic N-*myc* fragment (probe A). NAK36 and L176 showed an identical N-*myc* rearrangement. An additional cell line, CAK20, also showed a similar rearranged pattern of N-*myc* region (data not shown). By probing with 5′ (probe B, Fig. 1) as well as 3′ (probe C, Fig. 1) fragments of N-*myc*, it was concluded that MuLV insertion took place at sites near a unique *Hin*cII site which exists 61 bp downstream from the coding region. NAK36 had lost heterozygosity during the establishment of the cell line and only rearranged N-*myc* was left (Fig. 1). Cell lines which contained rearranged L-*myc* or B-*myc* gene were not found.

We also screened for rearrangement of other proto-oncogenes, such as *pim*-1, c-*abl*, *lck* and c-*fos*, and found four cell lines (CAK4.4, NAK34, NAK39 and L176) containing retroviral insertions near *pim*-1 (Table I).

DNA sequence analysis of the LTR-insertion sites near N-*myc* locus In order to analyze LTR-insertion sites in these cell lines, DNA fragments between upstream from termination codon of N-*myc* and MuLV LTR were amplified by PCR. The pattern obtained on agarose gel electrophoresis of amplified DNA fragments is shown in Fig. 2. When U3 antisense primer was used as one of the two primers for amplification, fragments of approximately 130 bp were specifically amplified from DNA of L176, NAK36 and CAK20 (Fig. 2, lanes 1, 2 and 3), but

not from NIH3T3 template (Fig. 2, lane 4). The U5 antisense primer served for amplification of various DNA fragments including an approximately 130 bp fragment. However, as the same fragments were amplified also from NIH3T3 template, it was unlikely that these fragments contained the N-*myc*/LTR junction. It is possible that some of these amplified DNA fragments were related to endogenous retroviruses. The most intense DNA band of approximately 1 kb had no relevance to MuLV, since this fragment was amplified from these four DNA preparations using only N-*myc* primer (5′ATGCCTTGCAAAATGGCGTT3′) (data not shown). On the basis of the above results, it seemed likely that MuLV were inserted in the same orientation as N-*myc*. The sites of LTR insertions into N-*myc* sequence are shown in Fig. 3A. In both L176 and NAK36, the insertion site was 18 bp downstream from the translational stop codon, and the site in CAK20 was 20 bp downstream from the stop codon. The LTR U3 sequences of L176 and NAK36 were identical. In contrast, the inserted LTR U3 sequence found in CAK20 was different from that of L176 and NAK36 at several positions; C→T(22), C→A(28), A→G(29), and A→G(34) (Fig. 3B).

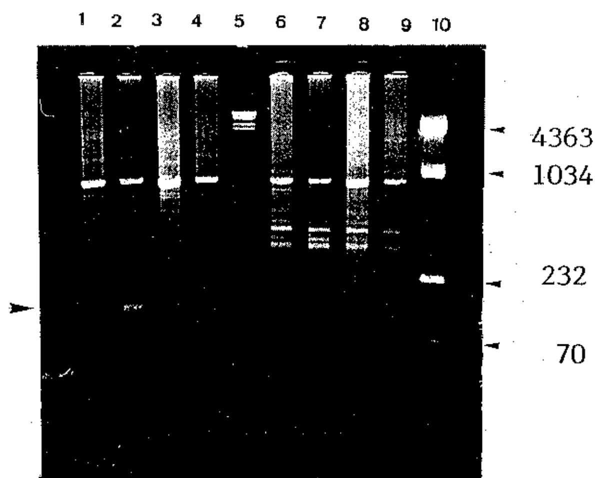

Fig. 2. Gel electrophoresis of PCR-amplified DNA fragments. After amplification, portions of the samples were electrophoresed in a 2% agarose gel. Lanes 1 and 6, CAK20; lanes 2 and 7, NAK36; lanes 3 and 8, L176; lanes 4 and 9, NIH3T3; lane 5, *Hin*dIII digested DNA; lane 10, *Pst*I-digested v-*fos* plasmid. Lanes 1–4 were DNA fragments amplified using an LTR U3 antisense primer and lanes 6–9 using an LTR U5 sense primer. An arrowhead indicates the position of a specifically amplified DNA fragment of approximately 130 bp. The lengths of *Pst*I-digested v-*fos* plasmid size marker are given in bp.

Expressions of N-*myc* and down regulation of c-*myc* genes Fig. 4 shows the steady-state expression and superinduction with CHX of both c-*myc* and N-*myc* mRNAs in thymic leukemia cell lines. N-*myc* mRNA expression was detected only in the cell lines which had retrovirus insertion at the 3′ region of N-*myc*. In these cell lines c-*myc* expression was undetectable. All other cell lines, irrespective of the presence of retroviral insertion near the c-*myc*, expressed c-*myc* mRNA. In Fig. 4, c-*myc* expression in CAK1.3 was not visible, though in other experiments the presence of c-*myc* mRNA was demonstrated clearly (data not shown).

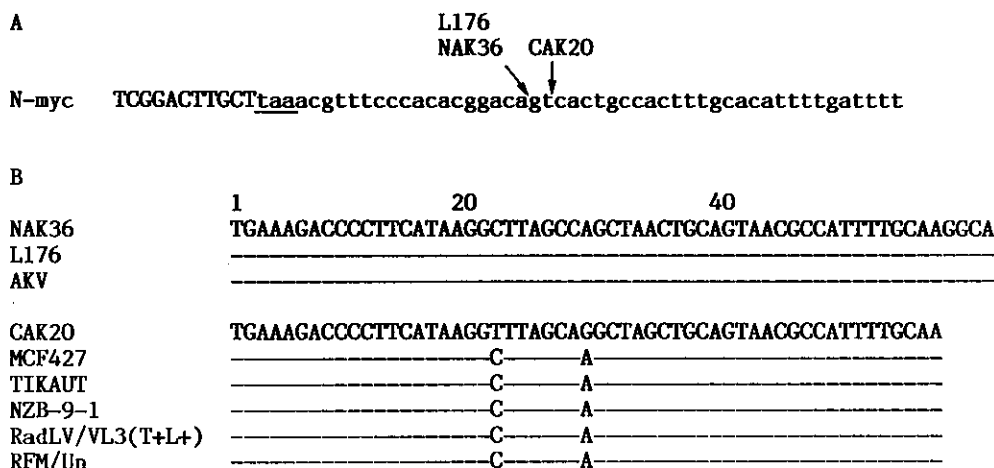

Fig. 3. DNA sequences around LTR insertion at the 3rd exon of N-*myc*. A. The sites of LTR insertion into N-*myc* sequence are shown. The N-*myc* sequence was taken from DePinho *et al.*[17] Upper case letters represent coding sequence and lower case letters, non-coding sequence. Arrows indicate the insertion sites. The underlined triplet is a stop codon. B. Partial sequences of inserted LTR. — indicates the same base as the top lines. Sequences of AKV, MCF247, Tikaut, NZB9-1, RadLV/VL3(T+L+), and RFM/Un were taken from references 33, 31, 7, 44, 43, and 42, respectively.

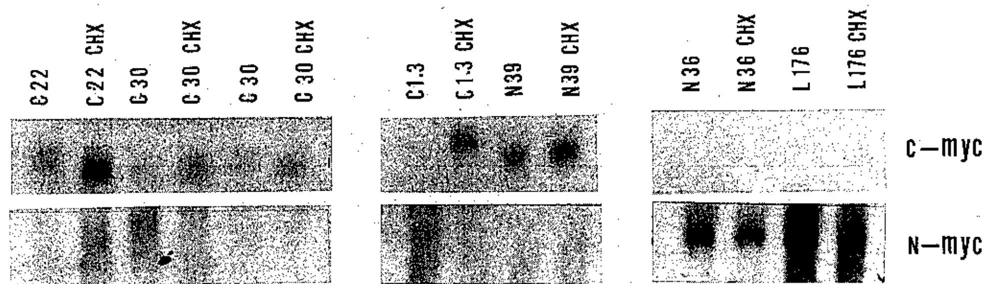

Fig. 4. Superinduction of c-*myc* and N-*myc* mRNA by CHX treatment. C22 and C30 are CAK22 and CAK30: they did not have LTR insertion around either c-*myc* or N-*myc*. C1.3 and N39 are CAK1.3 and NAK39: they had LTR insertion at the 3′ flanking region of the c-*myc* locus. N36 (NAK36) and L176 had LTR insertion at the 3rd exon of N-*myc*.

Upon CHX treatment, expression of c-*myc* mRNA was superinduced. The cell line which contained LTR insertion at 3′ of the N-*myc* gene was refractory to CHX treatment. N-*myc* mRNA was not superinduced and c-*myc* mRNA was not induced. The stability of N-*myc* mRNA may be caused by replacement of sequences responsible for the instability by a polyadenylation site within retroviral LTR and constitutive expression of N-*myc* gene caused down-regulation of c-*myc* expression.

DISCUSSION

Insertions of retroviruses around N-*myc* in the mouse genome have been reported from other laboratories. The inserted MuLV were either experimentally infected Moloney MuLV[12, 13] or Moloney-like MuLV resident in monocyte/plasmacytoma fusion cells.[35] The results of this report extend the findings to spontaneous thymic lymphomas.

Out of 18 spontaneous thymic lymphoma cell lines studied by Southern hybridization analyses, 4 lines showed *pim*-1 rearrangements, 3 c-*myc* rearrangements and 3 N-*myc* rearrangements. In NAK36, L176 and CAK20, MuLV LTR was inserted at the 3′ region of N-*myc*. The integration sites were close to each other and near the stop codon (Fig. 1), and the orientation of the inserted LTRs was the same as that of N-*myc* (Fig. 2). Partial sequence analysis revealed that the integration sites were 18 bp 3′ of the N-*myc* stop codon in both NAK36 and L176, and 20 bp downstream in CAK20 (Fig. 3). The integration sites in Moloney MuLV-induced thymic lymphomas reported by van Lohuizen *et al.*[12] were close to the stop codon of N-*myc* and one of the integration sites that was sequenced was located 21 bp downstream of the stop codon. Setoguchi *et al.*[35] reported an integration site of Moloney MuLV-like endogenous virus 18 bp upstream of the stop codon.

The mechanism of leukemogenesis through synergy of *myc*-group and *pim*-1 oncogenes[2, 6, 11] may also apply to spontaneously occurring thymic lymphoma in AKR mice as well as [BALB/c→AKR] bone marrow chimeras. Selten *et al.*[11] reported that BALB/c mice which were infected with Moloney MuLV at the new-born stage developed thymic lymphomas with high frequency of proviral integrations near *pim*-1 (46%) as well as near c-*myc* (50%). O'Donnell *et al.*[8] also reported a high incidence of LTR insertion upstream of the c-*myc* coding region in T-cell lymphomas which were induced by injection with MCF69Ll virus into new-born mice. In contrast, other groups reported relatively low frequencies of integration of MuLV near c-*myc*.[7, 36, 37] It is possible that the observed low frequency of LTR insertion close to the c-*myc* region may be underestimated, because, in this study, twelve cell lines which did not have LTR insertions near either N-*myc* or c-*myc* expressed c-*myc* mRNA. If the LTR was inserted at a site distant from the c-*myc* region, the insertion may not be identified by the digestion of cellular DNA with commonly used restriction enzymes such as *Bam*HI, *Eco*RI and *Hin*d III.

The strain and/or features of MuLV which caused thymic lymphomas in [BALB/c→AKR] bone marrow chimeras have not been studied as yet. LTR U3 sequences inserted into the 3′ region of N-*myc* in L176 and NAK36 were identical to the sequences that have been reported for AKV,[33] MCF-111A,[38] SL3-3,[39] FBR MuLV,[40] and Gross virus[41] (Fig. 3B). In contrast, the inserted LTR U3 sequence found in CAK20 was different from that of L176 or NAK36 at 4 positions (Fig. 3B) and no such sequence was found in the GenBank database version 7.06, although LTRs of RFM/Un,[42] RadLV/VL3 (T + L +),[43] MCF 247,[31] Tikaut[7] and NZB-9-1[44] were closer to CAK20 LTR than LTRs of L176 and CAK36. This finding suggests the possibility that a novel retrovirus infected thymic lymphocytes of

(BALB/c×B6)F_1 origin through AKR thymus epithelium. Complete sequence analysis of the provirus integrated in the genome of CAK20 may provide evidence for this hypothesis.

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