Frequent provirus insertional mutagenesis of \textit{Notch1} in thymomas of MMTV\textsuperscript{D}/myc transgenic mice suggests a collaboration of c-myc and Notch1 for oncogenesis

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The MMTV\textsuperscript{D}/myc transgenic mice spontaneously develop oligoclonal CD4\textsuperscript{+}CD8\textsuperscript{+} 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.

\textbf{[Key Words: Notch1; c-myc; transgenic mice; retrovirus; MuLV]}

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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; Rabbits 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\textsuperscript{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\textsuperscript{+}CD8\textsuperscript{+} (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
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, Bla1 [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 Lucht 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<sup>D</sup>/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<sup>D</sup>/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; Reame 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<sub>x</sub> trans-activator [Hsieh and Hayward 1995; Jarriault et al. 1995].

**Results**

Latency and characteristics of thymomas arising in Moloney MuLV-infected MMTV<sup>D</sup>/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<sup>D</sup>/myc mice that express the transgene in immature T cells. Newborn normal and MMTV<sup>D</sup>/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<sup>+</sup>CD8<sup>+</sup> T-cell thymomas spontaneously arising in MMTV<sup>D</sup>/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 ß [TCRß] gene or expressed T-cell-specific markers [data not shown]. Most of the tumors analyzed [17/29, 59%] were composed largely (>50%) of CD4<sup>+</sup>CD8<sup>+</sup> T cells, as determined by FACS analysis [data not shown]. The remaining tumors consisted of mixtures of CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, and single positive CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>+</sup>CD8<sup>+</sup> T cells.

Establishment and characterization of malignant T-cell lines from transplantable thymomas from Moloney MuLV-infected MMTV<sup>D</sup>/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

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*Figure 1.* Cumulative incidence of thymomas in transgenic MMTV<sup>D</sup>/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 [○, ▽] [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 TCRβ 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<sup>D</sup>/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<sup>D</sup>/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<sup>D</sup>/myc transgenic mice.

To search for new provirus insertion sites that contributed to thymoma development, we selected one tumor (T3481) with 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 Ah1 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).

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

| Locus screen | Restriction enzyme used | No. of rearranged tumors/ no. of tumors screened (%)<sup>b</sup> |
|--------------|-------------------------|---------------------------------------------------------------|
| Ah1          | EcoRV                   | 4/28 (14%)                                                   |
| Pim1         | EcoRV                   | 2/28 (7%)                                                    |
| Pal1         | HindIII                 | 1/28 (4%)                                                    |
| Bmi1         | EcoRI                   | 0/28 (<4%)                                                   |
| Vtn1/cyclin D2 | KpnI                  | 0/28 (<4%)                                                   |
| Gin1         | BamHI                   | 0/28 (<4%)                                                   |
| Mis1/Pvt1    | BamHII                  | 0/28 (<4%)                                                   |
| Int-3        | EcoRI                   | 0/27 (<4%)                                                   |
| Notch2       | EcoRV                   | 0/15 (<6%)                                                   |

<sup>a</sup>Tumor DNAs were screened by the Southern technique with <sup>32</sup>P-labeled probes, as described in Materials and methods.

<sup>b</sup>Each 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.

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 <sup>32</sup>P-labeled Mis6 probe D, Southern blotting identified SacI fragments of 16.5 kb in Mus spretus, 6.7 kb in *Mus musculus*, and 3.2 kb in NFS/N and C58/1 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 ± 1.3 (167)–Cchna–1.1 ± 0.8 (186)–Mis6–2.0 ± 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<sup>D</sup>/myc transgenic mice, we screened 61 EcoRV- or KpnI-digested tumor DNAs by the Southern technique, using <sup>32</sup>P-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
Figure 2. Schematic representation of the proviruses integrated within the Notch1 gene in T-cell tumors arising in Moloney MuLV-infected MMTV<sup>0</sup>/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) BamHI; (Bg) BglII; (K) KpnI; (P) PstI; (Pv) PvuII; (RV) EcoRV; (S) SacI, 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<sup>0</sup>/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<sup>0</sup>/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<sup>P</sup>/Myc transgenic mice

| Cell lines/ tissues<sup>a</sup> | Notch1 rearrang.<sup>b</sup> | 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 | 88-p110 | p330 | p280 | p330 |
| thymus                        | -            | -   | -     | -     | -     | -     | -    | -     | -     | -     | -     | -     |
| L7                            | -            | -   | -     | (+)   | -     | (+)   | -    | -     | -     | -     | -     | -     |
| L42                           | -            | -   | -     | (++)  | -     | (+)   | -    | -     | -     | -     | -     | -     |
| L61                           | -            | -   | -     | -     | -     | -     | -    | -     | -     | -     | -     | -     |
| T24-2                         | -            | -   | -     | -     | -     | -     | -    | -     | -     | -     | -     | -     |
| T3476                         | -            | -   | -     | (+)   | -     | (+)   | -    | -     | -     | -     | -     | -     |
| T3484                         | -            | -   | -     | (+)   | -     | (+)   | -    | -     | -     | -     | -     | -     |
| T4884                         | ++ 4 kb     | -   | (++)  | -     | -     | (+)   | 7 kb | (+)   | 6 kb  | N.D.  | N.D.  | N.D.  |
| T4889                         | (+) 4.5 kb  | -   | (+)   | -     | -     | (+)   | -    | -     | -     | N.D.  | N.D.  | N.D.  |
| T5067                         | ++ 4 kb     | -   | (+)   | -     | -     | (+)   | 6 kb | (+)   | (+)   | N.D.  | N.D.  | N.D.  |
| T5071                         | ++ 4.5 kb   | -   | (+)   | ++    | -     | (+)   | 6 kb | (+)   | (+)   | N.D.  | N.D.  | N.D.  |
| T14474                        | ++ 4.5 kb   | -   | (+)   | -     | -     | (+)   | -    | -     | -     | +     | +     | +     |
| L21                           | +            | -   | -     | (+)   | -     | (+)   | -    | -     | -     | +     | +     | +     |
| L45                           | +            | +   | (+)   | +     | -     | (+)   | -    | -     | -     | +     | +     | +     |
| L48                           | ++ 4 kb     | -   | (+)   | ++++  | 7.5 kb| N.D.  | ++    | ++++  | ++++  | ++++  | ++++  |
| L78                           | +++ 4 kb    | -   | -     | -     | -     | (+)   | 15 kb| ++++  | ++++  | ++++  | ++++  |
| L96                           | +++ 4 kb    | -   | -     | -     | -     | (+)   | 6 kb | ++++  | ++++  | ++++  | ++++  |
| L97                           | ++ 4.5 kb   | +   | (+)   | ++++  | 6 kb  | +     | +    | +     | +     | +     | +     |
| T3465                         | ++ 4.5 kb   | +   | (+)   | 20 kb | +     | (+)   | 13 kb| +     | +     | ++++  | ++++  |
| T3478                         | +++ 4 kb    | (+) | -     | (+)   | +     | (+)   | 7 kb | ++++  | (+)   | ++++  | (+)   |
| T3481                         | +++ 4 kb    | (+) | -     | (+)   | +     | (+)   | 6 kb | ++++  | ++     | ++++  |
| T3490                         | +++ 4.5 kb  | (+) | -     | (+)   | +     | (+)   | 7 kb | (+)   | -      | +     | -     |
| T4886                         | +++ 4.5 kb  | (+) | 12 kb | (+)   | +     | 18 kb | ++   | -      | (+)   |
| T4887                         | ++ 4.5 kb   | +   | (+)   | 14 kb | +     | (+)   | 14 kb| +     | +     | +     |
| T4898                         | +            | +   | (+)   | 7 kb  | +     | 7 kb  | +    | +     | +     | +     |
| T24-1                         | +            | +   | +     | (++)  | +     | (+)   | 7 kb | (+)   | +     | +     |
| T24-3                         | +            | +   | +     | (+)   | +     | (+)   | 13 kb| +     | +     | +     |
| T14419                        | +            | +   | (+)   | 12 kb | +     | (+)   | 13 kb| +     | +     | +     |
| T14422                        | +            | +   | (+)   | 7 kb  | +     | +     | (+) |
| T14428                        | +            | +   | (+)   | 7 kb  | +     | +     | +     |
| T14460                        | +            | +   | +     | (+)   | +     | (+)   | 17 kb| +     | +     | +     |

<sup>a</sup>L Cell line, (T) thymoma. Only monoclonal Notch1<sup>P</sup> thymomas are shown.<br><br><sup>b</sup>Each 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.<br><br><sup>c</sup>[+ | +] Lowest expression; (+ + +) highest expression.<br><br><sup>d</sup>N.D. Not determined.<br><br><sup>e</sup>110 detected only.<br><br><sup>f</sup>The intense 7.5-kb band did not allow detection of the 8.0-kb band.

An altered expression pattern was observed in almost all tumors and cell lines exhibiting Notch1 rearrangement (Notch1<sup>P</sup>) [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<sup>P</sup> 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
**Notch1 rearrangement in mouse T-cell lymphoma**

| Cell lines | Thymus | Unrearranged | Rearranged |
|------------|--------|--------------|------------|
| L7, L46, L54, L61, L42 | Thymus | 1, 2, 3, 4, 5 | 6, 7, 8, 9, 10, 11 |
| L21, L97, L48, L45, L87, L96 | Thymomas | 13, 14, 15, 16, 17, 18 | 19, 20, 21, 22, 23, 24 |
| Thymus | 12 | 25, 27, 28, 29, 30, 31 |

**Figure 3.** Northern blot analysis of representative tumors and established cell lines arising in Moloney MuLV-infected MMTV<sup>PP60</sup>/myc transgenic mice. RNAs (20 μg) were hybridized with <sup>32</sup>P-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, T14444, 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 Notch<sup>pp60</sup> 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 Notch<sup>pp60</sup> 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 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 L8 T-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-I/RBP-Jκ 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\(^{Lyt}/myc\) 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 four different CD10 repeats (Ab-intra-1) and the OPA motif (Ab-intra-2) of the intracellular domain, the Notch1-like 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...
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]. 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 Notch1iso 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
for truncated Notchl proteins
both mutant truncated transcripts and full-length pro-
cessed proteins contribute.

Discussion

The truncated Notchl 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 Notchl 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 Notchl 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 (Piml, Bm1, and Pall) [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 [Moroney] 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 Notchl may reflect the fact that the transgene is expressed in different lymphoid cell populations in MMTV\(^{D}\)/myc mice. Because Notchl 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 Notchl-related family members have been previously implicated in tumor formation in other systems. The human homolog of Notchl, TAN-1, was identified as a locus involved in the t(7:9)(q34;q34.3) chromosome translocation with the TcR\(^{\gamma}\) 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 Notchl-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 [Gallahen 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 Notchl deletion mutants have been highly selected by the oncogenic process in our T-cell tumors. Expression of similar Notchl 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 1987; 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 Notchl 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

Figure 7. Pulse-chase analysis of Notchl proteins in L48 cells.
Cells (2 x 10^7) were metabolically labeled with [\(^{35}\)S]methionine [200 \(\mu\)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-Notchl Ab-intra-1 [A] or Ab-extra-1 [B] antibodies and analyzed on 4%-8% SDS-polyacryl-
amide gels.
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, (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 Notch1 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\(^{\Delta}\)/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\(^{\Delta}\)/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\(^{\Delta}\)/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\(^{\Delta}\)/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 \textit{Notch1}\textsuperscript{enu} 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\textsuperscript{P2}/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 [10\textsuperscript{5} 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 \textsuperscript{32}P-labeled probes by the method of Southern were performed as described previously [Paquette et al. 1992].

**Molecular cloning procedures**

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

**Chromosome mapping**

\textit{Mis6} was mapped by analysis of two sets of multilocus genetic crosses: \textit{[NFS/N or CS\textsuperscript{S}1] \times M.\textit{musculus}] \times M.\textit{musculus}} and \textit{[NFS/N \times M.\textit{spretus}] \times M.\textit{spretus}} or CS\textsuperscript{8}1 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 \mu g of total RNA were reverse transcribed with Moloney MuLV reverse transcriptase according to the manufacturer’s specifications [GIBCO-BRL]. Samples [2 \mu 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, TAAGCTAGCTGGCAAAACCTACAGGTG; primer 112, CCCGAGCTCATAAAAAGAGCCCAAAAC, and primer 24, GAGTATGGACTCACC. The \textit{Notch1} specific primers were in exon C, primer 233, CTGGCTGGGAACTGGCAGCTC; primer 388, GAGTGGCCAGTTCGCCAGGAGG; in exon D, primer 234, CGACACGCTAGCAGAAACACT; in exon F, primer 299, ACAAGGACAGCGAAGGGCCCGG; and 3′ of exon F, primer 316, CCCACCTGCTTCTGATTTGCG; primer 303, GTCCACCTCGCTTGGTGTCAGCT; primer 252, GACATCCGACTGACGATCCAT. 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 [Wisstar].

For 5′ RACE, the cDNA was prepared with AMV reverse transcriptase [Pharmachem] as described above from poly(A)\textsuperscript{+} RNA of selected cell lines. The cDNA was purified on G-50 Spin columns then subjected to tailing reaction by use of dATP and terminal transferase [TdT] according to manufacturer’s specifications [Boehringer]. Purification through G-50 Spin columns was performed before the first round of RACE-PCR. The PCR were carried out with anchor T\textsubscript{7} and \textit{Notch1} specific primers. Four rounds of PCR using nested primers were performed.

**Probes**

The \textit{Notch1} probe D is a 0.9-kb BamHI–Sau3A genomic fragment. The \textit{Notch1} probe M corresponds to a 1.9-kb EcoRI–BamHI fragment from the cDNA clone MN4.0 (Rouau et al. 1992) and was subcloned in pUC18. The \textit{Notch1} probes K and A, respectively, were excised from the KS-Motch plasmid (Kopan and Weintraub 1993) or generated by RT-PCR [oligo 245: GTGGTGTCGCCCTAAGCTTCCGGGGCGG; the TCR\textsuperscript{R} RBL5 and Moloney U3 LTR fragment have been described previously [Paquette et al. 1992]. The DNA probes used to search for DNA rearrangements were: \textit{Vin1} (pro.6) [Hanna et al. 1993], \textit{Gnt1} [SS8] [Villeneur et al. 1987], \textit{Mis1} (pM1FR2) [Villeneueve et al. 1986], \textit{Bmi1} [van Lohuizen et al. 1991], \textit{Pai1} [p112] [van Lohuizen et al. 1991], \textit{Ahi1} [Poirier et al. 1988], and \textit{Pim1} [Selten et al. 1984]. All probes were labeled with \textsuperscript{32}PdCTP and \textsuperscript{32}PdATP 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 \texttimes 10\textsuperscript{5} \mu g \textsuperscript{3}H-mercautoethanol at a concentration of 2 \times 10\textsuperscript{5} cells/ml and incubated at 37°C in 5% CO\textsubscript{2}.
Growing cells were split twice a week. Lines generally became established after 2 to 4 weeks. Established lines have been passed 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-1 and Ab-extra-2, 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 Vinl/Notch1 chimeric proteins. The initiation codon was provided from the Vinl gene isolated from 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 microBCA 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].

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

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