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N-*myc* is frequently activated by proviral insertion in MuLV-induced T cell lymphomas

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We report a new common proviral insertion site in murine leukemia virus-induced T cell lymphomas to be N-*myc*. Proviral activation of N-*myc* was found in 35% of independently induced primary tumors. The vast majority of the proviral insertions occur within a small segment of the 3'-untranslated region of the N-*myc* gene, directly downstream of the protein-encoding domain. This results in an increased level of expression of a truncated N-*myc* mRNA. Together with the previously shown c-*myc* activation we now find involvement of *myc* genes in >75% of the primary T cell lymphomas induced by Moloney murine leukemia virus in C57BL10 and BALB/c mice, and show for the first time that N-*myc* can be over-expressed by a mechanism other than gene amplification.

Key words: Moloney murine leukemia virus/N-*myc*/proviral insertion

Introduction

Replication-competent murine leukemia viruses do not carry an oncogene in their genome, but rather cause tumors after a long latency period through the activation of cellular oncogenes (Nusse, 1986; Nusse and Berns, 1988). Previously we have shown that in Moloney murine leukemia virus (MuLV)-induced T cell lymphomas the *pim*-1 and c-*myc* genes are frequently activated by proviral insertion. In a group of 66 primary T cell lymphomas induced in BALB/c and C57BL mice, 45% carried proviral integrations near *pim*-1, while 40% had integrations near c-*myc*. Of the lymphomas carrying a proviral insertion near *pim*-1, 50% also had an integration near c-*myc* (Cuypers *et al.*, 1984; Selten *et al.*, 1984, 1985). These figures likely over-estimate the involvement of c-*myc* and *pim*-1 in these tumors as transplantation studies of lymphomas induced by Moloney MuLV in BALB/c and C57BL/10 often revealed their oligoclonal character, and proviral insertion probably occurred in only one of the subclones (Cuypers *et al.*, 1984). In other genetic backgrounds, in which predominantly monoclonal tumors were induced after a longer latency period, the contribution of provirally activated c-*myc* and *pim*-1 genes was considerably less (Mucenski *et al.*, 1987). Although other common insertion sites are also occupied in these tumors at low frequency, like *pvt*-1 (Graham *et al.*, 1985), *fis*-1(Silver and Butler, 1986), Mlvi's (Tsichlis *et al.*, 1985) and *Ick* (Voronova and Sefton, 1986), a significant fraction of the lymphomas do not bear proviruses at any of the known common insertion sites (Mucenski *et al.*, 1987). Here we report on the frequent activation of N-*myc* in MuLV-induced T cell lymphomas.

Results

N-myc functions as a common insertion site for Moloney MuLV

We have cloned a single somatically acquired provirus with flanking host sequences from a primary T cell lymphoma induced by Moloney MuLV in mice transgenic for the *pim*-1 gene (van Lohuizen *et al.*, 1989). A restriction map is shown in Figure 1a. Probe A, a 3.6 kbp long *Pst*I fragment from the cellular DNA flanking the provirus, was used to hybridize DNA blots of independent primary tumors (Figure 1). These tumors were induced by Moloney MuLV infection of newborn C57BL/10 and BALB/c mice, and have been analyzed for proviral integrations in the c-*myc* and *pim*-1 genes as described previously (Cuypers *et al.*, 1984; Selten *et al.*, 1984, 1985). Each tumor was isolated from a different mouse. Of 40 of such tumors analyzed, 15 displayed a novel 18 kb *Eco*RV band resulting from proviral integration, in addition to a 23.5 kb germ-line band. This indicated that the cloned region represented a frequently occupied common insertion site. Remarkably, all rearranged *Eco*RV fragments of individual tumors were nearly of the same size, indicating that the proviral integrations were clustered in a very narrow region of host DNA. This was confirmed using *Pvu*II digests that yielded rearranged fragments of 1.6 - 1.7 kbp in 12 out of 13 tumors analyzed (Figure 2a and b). In the one remaining tumor we could not unambiguously determine the position of the proviral insertion.

To examine whether this provirally activated gene showed homology to known genes, probe B, which recognized a transcript in tumors with proviral insertions in this locus (see below), was cloned in M13, and 350 bp were sequenced from both strands (see Figure 1a). An open reading frame was detected in one orientation spanning 87 amino acids. Screening of the SWISSPROT protein database revealed that the gene identified by the proviral insertion was mouse N-*myc*. Using mouse - Chinese hamster somatic cell hybrids, N-*myc* was mapped to mouse chromosome 12 (M.van Lohuizen and J.Hilkens, unpublished observations).

Proviral integration near N-myc results in enhanced expression of an aberrantly sized N-myc mRNA

When probe A was used on Northern blots containing 15 μg of total tumor RNA, a 2.5 kb transcript was detected in lymphomas containing a provirus in this region, whereas in tumors without proviral insertion no transcripts of this region were found. A 2.9 kb transcript was detected in RNA from the Abelson-transformed pre-B cell line 2M3 (see Figure 3).

To determine the nature of the aberrantly sized mRNA, we sequenced the junction fragment between the provirus

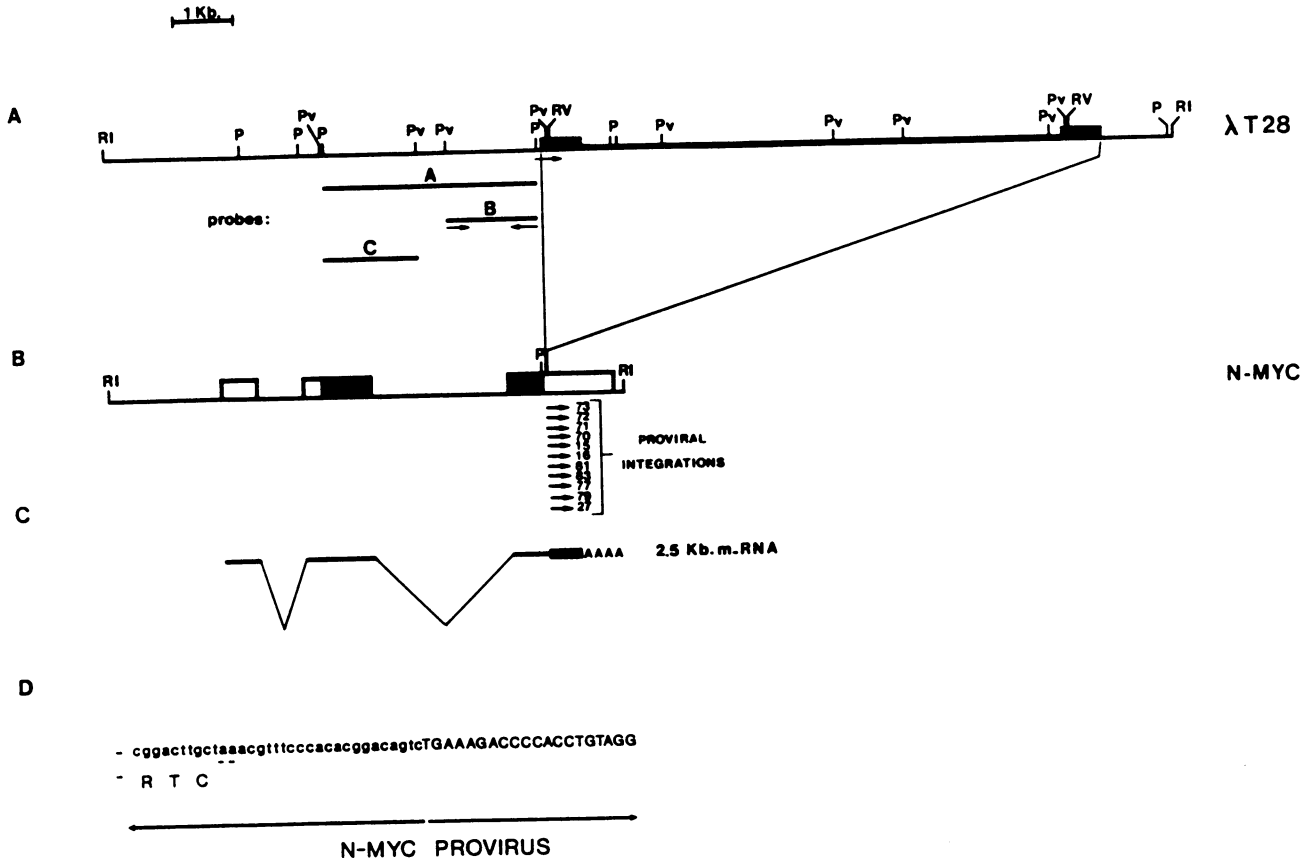

Fig. 1. Clustering or proviral integrations in 3′ untranslated region of N-*myc*. (**a**) Restriction map of the cloned provirus with flanking host sequences. The thick line bordered by the filled boxes (LTRs) represents the provirus. The thin line indicates flanking host sequences. Restriction endonucleases: RV, *Eco*RV; P, *Pst*I; PV, *Pvu*II. The specific probes used (A, B and C) are shown by lines under the restriction map. Thin arrows indicate regions that have been sequenced, using the Sanger dideoxy method (Sanger *et al.*, 1980). (**b**) Genomic organization of the mouse N-*myc* gene, as described previously (DePinho *et al.*, 1986; Taya *et al.*, 1986). Boxes represent exon sequences, solid boxes indicate N-*myc* protein coding regions. Arrows show the mapped proviral integrations. Numbers indicate independent primary lymphomas that were also used in previous studies (Cuypers *et al.*, 1984; Selten *et al.*, 1984, 1986). (**c**) Truncated 2.5 kb N-*myc* mRNA found in T cell lymphomas carrying a proviral integration. The filled box represents LTR sequences the thick line represents N-*myc* exon sequences. (**d**) The provirus−host junction fragment of the T28 clone was sequenced from a *Pst*I site as indicated in (a). Proviral sequences are given in captials. The last three amino acids of N-*myc* are indicated underneath. The N-*myc* translation termination codon (TAA) is underlined.

and flanking cellular sequence. This sequence analysis, as depicted in Figure 1d, indicated that the provirus was inserted directly downstream of the protein encoding domain in the same transcriptional orientation as N-*myc*. This explains why an N-*myc* transcript of 2.5 kb is found in these tumors instead of the normal 2.9 kb N-*myc* mRNA: 900 nucleotides of 3′-untranslated N-*myc* sequences are replaced by 500 nucleotides of 5′ long terminal repeat (LTR) sequences of the inserted provirus, as transcription, which probably starts at the normal N-*myc* promoter(s), is terminated in the 5′ LTR of the provirus. As a consequence most of the 3′ untranslated N-*myc* sequences are absent from the mRNA (see Figure 1c and d). We mapped 12 proviral integrations within an estimated 100 bp of the 3′ untranslated region of N-*myc* directly downstream of the protein encoding domain (see Figure 1b and d). All were inserted in the same transcriptional orientation as N-*myc*.

In the 40 lymphomas that were analyzed in this study, 15 carried an insertion in the N-*myc* gene, whereas 16 harbored a provirus near c-*myc*. In two of the three tumors that were clonal for a proviral integration in N-*myc* (tumor 15 and 71; compare intensities of the 1.7 and 5.0 kbp bands in Figure 2b), c-*myc* transcripts were almost completely absent (Selten *et al.*, 1984). RNA of the third monoclonal tumor (73) was not available for analysis. This suggests that high levels of N-*myc* might suppress the expression of c-*myc*. In a number of other tumors both elevated N-*myc* and c-*myc* mRNA levels were found. However, these latter tumors were not clonal with respect to a proviral integration in N-*myc*.

The clonality of the tumors was determined independently by hybridization with a T cell receptor beta-chain probe (H.T.Cuypers *et al.*, unpublished observations). To find out whether a mutually exclusive expression pattern between N-*myc* and c-*myc* is also observed in these T cell lymphomas it will be necessary to analyze independent subclones (e.g. by the selective outgrowth of single tumor clones after transplantation).

Discussion

We have shown that N-*myc* is frequently activated by proviral insertion in Moloney MuLV-induced T cell

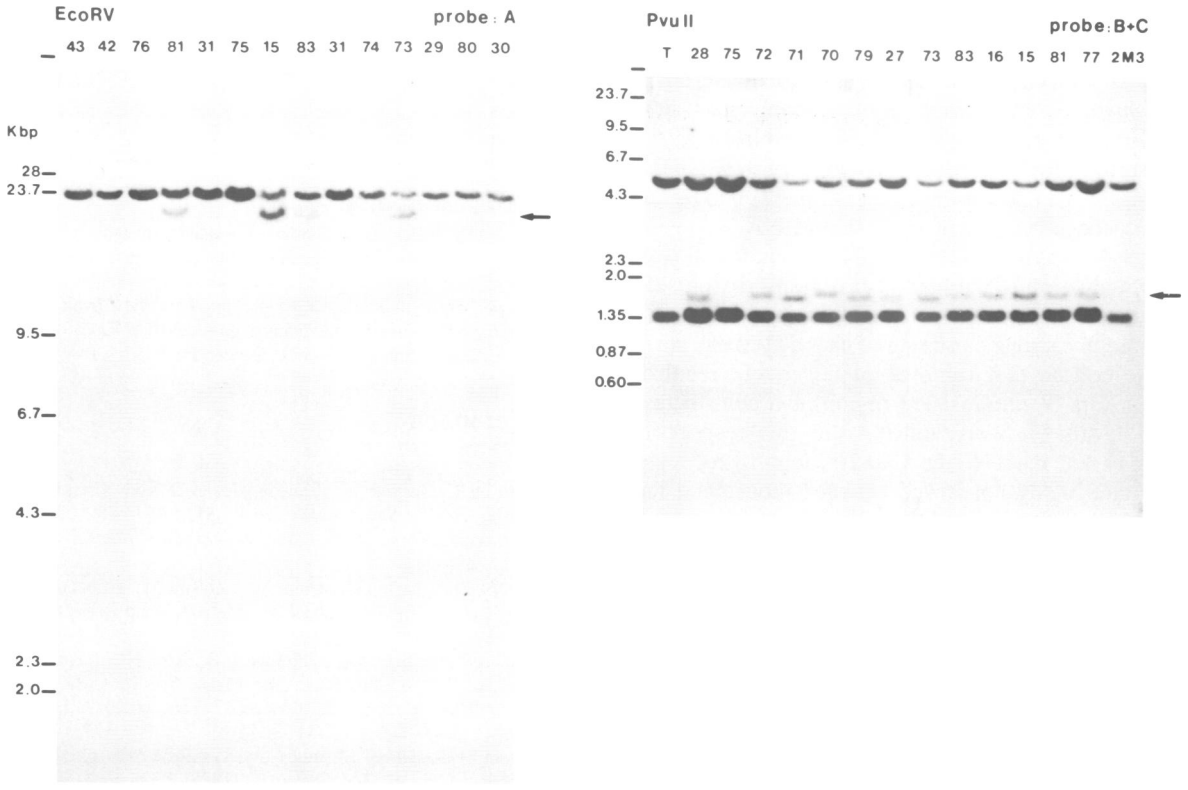

Fig. 2. Identification of a new common proviral integration site in Moloney MuLV-induced T cell lymphomas. (**a**) Tumor DNA (8 μg) was digested with *Eco*RV, separated on agarose gels, blotted to nitrocellulose and hybridized with probe A. The numbers indicate T cell lymphoma DNAs isolated from different BALB/c or C57BL/10 mice infected as newborns with Moloney MuLV (Cuypers *et al.*, 1984; Selten *et al.*, 1984, 1985). A 23.5 kbp germline band is seen in all tumor DNAs, whereas an 18 kbp band, representing a provirus−host junction fragment is detected in tumors 81, 15, 83 and 73 as indicated by arrows. (**b**) Fine mapping of the clustered proviral integrations. Tumor DNA was digested with *Pvu*II, separated by size, blotted in alkali to Genescreen+ (Chomczynska and Qasba, 1984) and probed with probes B and C. Numbers indicate independent T cell lymphomas. DNA from a T cell lymphoma cell line is loaded in lane T (M.van Lohuizen and A.Berns, unpublished). DNA from the Abelson MuLV-transformed pre-B cell line 2M3 is applied in the lane marked by 2M3, *Hin*dIII-digested lambda DNA, and *Hae*III-digested Φ×174 DNA were used as mol. wt markers.

lymphomas. Proviral insertions cluster in a narrow region of DNA directly downstream of the protein encoding domain of N-*myc*, resulting in enhanced expression of a truncated N-*myc* mRNA, encoding an unaltered N-*myc* protein.

The N-*myc* gene, together with c-*myc* and L-*myc* genes forming the *myc* gene family, was originally identified as a gene frequently amplified and over-expressed in human neuroblastomas (Kohl *et al.*, 1983; Schwab *et al.*, 1983). In contrast to the more widely expressed c-*myc* gene, high-level N-*myc* expression has been found to be highly tissue-specific and developmentally restricted. Expression was preferentially found in newborn brain, kidney and intestine of the mouse (Zimmerman *et al.*, 1986). This expression pattern correlates to some extent with the rather limited set of tumors in which N-*myc* can be over-expressed: human neuroblastoma, retinoblastoma and small cell lung carcinoma (Kohl *et al.*, 1984; Zimmerman *et al.*, 1986). Thus far gene amplification has been the only mechanism described for N-*myc* activation in tumors (Alt *et al.*, 1986; Taya *et al.*, 1986; DePinho *et al.*, 1987). Our results demonstrate for the first time that N-*myc* is also frequently activated by proviral insertion in MuLV-induced T cell lymphomas in mice.

Interestingly, all 12 mapped proviral integrations analyzed in this study are tightly clustered with ~100 bp of the 3′ untranslated region of N-*myc*, directly downstream of the

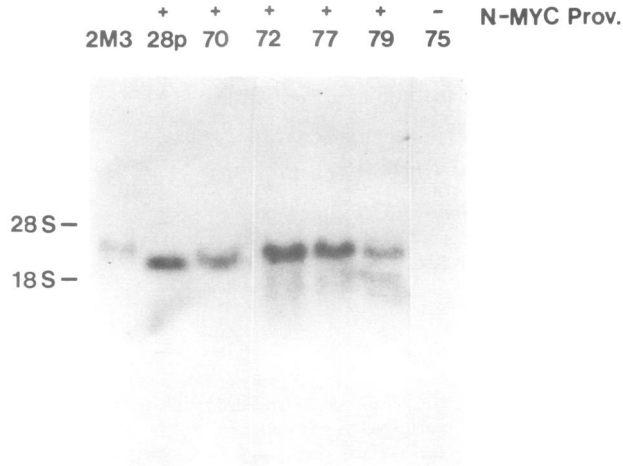

Fig. 3. Expression of N-*myc* mRNA in T cell lymphomas. Northern blot analysis of T cell lymphoma RNA. Numbers indicate different tumor RNAs. Lane 2M3 contained ~5 μg total RNA from the Abelson MuLV-transformed pre-B cell line 2M3. Lane with the tumor 28p contained 1 μg of poly(A)$^+$ selected RNA, isolated from the tumor out of which the integration was cloned, the other lanes contained 15 μg of total primary tumor RNA. The + and − signs indicate the presence or absence of a proviral integration in the N-*myc* gene.

protein encoding domain, in the same transcriptional orientation as N-*myc*. This explains why an N-*myc* transcript of 2.5 kb is found in these tumors instead of the normal 2.9 kb N-*myc* mRNA. Transcription probably starts at the normal N-*myc* promoter(s), but is terminated in the 5′ LTR of the inserted provirus, which provides a novel poly(A)-addition site. As a consequence most of the 3′ untranslated N-*myc* sequences are absent from the mRNA.

Because the inserted proviral LTR contains a strong enhancer, induction of the high N-*myc* transcription seems a likely mechanism of gene activation in these tumors. Furthermore, the strong clustering of integrations in a narrow area may indicate that removal of negatively regulating elements in the 3′ untranslated region also contributes to increased levels of N-*myc* mRNA. In this respect it is interesting to note that both the 5′ and 3′ untranslated regions of N-*myc* are highly conserved between mouse and man, whereas no conservation is detected between mouse N-*myc* 5′ or 3′ untranslated regions and c-*myc* or L-*myc* 5′ or 3′ untranslated regions (DePinho *et al.*, 1986, 1987; Kohl *et al.*, 1986; Legouy *et al.*, 1987). This had led to the suggestion that these sequences could be involved in the regulation of the differential expression patterns observed among members of the *myc* gene family (Zimmerman *et al.*, 1986). Remarkably, in tumors clonal for a proviral insertion near N-*myc*, endogenous c-*myc* mRNA was nearly completely absent, suggesting that N-*myc* might mediate suppression of transcription of other members of the *myc* family. An association between high level N-*myc* expression and the lack of c-*myc* expression has been reported for Wilm's tumors (Nisen *et al.*, 1986).

In summary, we have shown that 35% of the primary MuLV-induced murine T cell lymphomas carry proviral insertions near N-*myc*, whereas proviral insertion near c-*myc* was found in 40% of the tumors. In two monoclonal tumors that carried insertions in N-*myc*, the c-*myc* mRNA levels were almost below the detection level, corroborating the notion that over-expression of one member of the *myc* family can suppress expression of other members. These findings further extend the widely accepted importance of *myc* activations in lymphoid neoplasias.

Materials and methods

Lymphoma induction

Lymphomas were induced by injecting newborn mice of the C56BL/10 and BALB/c strains with 10^6 p.f.u. of Moloney MuLV clone 1A as described by Jaenisch *et al.* (1975). Mice were killed when moribund, and tumor tissues were frozen at −80°C.

DNA and RNA analysis

For Southern blot analysis, 8 μg of total genomic DNA of each tumor was digested with restriction enzymes as recommended by the supplier, separated on agarose gels and transferred to nitrocellulose. Filters were hybridized to ^{32}P-labeled probes and washed as described by Cuypers *et al.* (1984), the final wash was in 0.1 × SSC at 42°C.

The Moloney MuLV-specific U3LTR probe used for DNA analysis has been described by Cuypers *et al.* (1984). The N-*myc* probe is described below.

For Nothern blot analysis, 5−15 μg of total RNA, prepared by the LiCl−urea method was separated on 1% agarose formaldehyde gels (Maniatis *et al.*, 1982) and transferred to nylon membranes. N-*myc* probes: probe A, a 3.5 kbp *Pst*I fragment, extending over exon 2 and part of exon 3; probe B, a *Pvu*II−*Pst*I fragment; probe C, *Pst*I−*Pvu*II fragment, isolated from the lambda clone depicted in Figure 1. These probes were ^{32}P-labeled by nick translation; hybridization conditions were as described in Cuypers *et al.* (1984), the final wash was in 0.1 × SSC at 60°C.

Molecular cloning

The N-*myc* proviral integration site T28 was cloned from a primary tumor induced by Moloney MuLV in a *pim*-1 transgenic mouse. A charon 4A library was prepared from size-fractionated *Eco*RI digested tumor DNA as described by Cuypers *et al.* (1984). Four out of 10^5 recombinant phages screened, hybridized with a MoMuLV-specific U_3LTR probe (Cuypers *et al.*, 1984). All contained the same 15.8 kbp insert.

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