Frequent provirus insertional mutagenesis of *Notch1* in thymomas of MMTV^D/myc transgenic mice suggests a collaboration of *c-myc* and *Notch1* for oncogenesis

Luc Girard,¹ Zaher Hanna,^{1,2} Normand Beaulieu,¹ Caroline D. Hoemann,¹ Carole Simard,¹ Christine A. Kozak,³ and Paul Jolicoeur^{1,4,5,6}

¹Laboratory of Molecular Biology, Clinical Research Institute of Montréal, Montréal, Québec, Canada H2W 1R7;

²Département de Médecine et de ⁴Microbiologie et d'Immunologie, Université de Montréal, Montréal, Québec, Canada H3J 3J7; ³National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health, Bethesda, Maryland 20892, USA; ⁵Department of Experimental Medicine, McGill University, Montréal, Québec, Canada, H3G-1A4

The MMTV^D/myc transgenic mice spontaneously develop oligoclonal CD4⁺CD8⁺ T-cell tumors. We used provirus insertional mutagenesis in these mice to identify putative collaborators of *c-myc*. We found that *Notch1* was mutated in a high proportion (52%) of these tumors. Proviruses were inserted upstream of the exon coding for the transmembrane domain and in both transcriptional orientations. These mutations led to high expression of truncated *Notch1* RNAs and proteins (86–110 kD). In addition, many *Notch1*-rearranged tumors showed elevated levels of full-length *Notch1* transcripts, whereas nearly all showed increased levels of full-length (330-kD) or close to full-length (280-kD) *Notch1* proteins. The 5' end of the truncated RNAs were determined for some tumors by use of RT-PCR and 5' RACE techniques. Depending on the orientation of the proviruses, viral LTR or cryptic promoters appeared to be utilized, and coding potential began in most cases in the transmembrane domain. Pulse-chase experiments revealed that the 330-kD *Notch1* proteins were processed into 110- and 280-kD cleavage products. These results suggest that *Notch1* can be a frequent collaborator of *c-myc* for oncogenesis. Furthermore, our data indicate that *Notch1* alleles mutated by provirus insertion can lead to increased expression of truncated and full-length (330/280-kD) *Notch1* proteins, both being produced in a cleaved and uncleaved form.

[Key Words: *Notch1*; *c-myc*; transgenic mice; retrovirus; MuLV]

Received April 29, 1996; revised version accepted June 17, 1996.

The proto-oncogene *c-myc* is involved in growth regulation and differentiation of several cell types (for review, see Spencer and Groudine 1991). Although the steady-state levels of *c-myc* protein in exponentially growing cells are constant during the cell cycle (Hann et al. 1985; Rabbitts et al. 1985; Thompson et al. 1985), decreased levels achieved by antisense *c-myc* oligonucleotides (Heikkila et al. 1987) or by genetic disruption of one *c-myc* gene copy (Shichiri et al. 1993) have been reported to interfere with cell growth, indicating that *c-myc* is required for cell-cycle progression. Moreover, constitutive overexpression of *c-myc* in some cell types reduces their growth factor requirement in vitro (Kaczmarek et al. 1985) and predisposes to tumor formation in several experimental animal models (Corcoran et al. 1984; Li et al. 1984; Neil et al. 1984; Selten et al. 1984; Stewart et al. 1984; Adams et al. 1985; Leder et al. 1986; Spanopoulou

et al. 1989). In addition, deregulated expression of *c-myc* appears to be critical in the development of several human tumors (Bishop et al. 1987; Spencer and Groudine 1991).

We reported previously that overexpression of the *c-myc* gene under the regulation of the long terminal repeat (LTR) of a thymotropic, leukemogenic variant of the mouse mammary tumor virus (MMTV^D) in transgenic mice led to the development of thymoma in nearly 100% of the animals after 90–130 days (Paquette et al. 1992). The tumors are restricted to the thymus and consist of clonal or oligoclonal populations of immature CD4⁺CD8⁺ (double-positive) T cells. In a normal thymus, these double-positive immature T cells represent 80%–85% of all thymocytes and most are destined to die by apoptosis following negative selection or neglect, or for lack of positive selection (Blackman et al. 1990; Murphy et al. 1990). The relatively long latency needed before the appearance of these tumors and their clonality strongly suggest that the constitutive overexpression of

⁶Corresponding author.

the *c-myc* proto-oncogene is not sufficient for tumor induction, and that additional genetic events are required to collaborate with *c-myc* to fully transform the target T cells.

A few genes collaborating with *c-myc* for transformation have been identified to date in various *in vivo* tumor models, notably *v-Ha-ras* (Sinn et al. 1987; Alexander et al. 1989; Compere et al. 1989), *v-abl* (Rosenbaum et al. 1990; Haupt et al. 1993b), *v-raf* (Alexander et al. 1989), *p53* (Blyth et al. 1995), *Pim1* (Selten et al. 1984; van Lohuizen et al. 1989), *Bmi1*, *Pal1*, *Emi1*, *Blal* (Haupt et al. 1991; van Lohuizen et al. 1991; Levy and Lobelle-Rich 1992; Haupt et al. 1993a), *Fit1* (Tsujimoto et al. 1993), and *Pim2* (van der Lugt et al. 1995). These collaborators of *myc* for transformation are themselves interesting genes involved in growth regulation, differentiation, or apoptosis. Therefore, a *myc* complementation assay for transformation remains a powerful tool to identify important regulators of signal transduction in specific cell types.

To identify putative novel collaborators of *c-myc* for the development of thymomas in MMTV^D/*myc* transgenic mice, we used the provirus insertional mutagenesis approach. Several novel oncogenes and putative oncogenes have been identified previously by this technique (Peters 1990; Kung et al. 1991). We report here that the *Notch1* gene is a frequent target of provirus insertion in tumors arising in Moloney murine leukemia virus (MuLV)-infected MMTV^D/*myc* transgenic mice.

Notch1, the mammalian homolog of the *Drosophila* *Notch* gene, encodes a large transmembrane protein and has been shown to control cell-fate determination in several tissues (for review, see Artavanis-Tsakonas et al. 1995). In *Xenopus*, expression of a gain-of-function truncated mutant (harboring only the intracellular domain) of *X-Notch* affects normal development (Coffman et al. 1993) and prevents retinal cell differentiation (Dorsky et al. 1995). In mammals, three *Notch*-related genes have been identified: *Notch1*, *Notch2*, and *Notch3* (Ellisen et al. 1991; Weinmaster et al. 1991; Del Amo et al. 1992; Reaume et al. 1992; Weinmaster et al. 1992; Lardelli and Lendahl 1993; Kopan and Weintraub 1993; Lardelli et al. 1994), and *Notch1* also appears to control cell-fate determination (Kopan and Weintraub 1993; Kopan et al. 1994; Nye et al. 1994). Recent studies have shown that *Notch* signaling pathways have been preserved from *Drosophila* to mammals and involve binding to the CBF-1/RBP-J_κ trans-activator (Hsieh and Hayward 1995; Jarriault et al. 1995).

Results

*Latency and characteristics of thymomas arising in Moloney MuLV-infected MMTV^D/*myc* transgenic mice*

To identify novel genes that could collaborate with *c-myc* in tumor formation, we used the provirus insertional mutagenesis approach in MMTV^D/*myc* mice that express the transgene in immature T cells. Newborn normal and MMTV^D/*myc* transgenic mice were infected

with Moloney MuLV, a highly leukemogenic thymotropic retrovirus. Infected transgenic mice developed thymomas with a shorter latency (mean = 70 days) than uninfected control transgenic mice (mean = 115 days) (Fig. 1) or infected non-transgenic littermates (mean = 135 days) (data not shown), indicating that Moloney MuLV infection had somehow accelerated the oncogenic process in these mice.

In contrast to the CD4⁺CD8⁺ T-cell thymomas spontaneously arising in MMTV^D/*myc* transgenic mice, the thymomas developing in Moloney MuLV-infected transgenic mice appeared to be more disseminated and most of them (29/32, 91%) infiltrated the spleen and/or the peripheral lymph nodes. The detection of several newly acquired proviruses as discrete hybridizing bands in these tumors indicated that they were clonal or oligoclonal in origin. All the tumors screened (*n* = 58) belonged to the T-cell lineage because they had a rearrangement or a deletion of the T-cell receptor β (TCR β) gene or expressed T-cell-specific markers (data not shown). Most of the tumors analyzed (17/29, 59%) were composed largely (>50%) of CD4⁺CD8⁺ T cells, as determined by FACS analysis (data not shown). The remaining tumors consisted of mixtures of CD4⁺CD8⁺, CD4⁺CD8⁻, and single positive CD4⁺CD8⁻ or CD4⁻CD8⁺ T cells.

*Establishment and characterization of malignant T-cell lines from transplantable thymomas from Moloney MuLV-infected MMTV^D/*myc* mice*

To have access to permanent sources of cells harboring alleles mutated by provirus insertion and to facilitate the study of these mutations, we established permanent

Figure 1. Cumulative incidence of thymomas in transgenic MMTV^D/*myc* mice infected with Moloney MuLV. Two groups of newborn transgenic mice born at two different times (○, ● and ◇, ◆) were inoculated intraperitoneally with two different stocks of Moloney MuLV (● and ◆, respectively) (*n* = 41) or uninoculated (○, and ◇) (*n* = 38). The age represents the time of death from thymoma (○, ●, and ◇) or the age at which the mice were sacrificed because of terminal illness (◆).

T-cell lines in vitro. Primary thymomas were first transplanted intraperitoneally in CD1 nude mice. Of 29 transplanted thymomas, 26 (90%) proliferated. These were further transplanted (or not) once or twice in other nude mice before being dispersed in tissue-culture medium and incubated in vitro. Thirteen distinct cell lines were established from these tumors. All cell lines belonged to the T-cell lineage, as each exhibited TCR β gene rearrangement or deletion (data not shown). The cell-surface phenotype of these cell lines mimicked that of the primary thymomas for most of the markers examined (data not shown).

Identification of proviral insertion sites in thymomas of Moloney MuLV-infected MMTV^D/myc transgenic mice

Several loci have already been found to be the target of provirus insertion in MuLV-induced tumors, and some of them at high frequency. To determine whether some of these loci were also targeted by proviruses in T-cell tumors of Moloney MuLV-infected MMTV^D/myc transgenic mice, we first screened several of these thymoma DNAs with probes for known candidate target genes by the Southern technique. Interestingly, only a few of these genes were found to be rearranged in a low percentage of the tumors screened (Table 1). This was in contrast with the high frequency of provirus insertion within these genes in other experimental systems. These results suggested that novel genes could have been the target of provirus insertion in thymomas of Moloney MuLV-infected MMTV^D/myc transgenic mice.

To search for new provirus insertion sites that contributed to thymoma development, we selected one tumor (T3481) with only two newly acquired proviruses, and both proviruses flanked by adjacent cellular sequences were cloned in EMBL-3 phage arms using a U3 LTR-

specific probe. The two inserts (B28 and B33) were subcloned into plasmid vectors and mapped by restriction endonuclease digestion and Southern blotting with the U3 LTR probe (data not shown). Cellular fragments adjacent to each provirus were subcloned and those free of repetitive sequences were used as probes to screen other tumor DNAs for rearrangements, essentially as described before (Villemur et al. 1987; Poirier et al. 1988). By use of these probes, we identified several rearrangements in DNAs of other tumors. One probe derived from clone B33 mapped to Chr 10 and was found to belong to the *Ahi1* locus (Poirier et al. 1988; data not shown). The characterization of these integrations will be presented elsewhere. Another probe (probe D) (Fig. 2B) derived from clone B28, also detected rearrangements in several other thymoma DNAs (data not shown). This region, which represented a common provirus integration site, was designated *Mis6* (Moloney integration site 6).

Mis6 corresponds to the Notch1 gene and is a frequent target of provirus insertion

The chromosome location of *Mis6* was determined by analysis of two sets of multilocus crosses. With as probe the ³²P-labeled *Mis6* probe D, Southern blotting identified *SacI* fragments of 16.5 kb in *Mus spretus*, 6.7 kb in *Mus mus musculus*, and 3.2 kb in NFS/N and C58/J mice. Inheritance of the variant fragments was typed in the two sets of crosses and *Mis6* showed linkage to markers on proximal chromosome 2. Gene order and recombinational distances were determined to be as follows: *Gad2*, *Cchb2*–3.01 $\pm$ 1.3 (167)–*Cchna*–1.1 $\pm$ 0.8 (186)–*Mis6*–2.0 $\pm$ 1.0 (204)–*Abl*. Numbers in parentheses represent the total number of mice typed for adjacent markers. These results indicated that *Mis6* maps just proximal to *Abl*. To determine whether *Mis6* corresponded to a known gene, the probe D fragment, which hybridized to RNA transcripts in several tumors (see below), was sequenced. This sequencing revealed 100% identity with the mouse *Notch1* cDNA and allowed the positioning of probe D exon sequences in the middle of the *Notch1* gene (Fig. 2B). This chromosomal mapping of the *Mis6*/*Notch1* locus confirmed earlier work (Del Amo et al. 1993).

To determine the frequency at which *Notch1* was occupied by proviruses in other thymomas arising in MuLV-infected MMTV^D/myc transgenic mice, we screened 61 *EcoRV*- or *KpnI*-digested tumor DNAs by the Southern technique, using ³²P-labeled *Notch1* genomic probe D or cDNA probe K, respectively (Fig. 2B). Novel tumor-specific fragment(s), in addition to the normal germ-line fragment(s), were detected in 32 of the 61 tumors (52%) (Table 2; data not shown). In 19 tumors, the rearranged fragment was under-represented relative to the germ-line fragment, indicating that only a small proportion of cells (2%–32%) in these tumors harbored a provirus inserted within the *Notch1* gene. The same analysis performed on the 13 established cell lines showed that 6 of these lines harbored a rearranged *Notch1* allele. However, rearrangements of *Notch2* and

Table 1. Rearrangement of different loci in thymomas arising in Moloney MuLV-infected MMTV^D/Myc transgenic mice

Locus screen ^a	Restriction enzyme used	No. of rearranged tumors/no. of tumors screened (%) ^b
<i>Ahi1</i>	<i>EcoRV</i>	4/28 (14%)
<i>Pim1</i>	<i>EcoRV</i>	2/28 (7%)
<i>Pal1</i>	<i>HindIII</i>	1/28 (4%)
<i>Bmi1</i>	<i>EcoRI</i>	0/28 (<4%)
<i>Vin1/cyclin D2</i>	<i>KpnI</i>	0/28 (<4%)
<i>Gin1</i>	<i>BamHI</i>	0/28 (<4%)
<i>Mis1/Pvt1</i>	<i>BamHI</i>	0/28 (<4%)
<i>Int-3</i>	<i>EcoRI</i>	0/27 (<4%)
<i>Notch2</i>	<i>EcoRV</i>	0/15 (<6%)

^aTumor DNAs were screened by the Southern technique with ³²P-labeled probes, as described in Materials and methods.

^bEach rearrangement was confirmed either by digestion with more than one enzyme or by comigration of the rearranged fragment with a U3 LTR probe-hybridizing fragment.

Figure 2. Schematic representation of the proviruses integrated within the *Notch1* gene in T-cell tumors arising in Moloney MuLV-infected MMTV^D/myc transgenic mice. (A) A partial sequence of the *Notch1* gene with the sites of provirus insertion in some tumors (T) or cell lines (L) is shown. Sequences were obtained from PCR products generated from primers 110 or 112 (LTR), 233 (exon C), 234 (exon D). Nucleotides are typed in uppercase (exon) or lowercase (intron) letters and numbered according to the published cDNA sequence (Del Amo et al. 1993). (B) *Notch1* genomic DNA and cDNA are shown. (Solid line) Cellular intron sequences; (open boxes), exons arbitrarily numbered C, D, E, and F; (vertical arrows) sites of provirus integration giving rise to rearranged fragments which were equimolar (dark arrowheads) or under-represented (open arrowheads) relative to the germ-line fragments, respectively; (horizontal arrows) transcriptional orientation of provirus 5' to 3'. Restriction sites: (B) *Bam*HI, (Bg) *Bgl*II, (K) *Kpn*I, (P) *Pst*I, (Pv) *Pvu*II, (RV) *Eco*RV, (S) *Sac*I, T14441A and T14441B represent integrations of distinct proviruses in tumor T14441. Exon E is not localized precisely. (Bottom) (EGF) EGF repeats; (NLR) *Notch/lin-12* repeats; (TM) transmembrane domain; (ANK) ankyrin repeats. OPA and PEST motifs and fragments used for probes or for raising antibodies are indicated.

int3 (a *Notch*-related gene) were undetectable in these tumors (Table 1). The same analysis was also performed on tumors from MMTV^D/myc transgenic mice not infected with Moloney MuLV ($n = 13$), on tumors induced by Moloney MuLV in littermate nontransgenic CD-1 mice ($n = 12$), on tumors induced by a Moloney MuLV variant (harboring SupF in its LTR) in NIH/Swiss mice ($n = 9$), on tumors induced by Gross passage A MuLV ($n = 11$) or by BL/VL3 radiation leukemia virus (RadLV) ($n = 11$), respectively, in SIM.S and SIM.R mice. No *Notch1* rearrangement could be detected with the *Notch1* probe D in any of these tumors (data not shown).

To confirm that the rearrangements detected within the *Notch1* gene reflected the presence of MuLV proviruses, to map the sites of integration precisely, and to determine the transcriptional orientation of the proviruses, a restriction analysis was performed with various restriction endonucleases using the *Notch1* probe D and the U3 LTR-specific probe, essentially as carried out previously with other common provirus integration sites (Villeneuve et al. 1986; Poirier et al. 1988). The position

and orientation of MuLV proviruses in the *Notch1* gene of 17 primary tumors and four cell lines are shown schematically in Figure 2B. The precise LTR-*Notch1* junction was confirmed for seven tumors and four cell lines by sequencing a PCR fragment amplified directly from the tumor or cell line DNA (Fig. 2A). Significantly, most proviruses had integrated between genomic sequences coding for the last *Notch/lin-12* repeat and the transmembrane domain of the *Notch1* gene.

Northern blot analysis of *Notch1* RNA in T-cell tumors arising in Moloney MuLV-infected MMTV^D/myc transgenic mice

To determine whether provirus insertion affected *Notch1* gene expression, a Northern blot analysis was performed with probes corresponding to the intracellular (probe K) or the extracellular (probes A, M, and D) region of *Notch1* (Fig. 2B). This analysis was performed on all cell lines and on those tumors whose *Notch1*-rearranged fragment was equimolar to the germ-line fragment. An

Table 2. RNA and protein expression in thymomas and established cell lines of Moloney MuLV-infected MMTV^D/Myc transgenic mice

Cell lines/ tissues ^a	<i>Notch1</i> rearrang. ^b	RNA ^{c,d}							Proteins ^{c,d}			
		K (intra)				M (extra)			Ab-intra-1		Ab-extra-1	
		3.5–4.5 kb	8 kb	10 kb	novel	8 kb	10 kb	novel	p86–p110	p330	p280	p330
thymus	-	-	(+)	+	-	(+)	+	-	-	-	+	-
L7	-	-	-	(+)	-	-	(+)	-	+ ^e	(+)	++	(+)
L42	-	-	(+)	++	-	(+)	++	-	+++ ^e	++	+++	++
L61	-	-	-	-	-	-	-	-	-	-	-	-
T24-2	-	-	-	-	-	-	-	-	-	-	-	-
T3476	-	-	-	(+)	-	-	(+)	-	-	(+)	+	(+)
T3484	-	-	-	(+)	-	-	(+)	-	-	-	(+)	-
T4884	-	++ 4 kb	-	(+)	-	-	(+)	(+) 7 kb (+) 6 kb	N.D.	N.D.	N.D.	N.D.
T4889	-	(+) 4.5 kb (+) 4 kb	-	(+)	-	-	(+)	-	N.D.	N.D.	N.D.	N.D.
T5067	-	+ 4 kb	-	(+)	-	-	(+)	(+) 6 kb	+	(+)	++	(+)
T5071	-	+ 4.5 kb + 4 kb	(+)	++	-	(+)	++	-	N.D.	N.D.	N.D.	N.D.
T14474	-	+ 4.5 kb + 4 kb	(+)	+	-	(+)	+	-	+	+	++	+
L21	+	-	-	(+)	-	-	(+)	-	(+)	(+)	+	(+)
L45	+	+ 4.5 kb (+) 4 kb	+	++	-	+	++	+ 6 kb	+++	++	+++	++
L48	+	++ 4 kb + 3.5 kb	N.D. ^f	+	+++ 7.5 kb	N.D. ^f	+	+++ 7.5 kb	+++	+++	+++	+++
L87	+	+++ 4 kb	-	-	-	-	-	-	++	-	-	-
L96	+	+++ 4 kb	-	-	-	-	-	(+) 15 kb +++ 6 kb	+++	-	+++	-
L97	+	+ 4.5 kb + 4 kb + 3.5 kb	-	(+)	-	-	(+)	-	+++	(+)	+	(+)
T3465	+	++ 4.5 kb ++ 4 kb	++	+	+ 20 kb + 13 kb	++	+	+ 20 kb	++	++	+++	++
T3478	+	+++ 4 kb	(+)	+	-	(+)	+	(+) 7 kb	+++	(+)	++	(+)
T3481	+	+++ 4 kb	(+)	(+)	-	-	(+)	+ 6 kb	+++	-	++	-
T3490	+	+++ 4 kb	-	(+)	-	-	(+)	(+) 7 kb	(+)	-	+	-
T4886	+	+++ 4.5 kb ++ 4 kb	(+)	+	+ 12 kb	+	(+)	+ 18 kb + 7 kb	++	-	(+)	-
T4887	+	++ 4.5 kb +++ 4 kb	+	++	(+) 14 kb + 7.5 kb + 7 kb	+	++	(+) 14 kb + 7 kb	++	+	+	(+)
T4898	+	+ 4 kb	(+)	+	-	(+)	+	+ 7 kb	(+)	+	++	+
T24-1	+	+ 4.5 kb + 4 kb	+	+	-	+	+	-	(+)	+	++	+
T24-3	+	+++ 4 kb	(+)	++	+ 12 kb	+++	++	+ 12 kb	++	(+)	+++	(+)
T14419	+	+++ 4 kb	(+)	++	(+) 13 kb	+++	+++	+++ 15 kb +++ 13 kb	++	(+)	+++	(+)
T14422	+	+++ 4 kb	(+)	+	-	(+)	+	(+) 7 kb	(+)	-	+	-
T14428	+	++ 4 kb	(+)	(+)	-	(+)	(+)	-	+	+	++	+
T14460	+	+++ 4 kb	-	+	-	-	+	(+) 17 kb	++	-	++	-

^a[L] Cell line; [T] thymoma. Only monoclonal *Notch1*^{pro} thymomas are shown.^bEach rearrangement (except L97) was confirmed either by digestion with more than one enzyme or by comigration of the rearranged fragment with a U3 LTR probe-hybridizing fragment.^c[+] Lowest expression; (+++) highest expression.^d[N.D.] Not determined.^ep110 detected only.^fThe intense 7.5-kb band did not allow detection of the 8.0-kb band.

altered expression pattern was observed in almost all tumors and cell lines exhibiting *Notch1* rearrangement (*Notch1*^{pro}) (Fig. 3). High levels of 3.5- to 4.5-kb transcripts were detected with the 3' end probe K in nearly

all ($n = 18/19$) *Notch1*^{pro} tumors and cell lines analyzed (Fig. 3A; Table 2). These truncated transcripts were also detected in five unrearranged tumors studied (T4884, T4889, T5067, T5071, T14474), indicating either that

Figure 3. Northern blot analysis of representative tumors and established cell lines arising in Moloney MuLV-infected MMTV^D/myc transgenic mice. RNAs (20 μ g) were hybridized with ³²P-labeled *Notch1* intracellular (probe K) (A) or extracellular (probe M) (B) fragment. (Lanes 1–5) Unrearranged cell lines L7, L46, L54, L61, and L42; (lanes 6–11) rearranged cell lines L21, L97, L48, L45, L87, and L96; (lane 12) thymus from a 28-day-old normal CD1 mouse; (lanes 13–18) unrearranged thymomas T5067, T24-2, T14424, T14442, T14454, and T14474; (lanes 19–31) rearranged thymomas T24-1, T14428, T3490, T3478, T4898, T14422, T3465, T3481, T4887, T4886, T24-3, T14460, and T14419. (C) The same blot was rehybridized with a 18S ribosomal probe. Lanes 14–24 in A and lane 31 in B were underexposed two and five times, respectively.

these tumors harbor a yet undetected provirus insertion in a more distant part of the gene, or that these transcripts arose by another mechanism. In some *Notch1*^{pro} tumors and cell lines, overexpression of full-length (8/10-kb) transcripts could also be detected with this probe (Fig. 3A, lanes 9,25,27,29,31). In seven *Notch1*^{pro} tumors or cell lines, probe K (and/or probe M) detected longer transcripts (12–20 kb) (Fig. 3A,B; lanes 25,27,28,29,31, data not shown), which may be aberrant spliced variants that have not been studied further. Interestingly, the high level expression of the 8/10-kb *Notch1* RNA was seen almost exclusively in cells overexpressing truncated *Notch1* RNA.

The same analysis carried out with the 5' end probe M (Fig. 3B) confirmed the expression pattern of the 8/10-kb transcripts initially observed with probe K. In addition, probe M detected novel shorter transcripts of different lengths (6–7.5 kb) in several tumors (Fig. 3B, lanes 9,11,21–24,26–28). The size of these novel transcripts perfectly paralleled the distance between the 5' end of the published *Notch1* cDNA (Del Amo et al. 1993) and the localization of the respective inserted proviruses (Fig. 2B). In almost all of the tumors studied, probes A and D revealed the same hybridizing RNA species as probe M (data not shown). Moreover, the 5' end probes A, M, and, in most cases, D, did not detect the 3.5- to 4.5-kb tran-

scripts recognized by the 3' end probe K, indicating that these truncated RNAs contain mainly sequences coding for the *Notch1* intracellular domain.

Characterization of the 5' end of the truncated 3.5- to 4.5-kb transcripts: coding potential begins at the Notch1 trans-membrane domain

Because the length of the 3.5- to 4.5-kb transcripts roughly corresponded to the distance between the sites of proviral integration and the 3' end of the cDNA, it was conceivable that the synthesis of these RNA species initiated at, or close to, the site of provirus insertion. To determine the structure of the 5' end of these 3.5- to 4.5-kb transcripts and possibly their mode of production, we used reverse transcription (RT)-PCR and 5' rapid amplification of cDNA ends (RACE) amplification techniques. In tumors where the provirus had integrated in the same direction as the *Notch1* gene, the proviral LTR was expected, from previous studies (Peters 1990; Kung et al. 1991), to serve as a promoter driving the expression of the (truncated) downstream *Notch1* gene. This was indeed the case for three tumors and one cell line (L96)

analyzed by RT-PCR, with a 5' oligonucleotide derived from the U5 LTR and 3' oligos derived from the *Notch1* cDNA between the transmembrane and the ankyrin domain (Fig. 4A). As an example, the RT-PCR product obtained from tumor T4898 was a DNA fragment of 0.7 kb whose sequence corresponded to a chimeric viral/*Notch1* RNA that started in the LTR (from primer 24) followed by 51 nucleotides of *Notch1* intron sequences where it was spliced, at a cryptic splice donor site, to the next exon (E) of the *Notch1* gene corresponding to nucleotide 5067 of the published cDNA (Del Amo et al. 1993). The first potential in-frame ATG was located at nucleotide 5257 of the *Notch1* cDNA sequence within the trans-membrane domain. This RNA would have the capacity to code for an estimated 103-kD protein. The same analysis carried out with the three other *Notch1*^{pro} tumor cells yielded fragments with similar structures (Fig. 4A).

To study the structure of the 5' end of these transcripts in tumors where the provirus had integrated in opposite transcriptional orientation to the *Notch1* gene, 5' RACE amplifications were carried out on two cell-line RNAs. The 5' end of the RACE product from cell-line L87 RNA

Figure 4. Analysis of the 5' end of the 3.5- to 4.5-kb truncated *Notch1* transcripts by RT-PCR and 5' RACE amplification. Provirus inserted in sense (A) or antisense (B) orientation. The PCR products generated with the indicated primers (thick horizontal arrows) were cloned and sequenced. Nucleotides of the published cDNA sequence (Del Amo et al. 1993) are indicated. (Bold letters) Moloney proviral sequences; (shaded boxes) PCR-amplified sequences; (M) potential methionine translation start point; in all products, except RC/L45 and RT/L87, this methionine was preceded by an in-frame stop codon; other symbols as in Fig. 2. Primers 252, 299, 303, and 316 are located in the *Notch1* cDNA between the trans-membrane domain and the ankyrin repeats. RT and RC designate the RT-PCR or 5' RACE products, respectively. RC/L96 starts at the U3/R junction. RT/L21 and RT/T3490 products were identical.

contained 84 nucleotides of *Notch1* intron sequences located 133 nucleotides downstream from the integration site. These sequences were then spliced to the next exon (exon E) at nucleotide 5067 of the *Notch1* cDNA, utilizing a cryptic splice donor site in the intron (Fig. 4B). This result suggested that, as a result of the provirus integration, a cryptic promoter within the intronic *Notch1* sequence upstream of exon E was activated by the LTR enhancer to yield the truncated transcript. RT-PCR amplification performed on the same L87 cell line, however, yielded a chimeric cDNA product containing U3 LTR sequences plus 97 nucleotides of *Notch1* intronic sequences. The RNA was then spliced at a different cryptic donor site from the site of the RACE product to the acceptor site in exon E (Fig. 4B). This result indicated that distinct truncated RNA variants were produced and suggested that some may originate from a cryptic promoter within the provirus itself. The same analysis performed with L45, L21, and T3490 tumor cell RNA yielded products with similar structures (Fig. 4B).

Therefore, it appeared that provirus insertion within the *Notch1* gene led to the synthesis of novel truncated 3.5- to 4.5-kb transcripts from viral LTR or cryptic promoters. This is likely to represent the most important functional consequence of the provirus insertions in *Notch1*. Regardless of the precise integration site, all of these 3' truncated transcripts have retained the capacity to code for truncated proteins containing part of the *trans*-membrane domain, the recently described binding site for the CBF-1/RBP-J_κ transcription factor (Hsieh et al. 1996), the ankyrin repeats and the more distal domains (OPA, PEST). This suggests that some or all of these domains may be necessary for full oncogenic potential of the truncated Notch1 proteins.

Analysis of the 3' end of the truncated transcripts harboring only sequences coding for the extracellular domain of Notch1

Northern blot analysis has revealed a class of truncated

Notch1 RNAs that hybridized exclusively with probes specific to the extracellular domain of *Notch1* (probes A, M, D) and whose length correlated with the site of provirus integration. This suggested that these transcripts originated at the 5' end of the gene and terminated at the site of provirus integration. To confirm the structure of the 3' end of these transcripts, we performed a RT-PCR analysis on RNA of one cell line and one tumor, by use of primers from *Notch1* exon C and from the Moloney LTR. As expected, the PCR products contained exon C and D *Notch1* sequences fused to viral LTR sequences (Fig. 5). Translation termination codons were found in all three reading frames within the viral LTR. This result is consistent with a premature termination of these transcripts at a site within the viral genome. These transcripts have the capacity to code for proteins containing only the extracellular domain, up to, but not including, the *trans*-membrane domain.

Detection of multiple forms of Notch1 proteins in T-cell tumors arising in Moloney MuLV-infected MMTV^D/myc transgenic mice

To study the pattern of expression of Notch1 proteins in the same tumors (or cell lines) analyzed for RNA, four different polyclonal antibodies (Ab) were raised against most of the cdc10 repeats (Ab-intra-1) and the OPA motif (Ab-intra-2) of the intracellular domain, the *Notch/lin-12* repeats (Ab-extra-1) and the EGF-like repeats (Ab-extra-2) of the extracellular domain (Fig. 2B). Notch1 (but not Notch2 or Notch3) proteins were most likely detected in our study. First, none of the tumors or cell lines analyzed exhibited overexpression of *Notch2* or *Notch3* transcripts comparable to that of *Notch1* (data not shown). Second, in all tumors or cell lines, overexpression of the detected Notch proteins closely paralleled the increased *Notch1* RNA expression (Table 2). Third, the three antibodies did not detect any Notch proteins in L54 cells (Fig. 6, lane 3; data not shown), a line that

Figure 5. Analysis of the 3' end of truncated transcripts by RT-PCR amplification. The PCR products were cloned and sequenced. (*) In-frame stop codon. Other symbols as in Fig. 4.

Figure 6. Detection of Notch1 proteins in representative tumors and established cell lines arising in Moloney MuLV-infected MMTV^D/myc transgenic mice. Protein extracts (100 μ g) from tumors or cell lines were separated on 4%–8% SDS-polyacrylamide gels and analyzed by the Western blotting procedure, using the primary rabbit antibodies Ab-intra-1 (A) or Ab-extra-1 (B). The order of the samples is the same as the order of the RNA shown in Fig. 5. (Lanes 1–5) Unrearranged cell lines L7, L46, L54, L61, and L42; (lanes 6–11) rearranged cell lines L21, L97, L48, L45, L87, and L96; (lane 12) thymus from a 28-day old normal CD1 mouse; (lanes 13–18) unrearranged thymomas T5067, T24-2, T14424, T14442, T14454, and T14474; (lanes 19–31) rearranged thymomas T24-1, T14428, T3490, T3478, T4898, T14422, T3465, T3481, T4887, T4886, T24-3, T14460, and T14419. (Asterisk) Protein detected nonspecifically.

showed no *Notch1* RNA expression while expressing the highest levels of full-length *Notch2* RNA (not shown). Fourth, full-length (330/280-kD) Notch proteins were not detected in L87 cells (Fig. 6, lane 10) which do not express full-length (8/10-kb) *Notch1* RNA (Fig. 3, lane 10), despite the fact that these cells were among those expressing the higher levels of full-length *Notch3* RNA (data not shown).

Interestingly, several different forms of Notch1 proteins were detected with our antibodies. The Ab-extra-1 and Ab-extra-2 (data not shown) reagents detected two large proteins of 330 and 280 kD in tumor cells and in normal thymus, most likely encoded by the full-length (8/10 kb) *Notch1* RNA (Fig. 6B). The 280-kD species was overexpressed in most tumors or cell lines exhibiting overexpression of truncated 3' *Notch1* transcripts (Fig. 6B; Table 2). Overexpression of the 330/280-kD *Notch1* proteins was also observed in some unrearranged tumors and cell lines (Fig. 6B, lanes 1,5,13,18) consistent with RNA expression data. In general, the levels of the 330-kD were lower than those of 280-kD proteins. Whereas the levels of the 330-kD proteins correlated well with the presence of the 8/10-kb *Notch1* RNA, such a correlation was not evident for the 280-kD proteins (see e.g. L96, Fig. 3,6, lane 11).

The antibody Ab-intra-1 revealed a number of novel abundant truncated Notch1 proteins (86–110 kD) in all tumors or cell lines exhibiting high levels of truncated

Notch1 3' transcripts (Fig. 6A; Table 2). These truncated proteins were not detected with Ab-extra-1 (Fig. 6B) or Ab-extra-2 (data not shown), suggesting that they contain almost exclusively the intracellular domain. These truncated proteins were heterogeneous in size, but truncated Notch1 proteins of common size could be observed in several distinct tumors. The *Notch1* Ab-intra-1 antibodies also reacted with the 330-kD proteins detected previously with the antibodies against the extracellular domain. The same Ab-intra-1, however, did not react with the 280-kD forms of *Notch1*, on Western blots while they detect these 280-kD proteins in immunoprecipitation (Fig. 7). Finally, there was a good correlation in most tumors and cell lines between the presence of intracellular 86- to 110-kD truncated proteins and the overexpression of 280-kD proteins (Fig. 6A; Table 2). The same analysis performed with Ab-intra-2 yielded patterns of Notch1 proteins virtually identical to those detected with Ab-intra-1. Because the OPA domain of Notch1 is not present in Notch2 and Notch3 proteins, this confirmed that the proteins detected were encoded by the *Notch1* gene.

To study further the various Notch1 proteins in our system, we performed a pulse-chase experiment with *Notch1*^{pro} L48 cells, followed by immunoprecipitation with *Notch1* Ab-intra-1 or Ab-extra-1. As demonstrated recently by others (Aster et al. 1994; Zagouras et al. 1995), we confirmed that the 330-kD protein can be

Figure 7. Pulse-chase analysis of Notch1 proteins in L48 cells. Cells (2×10^7) were metabolically labeled with [35 S]methionine (200 μ Ci/ml) for 15 min and incubated in medium containing excess cold methionine for different times: (Lane 1) 0 hr; (lane 2) 0.5 hr; (lane 3) 1 hr; (lane 4) 2 hr; (lane 5) 4 hr. Proteins were immunoprecipitated with anti-Notch1 Ab-intra-1 (A) or Ab-extra-1 (B) antibodies and analyzed on 4%–8% SDS-polyacrylamide gels.

chased into a 110-kD product (Fig. 7). In addition, we found that the 330-kD protein was chased into prominent 280- and 86-kD cleavage products in these cells (Fig. 7).

The truncated 3.5- to 4.5-kb Notch1 transcripts detected in tumor cells have the capacity to code for truncated Notch1 proteins

Although pulse-chase analysis indicated that the 110-kD truncated Notch1 proteins were generated from the precursor 330-kD proteins (Fig. 7), our Northern and PCR analysis indicated that the truncated 3.5- to 4.5-kb transcripts have the capacity to code for truncated proteins of about 86- to 110-kD harboring the intracellular domain of Notch1. In addition, in all tumor cells overexpressing the truncated *Notch1* RNA, high levels of truncated proteins were also detected. Finally, direct evidence that truncated transcripts can generate truncated proteins has been offered by analysis of one cell line, L87. In this line, only the 3.5- to 4.5-kb RNAs are overexpressed, whereas only faint expression of other *Notch1* RNA species could be detected [Fig. 3, lane 10]. These cells express only the truncated 86- to 110-kD Notch1 proteins (Fig. 6A,B, lane 10), strongly suggesting that the truncated 3.5- to 4.5-kb *Notch1* RNA are indeed authentic templates for the truncated proteins. Therefore, it appears that the truncated 86- to 110-kD proteins represent a pool, to which both mutant truncated transcripts and full-length processed proteins contribute.

Discussion

The truncated Notch1 gene can be a frequent collaborator of c-myc in T-cell tumors

We found that Moloney MuLV infection of MMTV^D/myc transgenic mice accelerated the development of

T-cell lymphoma and that a high proportion (up to 52%) of these tumors exhibited a provirus insertion mutation within the *Notch1* gene. Given the high frequency of this event, the clonality of the emerging malignant tumors, despite the random nature of provirus integration, and the shorter latencies of tumor development compared to uninoculated transgenic mice, the observed in vivo-selected class of *Notch1* mutations are likely to represent a genetic event collaborating with *c-myc* for T-cell transformation. Other known genes identified previously as frequent collaborators of *c-myc* in T- or B-cell tumors (*Pim1*, *Bmi1*, and *Pal1*) (Haupt et al. 1991; van Lohuizen et al. 1989, 1991) were infrequently targeted by provirus in tumors of the MMTV^D/myc mice, despite the fact that the same retrovirus strain (Moloney) was used. This difference may reflect the different mouse background (CD1) in which the MMTV^D/myc transgene was bred, as compared to previous studies. It is known that strain differences affect the frequency at which provirus insertion of targeted genes is observed (Selten et al. 1984). Alternatively, the preferential provirus insertions within *Notch1* may reflect the fact that the transgene is expressed in different lymphoid cell populations in MMTV^D/myc mice. Because *Notch1* did not appear to be targeted in 100% of the tumors studied, other unknown genes are likely to represent additional collaborators of *c-myc* in several other tumors.

Mutation of *Notch1*-related family members have been previously implicated in tumor formation in other systems. The human homolog of *Notch1*, *TAN-1*, was identified as a locus involved in the t(7;9)(q34;q34.3) chromosome translocation with the TcR β gene (Ellisen et al. 1991) in a few sporadic cases of T-cell leukemia. The *TAN-1* gene was found to be truncated at sites strikingly close (100–300 bp) to some of the sites of provirus insertion found in our tumors (L45, T4896, T4879, T3487). Another *Notch*-related gene, *Int3*, has been implicated in MMTV-induced tumor formation. In these tumors, the *Int3* gene was truncated and activated by provirus insertion yielding truncated *Int3* transcripts with coding potential for the cytoplasmic domain (Gallahan and Callahan 1987; Robbins et al. 1992). Therefore, specific truncations of the *Notch* family genes may be required to activate their oncogenic potential.

A wide array of *Notch1* deletion mutants have been highly selected by the oncogenic process in our T-cell tumors. Expression of similar *Notch* deletion mutants appears to give rise to a gain-of-function phenotype in *Drosophila* (Lieber et al. 1993; Rebay et al. 1993), in *Caenorhabditis elegans* (Struhl et al. 1993), in *Xenopus* embryos (Coffman et al. 1993; Dorsky et al. 1995) and in mammalian cells (Kopan and Weintraub 1993; Kopan et al. 1994; Nye et al. 1994). One such construct, expressing a transcript similar to those observed in our tumors, except for the presence of a leader signal sequence, was found to be oncogenic for T cells in vivo (Aster et al. 1994). Therefore, the overexpressed 3.5- to 4.5-kb truncated *Notch1* transcripts detected in our T-cell tumors are likely to participate in tumor formation, as expected for a gain-of-function phenotype. The structure of their

encoded truncated proteins may be optimal for transformation.

Analysis of several of these *Notch1* mutated alleles has allowed us to define the minimal sequences of the truncated *Notch1* protein which may be essential for oncogenicity. The fact that no provirus integration has been detected downstream of the exons coding for the recently described binding site for CBF-1/RBP-J κ (Hsieh et al. 1996), suggests that these sequences may be essential for a fully oncogenic protein. Furthermore, the lack of integration downstream of the exon coding for the *trans*-membrane domain suggests that retention of most of the *trans*-membrane domain is selected for, even though the membrane-targeting sequences (leader peptide) have been deleted. Previous experiments in *Drosophila* have shown that a *Notch* null mutant could be rescued by a deletion mutant harboring only cytoplasmic sequences, whereas the ability to rescue was lost when the *trans*-membrane domain and limited extracellular sequences were added (Lieber et al. 1993). Moreover, truncated *Notch* mutants have been found to be much more active than full-length *Notch* constructs in eliciting abnormal *Drosophila* development (Fortini et al. 1993; Rebay et al. 1993), or in suppressing neurogenesis or myogenesis in mammalian cells (Kopan et al. 1994, 1996; Nye et al. 1994). In all of the truncated *Notch1* cDNAs that we analyzed, most extracellular sequences were absent. Together, these results indicate that the truncated *Notch/lin-12* proteins deleted of most of their extracellular domain are more effective in signaling than the full-length proteins, both in lower eukaryotes and in mammalian cells.

Our results represent the first indication that such mutated *Notch1* alleles can collaborate with *c-myc* for transformation. At this point, it is not clear how this collaboration occurs. Whatever the nature of the apparent *Notch1/c-myc* collaboration, the Moloney MuLV-infected MMTV^D/myc mice described here represent a powerful biological system to analyze the various transforming (gain-of-function) alleles of *Notch1* in T cells. In addition, the availability of several T-cell lines exhibiting *Notch1* gain-of-function mutations may be instrumental in developing assays for *Notch1* signaling in T cells.

Notch1 provirus insertional mutations induce a complex molecular expression phenotype

The presence of mutated *Notch1* alleles in the MMTV^D/myc tumors leads to a complex *Notch1* expression phenotype regardless of the orientation of the provirus. We have detected a novel class of highly expressed 3.5- to 4.5-kb RNA transcripts, coding for the 3' end of *Notch1*, in most *Notch1*-rearranged tumors. Similar transcripts have also been detected in a few tumors that do not exhibit an apparent *Notch1* gene rearrangement, suggesting that other types of mutation may induce the expression of these truncated transcripts. These transcripts are initiated either at the viral LTR or at cryptic promoters, depending on whether the provirus is integrated in the

sense or antisense orientation, respectively. These 3.5- to 4.5-kb RNA transcripts (viral U5/*Notch1* fusion and cryptic promoter-initiated) carry, in most cases, the first potential in-frame internal ATG in the *trans*-membrane domain. Our results indicate that these truncated RNAs can code for the 86- to 110-kD truncated proteins detected. The elevated expression of the truncated proteins in MMTV^D/myc tumors correlates well with *Notch1* rearrangement and overexpression of the 3.5- to 4.5-kb transcripts. Furthermore, we confirmed a precursor-product relationship of the 330/110-kD proteins, in agreement with the data presented by Aster et al. (1994) who showed that a 120-kD species (referred to as 110 kD here) is produced by cleavage of the 330-kD *Notch1* precursor. Similar transcripts also coding for truncated proteins have been observed in one cell line (SUP-T1) harboring a translocation of the human *Notch1* homolog, *TAN-1* (Ellisen et al. 1991; Aster et al. 1994). In addition, heterogeneous shorter *Notch1* proteins similar to the 86- to 110-kD species observed in our tumors have been detected previously in cells transfected with various *Notch1* constructs truncated of most of their extracellular domains (Kopan et al. 1996). Some of these truncated proteins (referred to as 70 and 63 kD) were found to be generated by proteolytic cleavage of a slightly larger membrane-tethered product (81 kD) (Kopan et al. 1996). It is possible that some of the truncated 86- to 110-kD proteins detected in our tumors are generated similarly from cleavage of a slightly longer product. Their heterogeneity may also reflect the heterogeneity of the 5' end of the truncated transcripts presumably coding for some of these proteins and/or initiation at several different methionines within the *trans*-membrane domain and more distally. Therefore, in MMTV^D/myc tumors in which the full-length and the 3.5- to 4.5-kb *Notch1* RNAs are both highly expressed, the 86- to 110-kD proteins appear to be generated by two distinct mechanisms; by cleavage from the 330-kD (and smaller) *Notch1* proteins and by synthesis from the 3.5- to 4.5-kb truncated RNAs.

The second class of novel transcripts detected in some tumors harboring the mutated *Notch1* alleles are of distinct length in each tumor and harbor only 5' sequences. These transcripts appear to be initiated at the *Notch1* promoter and to terminate at the provirus insertion site. Our inability to detect the putative *Notch1* proteins (~185 kD) encoded by these transcripts in cell extracts or in cell supernatants could be caused by their localization within the extracellular matrix or their instability. There have not been any reports of *Notch* mutants with such a coding capacity in other systems, nor have any such mutants been constructed to test for specific phenotypes.

The third class of highly expressed transcripts detected in tumors exhibiting *Notch1* insertional mutation are 8/10-kb transcripts, apparently full length, hybridizing with both intracellular and extracellular *Notch1* probes. These transcripts appear to initiate at the *Notch1* promoter and are overexpressed in many tumors. The structure of the 8/10-kb transcripts has not been investigated

in great detail. They may represent normal splicing variants, as they have also been detected in normal tissues (thymus, lung) and in cells in culture (Rat-1) (data not shown). The 8/10-kb transcripts have the capacity, and are likely to code for the 330-kD proteins detected. We found, in our pulse-chase experiments, that the 330-kD protein is the precursor of the 110-kD protein. More strikingly, the 330-kD protein is also processed into a 280-kD protein. As a processed product, it is possible that p280 is, in fact, the only Notch1 protein active in ligand recognition. Overexpression of this 280-kD Notch1 protein species correlates very well with the presence of the truncated Notch1 proteins in most tumors. Given the low levels of the precursor p330 in several tumors, these results suggest that the presence of these truncated proteins may play a role in the stabilization of the 280-kD proteins. Because nearly every *Notch1*^{pro} tumor and cell line overexpressed the Notch1 p280, it is possible that its high expression plays an essential role in transformation. It remains to be elucidated how the different forms of the Notch1 proteins detected in our tumors participate in tumor formation and whether they each activate common or distinct pathways.

Materials and methods

Mice and viruses

The MMTV^D/myc transgenic mice were bred on CD1 background and have been described previously (Paquette et al. 1992). Newborn (<48 hr) mice were inoculated with Moloney MuLV (10⁵ PFU/ml) intraperitoneally, as described previously (Villeneuve et al. 1986).

DNA extraction and restriction endonuclease digestion

DNA extraction, digestion with restriction endonucleases, separation of DNA fragments by agarose gel electrophoresis and hybridization with ³²P-labeled probes by the method of Southern were performed as described previously (Paquette et al. 1992).

Molecular cloning procedures

A genomic library of *Bam*HI-digested DNA from tumor T3481 was constructed by ligation into the arms of λ phage EMBL-3 vector and screened by hybridization with ³²P-labeled Moloney U3 LTR-specific probe, essentially as described previously (Villemur et al. 1987; Poirier et al. 1988). The *Notch1* genomic sequences used for mapping exons E and F were isolated from a normal BALB/c genomic λ library. Cloning in plasmid pBR322, GEM-3, or pUC18 vectors was carried out as described (Poirier et al. 1988).

Chromosome mapping

Mis6 was mapped by analysis of two sets of multilocus genetic crosses: (NFS/N or C58/J $\times$ *M.m. musculus*) $\times$ *M.m. musculus* and (NFS/N $\times$ *M. spretus*) $\times$ *M. spretus* or C58/J and were typed for chromosome 2 markers as described previously (Chin et al. 1995). Data were stored and analyzed by use of the program LOCUS designed by C.E. Buckler (NIAID, Bethesda, MD). Recombinational distances and standard errors were calculated according to Green (1981).

RNA analysis

RNA was isolated, separated on 1% formaldehyde-agarose gels, transferred by the Northern blot procedure to Hybond-N membranes (Amersham Co) and hybridized as described previously (Paquette et al. 1992).

RT-PCR, 5' RACE-PCR and DNA sequencing

For RT-PCR, 5 μ g of total RNA were reverse transcribed with Moloney MuLV reverse transcriptase according to the manufacturer's specifications (GIBCO-BRL). Samples (2 μ l) of these reverse transcription reactions were amplified by PCR with the indicated primers. The sequences of the Moloney MuLV LTR oligonucleotides used were primer 110, TAAGCTAGCTTGC-CAAACCTACAGGTG; primer 112, CCCGAGCTCAATAAAGAGCCCACAAC; and primer 24, GAGTGATTGACTACC. The *Notch1*-specific primers were in exon C, primer 233, CTG-GCTGGGAAGTGGCACTC; primer 388, GAGTGCCAGTTC-CCAGCCAG; in exon D, primer 234, CGACCAGCTACCGAACAAGTTC; in exon F, primer 299, ACAGGAGCACGAAG-GCGGCCGC; and 3' of exon F, primer 316, CCCCACTCGT-TCTGATTGTCG; primer 303, GTCCACTGCCTGTGGT-CAGTC; primer 252, GACATCCATGCAGTCAGCATCC. The amplified DNA fragments were purified on agarose gels and cloned in a T-vector (Marchuk et al. 1991). Nucleotide sequencing was performed by the Sanger method, as described (Hanna et al. 1993). Computer sequencing analysis was performed by use of GCG package (Wistar).

For 5' RACE, the cDNA was prepared with AMV reverse transcriptase (Pharmacia) as described above from poly(A)⁺ RNA of selected cell lines. The cDNA was purified on G-50 Spun columns then subjected to tailing reaction by use of dATP and terminal transferase (TdT) according to manufacturer's specifications (Boehringer). Purification through G-50 Spun columns was performed before the first round of RACE-PCR. The PCR were carried out with anchor T₁₇ and *Notch1* specific primers. Four rounds of PCR using nested primers were performed.

Probes

The *Notch1* probe D is a 0.9-kb *Bam*HI–*Sac*I genomic fragment. The *Notch1* probe M corresponds to a 1.9-kb *Eco*RI–*Bam*HI fragment from the cDNA clone MN4.0 (Reaume et al. 1992) and was subcloned in pUC18. The *Notch1* probes K and A, respectively, were excised from the KS-Motch plasmid (Kopan and Weintraub 1993) or generated by RT-PCR [oligo 245: GTGGT-GTGGCTCAACGTCCTG and oligo 304: GTATGAAGACTCAAAGGGCAG]. The TCR β RBL5 and Moloney U3 LTR fragment have been described previously (Paquette et al. 1992). The DNA probes used to search for DNA rearrangements were: *Vin1* (pro.6) (Hanna et al. 1993), *Gin1* (SS8) (Villemur et al. 1987), *Mis1* (pM1FR2) (Villeneuve et al. 1986), *Bmi1* (van Lohuizen et al. 1991), *Pal1* (p11a2) (van Lohuizen et al. 1991), *Ahi1* (Poirier et al. 1988), and *Pim1* (Selden et al. 1984). All probes were labeled with [³²P]dCTP and [³²P]dATP by the random primer method, as described previously (Hanna et al. 1993).

Establishment of cells in culture

Thymoma cells were dispersed in RPMI-1640 medium and inoculated intraperitoneally into CD1 nude mice. One primary tumor, or tumors growing in nude mice after one or two passages, were dispersed in RPMI-1640 containing 10% fetal calf serum (Hyclone) and 5 $\times$ 10^{−5} M β -mercaptoethanol at a concentration of 2 $\times$ 10⁶ cells/ml and incubated at 37°C in 5% CO₂.

Growing cells were split twice a week. Lines generally became established after 2 to 4 weeks. Established lines have been passaged for more than a year.

Production of antibodies

The regions of the *Notch1* intracellular (nucleotides 5799–6670 and 7066–7509) (respectively Ab-intra-1 and Ab-intra-2) and extracellular (nucleotides 4204–4681 and 2706–3607) (Ab-extra-2 and Ab-extra-1, respectively) domains were subcloned in the pGEX-2T or pGEX-3X vector (Pharmacia). Proteins were produced in bacteria and used to immunize rabbits as described (Huang and Jolicoeur 1990). Polyclonal rabbit antisera were tested for their ability to immunoprecipitate [³⁵S]methionine-labeled in vitro-synthesized *Vin1/Notch1* chimeric proteins. The initiation codon was provided from the *Vin1* gene isolated by our lab (Hanna et al. 1993). The antisera were used at a 1:500 dilution for Western blots.

Protein immunoblotting and immunoprecipitation

All protein extractions were done in RIPA buffer containing 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin, 50 µg/ml TLCK, and 100 µg/ml PMSF. Western immunoblotting was performed as described previously (Jolicoeur et al. 1992) except that HRP-conjugated anti-rabbit (Dakko) and Renaissance chemiluminescent substrate (Dupont) were used for immunodetection. Protein concentrations were determined by a micro-BCA protein assay (Pierce). Equal amounts of proteins from each tumor or cell line were loaded. After transfer, the membranes were also stained with Ponceau red to confirm equal loading. For the pulse-chase analysis, cells were incubated for 10 min in medium containing 300 µCi/ml of [³⁵S]-methionine (Dupont) followed by a chase in medium containing excess unlabeled methionine for the indicated periods of time. Immunoprecipitations were performed with antisera-preloaded protein A-Sepharose, washes were done with RIPA containing PMSF, as before (Huang and Jolicoeur 1990).

Acknowledgments

This work was supported by grants from the Medical Research Council of Canada, the National Cancer Institute of Canada, and the Cancer Research Society Inc. to Paul Jolicoeur. We are grateful to Janet Rossant and Harold Weintraub (deceased) for providing *Notch1* cDNA clones, to Anton Berns and Jerry Adams for providing *Bmi1* and *Pal1* probes. We thank Yves Paquette for providing transgenic mice for assessment of tumor latencies. We thank Benoît Laganière and Ginette Massé for their excellent technical assistance. Luc Girard was a recipient of a Fellowship from FCAR.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Adams, J.M., A.W. Harris, C.A. Pinkert, L.M. Corcoran, W.S. Alexander, S. Cory, R.D. Palmiter, and R.L. Brinster. 1985. The *c-myc* oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* **318**: 533–538.
- Alexander, W.S., J.M. Adams, and S. Cory. 1989. Oncogene cooperation in lymphocyte transformation: Malignant conversion of Eµ-*myc* transgenic pre-B cells *in vitro* is enhanced by *v-H-ras* or *v-raf* but not *v-abl*. *Mol. Cell. Biol.* **9**: 67–73.
- Artavanis-Tsakonas, S., K. Matsuno, and M.E. Fortini. 1995. *Notch* signaling. *Science* **268**: 225–232.
- Aster, J., W. Pear, R. Hasserjian, H. Erba, F. Davi, B. Luo, M. Scott, D. Baltimore, and J. Sklar. 1994. Functional analysis of the *TAN-1* gene, a human homolog of *Drosophila Notch*. *Cold Spring Harbor Symp. Quant. Biol.* **59**: 125–136.
- Bishop, J.M. 1987. The molecular genetics of cancer. *Science* **235**: 305–311.
- Blackman, M., J. Kappler, and P. Marrack. 1990. The role of the T cell receptor in positive and negative selection of developing T cells. *Science* **248**: 1335–1341.
- Blyth, K., A. Terry, M. O'Hara, E.W. Baxter, M. Campbell, M.D. Stewart, L.A. Donehower, D.E. Onions, J.C. Neil, and E.R. Cameron. 1995. Synergy between a human *c-myc* transgene and *p53* null genotype in murine thymic lymphomas: Contrasting effects of homozygous and heterozygous *p53* loss. *Oncogene* **10**: 1717–1723.
- Chin, H., O.-J. Kwon, H.H. Jung, D.S. Kim, and C.A. Kozak. 1995. Genetic mapping of the mouse genes encoding the voltage-sensitive calcium channel subunits. *Genomics* **28**: 592–595.
- Coffman, C.R., P. Skoglund, W.A. Harris, and C.R. Kintner. 1993. Expression of an extracellular deletion of *Xotch* diverts cell fate in *Xenopus* embryos. *Cell* **73**: 659–671.
- Compere, S.J., P. Baldacci, A.H. Sharpe, T. Thompson, H. Land, and R. Jaenisch. 1989. The *ras* and *myc* oncogenes cooperate in tumor induction in many tissues when introduced into midgestation mouse embryos by retroviral vectors. *Proc. Natl. Acad. Sci.* **86**: 2224–2228.
- Corcoran, L.M., J.M. Adams, A.R. Dunn, and S. Cory. 1984. Murine T lymphomas in which the cellular *myc* oncogene has been activated by retroviral insertion. *Cell* **37**: 113–122.
- Del Amo, F.F., D.E. Smith, P.J. Swiatek, M. Gendron-Maguire, R.J.M. Greenspan, A.P. McMahon, and T. Gridley. 1992. Expression pattern of *Notch*, a mouse homolog of *Drosophila Notch*, suggests an important role in early postimplantation mouse development. *Development* **115**: 737–744.
- Del Amo, F.F., M. Gendron-Maguire, P.J. Swiatek, N.A. Jenkins, N.G. Copeland, and T. Gridley. 1993. Cloning, analysis, and chromosomal localization of *Notch-1*, a mouse homolog of *Drosophila Notch*. *Genomics* **15**: 259–264.
- Dorsky, R.I., D.H. Rapaport, and W.A. Harris. 1995. *Xotch* inhibits cell differentiation in the *Xenopus* retina. *Neuron* **14**: 487–496.
- Ellisen, L.W., J. Bird, D.C. West, A.L. Soreng, T.C. Reynolds, S.D. Smith, and J. Sklar. 1991. *TAN-1*, the human homolog of the *Drosophila notch* gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**: 649–661.
- Fortini, M.E., I. Rebay, L.A. Caron, and S. Artavanis-Tsakonas. 1993. An activated *notch* receptor blocks cell-fate commitment in the developing *drosophila* eye. *Nature* **365**: 555–557.
- Gallahan, D. and R. Callahan. 1987. Mammary tumorigenesis in feral mice: Identification of a new *int* locus in mouse mammary tumor virus (Czech II)-induced mammary tumors. *J. Virol.* **61**: 66–74.
- Green, E.L. 1981. Breeding system. In *The mouse in biomedical research* [ed. H.L. Foster, J.D. Smith, and J.G. Fox], Vol. 1, pp. 91–104. Academic Press, New York, NY.
- Hann, S.R., C.B. Thompson, and R.N. Eisenman. 1985. *c-myc* oncogene protein synthesis is independent of the cell cycle in human and avian cells. *Nature* **314**: 366–369.
- Hanna, Z., M. Jankowski, P. Tremblay, X. Jiang, A. Milatovich,

- U. Francke, and P. Jolicoeur. 1993. The *Vin-1* gene, identified by provirus insertional mutagenesis, is the cyclin D2. *Oncogene* **8**: 1661–1666.
- Haupt, Y., W.S. Alexander, G. Barri, S.P. Klinken, and J.M. Adams. 1991. Novel zinc finger gene implicated as *myc* collaborator by retrovirally accelerated lymphomagenesis in $\text{E}\mu$ -*myc* transgenic mice. *Cell* **65**: 753–763.
- Haupt, Y., M.L. Bath, A.W. Harris, and J.M. Adams. 1993a. *bmi-1* transgene induces lymphomas and collaborates with *myc* in tumorigenesis. *Oncogene* **8**: 3161–3164.
- Haupt, Y., A.W. Harris, and J.M. Adams. 1993b. Moloney virus induction of T-cell lymphomas in a plasmacytomagenic strain of $\text{E}\mu$ -*v-abl* transgenic mice. *Int. J. Cancer* **55**: 623–629.
- Heikkilä, R., G. Schwab, E. Wickstrom, S.L. Loke, D.H. Pluznik, R. Watt, and L.M. Neckers. 1987. A *c-myc* antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G0 to G1. *Nature* **328**: 445–449.
- Hsieh, J. and S.D. Hayward. 1995. Masking of the CBF1/RBPJ κ transcriptional repression domain by Epstein-Barr virus EBNA2. *Science* **268**: 560–563.
- Hsieh, J.D., T. Henkel, P. Salmon, E. Robey, M.G. Peterson, and S.D. Hayward. 1996. Truncated mammalian *Notch1* activates CBF1/RBPJ κ -repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. *Mol. Cell. Biol.* **16**: 952–959.
- Huang, M. and P. Jolicoeur. 1990. Characterization of the gag/fusion protein encoded by the defective Duplan retrovirus inducing murine acquired immunodeficiency syndrome. *J. Virol.* **64**: 5764–5772.
- Jarriault, S., C. Brou, F. Logeat, E.H. Schroeter, R. Kopan, and A. Israel. 1995. Signalling downstream of activated mammalian *notch*. *Nature* **377**: 355–358.
- Jolicoeur, P., A. Laperrière, and N. Beaulieu. 1992. Efficient production of human immunodeficiency virus proteins in transgenic mice. *J. Virol.* **66**: 3904–3908.
- Kaczmarek, L., J.K. Hyland, R. Watt, M. Rosenberg, and R. Baserga. 1985. Microinjected *c-myc* as a competence factor. *Science* **228**: 1313–1315.
- Kopan, R. and H. Weintraub. 1993. Mouse *notch*: Expression in hair follicles correlates with cell fate determination. *J. Cell Biol.* **121**: 631–641.
- Kopan, R., J.S. Nye, and H. Weintraub. 1994. The intracellular domain of mouse *Notch*: A constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development* **120**: 2385–2396.
- Kopan, R., E.H. Schroeter, H. Weintraub, and J.S. Nye. 1996. Signal transduction by activated *mNotch*: Importance of proteolytic processing and its regulation by the extracellular domain. *Proc. Natl. Acad. Sci.* **93**: 1683–1688.
- Kung, H.J., C. Boerkoel, and T.H. Carter. 1991. Retroviral mutagenesis of cellular oncogenes: A review with insights into the mechanisms of insertional activation. *Curr. Top. Microbiol. Immunol.* **171**: 1–25.
- Lardelli, M. and U. Lendahl. 1993. *Motch A* and *Motch B*—Two mouse *Notch* homologues coexpressed in a wide variety of tissues. *Exp. Cell Res.* **204**: 364–372.
- Lardelli, M., J. Dahlstrand, and U. Lendahl. 1994. The novel *notch* homologue mouse *notch3* lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mech. Dev.* **46**: 123–136.
- Leder, A., P.K. Pattengale, A. Kuo, T.A. Stewart, and P. Leder. 1986. Consequences of widespread deregulation of the *c-myc* gene in transgenic mice: Multiple neoplasms and normal development. *Cell* **45**: 485–495.
- Levy, L.S. and P.A. Lobelle-Rich. 1992. Insertional mutagenesis of *flvi-2* in tumors induced by infection with LC-FeLV, a *myc*-containing strain of feline leukemia virus. *J. Virol.* **66**: 2885–2892.
- Li, Y., C.A. Holland, J.W. Hartley, and N. Hopkins. 1984. Viral integration near *c-myc* in 10–20% of mcf 247-induced AKR lymphomas. *Proc. Natl. Acad. Sci.* **81**: 6808–6811.
- Lieber, T., S. Kidd, E. Alcamo, V. Corbin, and M.W. Young. 1993. Antineurogenic phenotypes induced by truncated *Notch* proteins indicate a role in signal transduction and may point to a novel function for *Notch* in nuclei. *Genes & Dev.* **7**: 1949–1965.
- Marchuk, D., M. Drumm, A. Saulino, and F.S. Collins. 1991. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Res.* **19**: 1154.
- Murphy, K.M., A.B. Heimberger, and D.Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4⁺CD8⁺TCR^{lo} thymocytes *in vivo*. *Science* **250**: 1720–1723.
- Neil, J.C., D. Hughes, R. McFarlane, N.M. Wilkie, D.E. Onions, G. Lees, and O. Jarrett. 1984. Transduction and rearrangement of the *myc* gene by feline leukaemia virus in naturally occurring T-cell leukaemias. *Nature* **308**: 814–820.
- Nye, J.S., R. Kopan, and R. Axel. 1994. An activated *Notch* suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells. *Development* **120**: 2421–2430.
- Paquette, Y., L. Doyon, A. Laperrière, Z. Hanna, J. Ball, R.P. Sekaly, and P. Jolicoeur. 1992. A viral Long Terminal Repeat expressed in CD4⁺CD8⁺ precursors is downregulated in mature peripheral CD4⁺CD8⁺ or CD4⁺CD8⁺ T cells. *Mol. Cell. Biol.* **12**: 3522–3530.
- Peters, G. 1990. Oncogenes at viral integration sites. *Cell Growth Differ.* **1**: 503–510.
- Poirier, Y., C. Kozak, and P. Jolicoeur. 1988. Identification of a common helper provirus integration site in Abelson murine leukemia virus-induced lymphoma DNA. *J. Virol.* **62**: 3985–3992.
- Rabbitts, P.H., J.V. Watson, A. Lamond, A. Forster, M.A. Stinson, G. Evan, W. Fischer, E. Atherton, R. Sheppard, and T.H. Rabbitts. 1985. Metabolism of *c-myc* gene products: *c-myc* mRNA and protein expression in the cell cycle. *EMBO J.* **4**: 2009–2015.
- Reaume, A.G., R.A. Conlon, R. Zirngibl, T.P. Yamaguchi, and J. Rossant. 1992. Expression analysis of a *Notch* homologue in the mouse embryo. *Dev. Biol.* **154**: 377–387.
- Rebay, I., R.G. Fehon, and S. Artavanis-Tsakonas. 1993. Specific truncations of *Drosophila Notch* define dominant activated and dominant negative forms of the receptor. *Cell* **74**: 319–329.
- Robbins, J., B.J. Blondel, D. Gallahan, and R. Callahan. 1992. Mouse mammary tumor gene *int-3*: A member of the *notch* gene family transforms mammary epithelial cells. *J. Virol.* **66**: 2594–2599.
- Rosenbaum, H., A.W. Harris, M.L. Bath, J. McNeall, E. Webb, J.M. Adams, and S. Cory. 1990. An $\text{E}\mu$ -*v-abl* transgene elicits plasmacytomas in concert with an activated *myc* gene. *EMBO J.* **9**: 897–905.
- Selten, G., H.T. Cuypers, M. Zijlstra, C. Melief, and A. Berns. 1984. Involvement of *c-myc* in MuLV-induced T cell lymphomas in mice: Frequency and mechanisms of activation. *EMBO J.* **3**: 3215–3222.
- Shichiri, M., K.D. Hanson, and J.M. Sedivy. 1993. Effects of *c-myc* expression on proliferation, quiescence, and the G0 to G1 transition in nontransformed cells. *Cell Growth Differ.* **4**: 93–104.
- Sinn, E., W. Muller, P. Pattengale, I. Tepler, R. Wallace, and P. Leder. 1987. Coexpression of MMTV/*v-Ha-ras* and MMTV/

- c-myc* genes in transgenic mice: Synergistic action of oncogenes *in vivo*. *Cell* **49**: 465–475.
- Spanopoulou, E., A. Early, J. Elliott, N. Crispe, H. Ladyman, M. Ritter, S. Watt, F. Grosveld, and D. Kioussis. 1989. Complex lymphoid and epithelial thymic tumours in Thyl-*myc* transgenic mice. *Nature* **342**: 185–189.
- Spencer, C.A. and M. Groudine. 1991. Control of *c-myc* regulation in normal and neoplastic cells. *Adv. Cancer Res.* **56**: 1–48.
- Stewart, T.A., P.K. Pattengale, and P. Leder. 1984. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/*myc* fusion genes. *Cell* **38**: 627–637.
- Struhl, G., K. Fitzgerald, and I. Greenwald. 1993. Intrinsic activity of the Lin-12 and Notch intracellular domains *in vivo*. *Cell* **74**: 331–345.
- Thompson, C.B., P.B. Challoner, P.E. Neiman, and M. Groudine. 1985. Levels of *c-myc* oncogene mRNA are invariant throughout the cell cycle. *Nature* **314**: 363–366.
- Tsujimoto, H., R. Fulton, K. Nishigaki, Y. Matsumoto, A. Hasegawa, A. Tsujimoto, S. Cevario, S.J. O'Brien, A. Terry, D. Onions, and J.C. Neil. 1993. A common proviral integration region, *fit-1*, in T-cell tumors induced by *myc*-containing feline leukemia viruses. *Virology* **196**: 845–848.
- van der Lugt, N.M.T., J. Domen, E. Verhoeven, K. Linders, H. van der Gulden, J. Allen, and A. Berns. 1995. Proviral tagging in $\epsilon\mu$ -*myc* transgenic mice lacking the *Pim-1* proto-oncogene leads to compensatory activation of *Pim-2*. *EMBO J.* **14**: 2536–2544.
- van Lohuizen, M., S. Verbeek, P. Krimpenfort, J. Domen, C. Saris, T. Radaszkiewicz, and A. Berns. 1989. Predisposition to lymphomagenesis in *pim-1* transgenic mice: Cooperation with *c-myc* and *N-myc* in murine leukemia virus-induced tumors. *Cell* **56**: 673–682.
- van Lohuizen, M., S. Verbeek, B. Scheijen, E. Wientjens, H. van der Gulden, and A. Berns. 1991. Identification of cooperating oncogenes in $\epsilon\mu$ -*myc* transgenic mice by provirus tagging. *Cell* **65**: 737–752.
- Villemur, R., Y. Monczak, E. Rassart, C. Kozak, and P. Jolicoeur. 1987. Identification of a new common provirus integration site in Gross passage A murine leukemia virus-induced mouse thymoma DNA. *Mol. Cell. Biol.* **7**: 512–522.
- Villeneuve, L., E. Rassart, P. Jolicoeur, M. Graham, and J.M. Adams. 1986. Proviral integration site *Mis-1* in rat thymomas corresponds to the pvt-1 translocation breakpoint in murine plasmacytomas. *Mol. Cell. Biol.* **6**: 1834–1837.
- Weinmaster, G., V.J. Roberts, and G. Lemke. 1991. A homolog of *Drosophila Notch* expressed during mammalian development. *Development* **113**: 199–205.
- Weinmaster, G., V.J. Roberts, and G. Lemke. 1992. *Notch2*: A second mammalian Notch gene. *Development* **116**: 931–941.
- Zagouras, P., S. Stifani, C.M. Blaumueller, M.L. Carcangiu, and S. Artavanis-Tsakonas. 1995. Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc. Natl. Acad. Sci.* **92**: 6414–6418.

Frequent provirus insertional mutagenesis of Notch1 in thymomas of MMTVD/myc transgenic mice suggests a collaboration of c-myc and Notch1 for oncogenesis.

L Girard, Z Hanna, N Beaulieu, et al.

Genes Dev. 1996, **10**:

Access the most recent version at doi:[10.1101/gad.10.15.1930](https://doi.org/10.1101/gad.10.15.1930)

References

This article cites 68 articles, 30 of which can be accessed free at:
<http://genesdev.cshlp.org/content/10/15/1930.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

Focused on your science.

USC
SCIENTIFIC
SIDE BY SCIENCE