Perturbation of Ikaros isoform selection by MLV integration is a cooperative event in Notch^{IC}-induced T cell leukemogenesis

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Summary

The chromosomal translocation t(7;9)(q34;q34.3) in human T cell acute lymphoblastic leukemia (T-ALL) results in the aberrant expression of the intracellular domain of Notch (N^{ic}). Consistent with the current multistep model for tumorigenesis, mice that express N^{ic} in T cell progenitors develop a T-ALL-like disease with a lengthened latency. Proviral insertional mutagenesis greatly accelerated the onset of leukemia in N^{ic} transgenic mice. We demonstrate that the Ikaros (Ik) locus is a common target of proviral integration in N^{ic} transgenic mice, which results in the loss of Ik DNA binding activity through altered isoform expression. We propose that cooperative leukemogenesis occurs in cells that have constitutive N^{ic} and altered Ik isoform expression because genes normally repressed by Ik become activated by N^{ic}/CSL.

Introduction

Notch1 signaling is critically important for cell fate decisions during hematopoiesis (for review see Allman et al., 2002; Deftos and Bevan, 2000; Kojika and Griffin, 2001; Ohishi et al., 2002). Several lines of evidence indicate that Notch signaling plays a direct role during many of the bifurcation points during hematopoiesis, including the transition of a common lymphoid progenitor cell to a mature T cell. Expression of constitutively active Notch1 (N^{ic}) in bone marrow (BM) hematopoietic stem cells (HSC) not only directs lineage commitment toward T cells in a thymus-independent manner, but also blocks the development of B cells (Pui et al., 1999). In addition, targeted disruption of Notch using an interferon inducible cre/lox system in postnatal mice results in the early arrest of T cells and causes an accumulation of immature B cells in the thymus (Radtke et al., 1999). In addition, expression of Lunatic Fringe, which is a negative regulator of Notch signaling, in thymic progenitors also leads to an accumulation of B cells in the thymus (Koch et al., 2001). These data indicate that Notch functions, in part, to direct T cell commitment by suppressing B cell differentiation. However, the role of Notch signaling in cells that have committed to a T cell fate is, at this point, not completely understood. Targeted disruption of the Notch1 gene in immature ($CD25^+CD44^-$) T cell precursors did not effect the maturation of these cells to become either a CD4+ or a CD8+ single positive (SP) T cell, providing data that active Notch1 signaling is not required in the maturation decisions of committed T cell precursors (Wolfer et al., 2001). One possible explanation for this finding is functional redundancy among Notch family members. However, there is currently no compelling evidence that supports a model for compensation of Notch1 by related proteins. In contrast, multiple studies using retroviral transduction and transgenics have provided evidence indicating that Notch1 signaling does have an active role in T cell maturation. Transgenic mice that express alleles of N^{ic} in immature T cells have an accumulation of either CD8+ SP T cells or both CD4+ and CD8+ SP mature T cells (Deftos et al., 2000; Robey et al., 1996). Additionally, infection of bone marrow cells with virus expressing N^{ic} impairs the maturation of immature CD4+/CD8+ double positive to SP T cells when the infected cells are used to reconstitute lethally irradiated mice (Izon et al., 2001). Moreover, it has been suggested that Notch1 is able to inhibit TCR-induced apoptosis of CD4+/CD8+ DP T cells by interaction with, and inhibition of, Nurr77 (Jehn et al., 1999). This data could, at least in part, explain the accumulation of T cells observed in each of these cases. Therefore, it appears that a lack of Notch1 signaling does not alter the maturation of committed T cells, but constitutive Notch1 signaling does perturb the maturation of T cells by directing cells to adopt one fate over another.

The mammalian Notch gene family consists of four closely related members (Notch1–4). Substantial evidence now exists that Notch1, Notch2, and Notch3 play direct roles in leukemogenesis (Aster and Pear, 2001; Aster et al., 2000; Bellavia et al.,

SIGNIFICANCE

Elucidation of the cooperative networks involved in leukemogenesis will have significant impact on the treatment of disease by providing multiple targets for drug intervention and prognostic markers. Here we provide evidence that mutations in Notch and Ik cooperate in leukemogenesis in mice and, perhaps, humans. Ik is commonly mutated in pediatric B- and T-ALLs; however, the t(7;9)(q34;q34.3) translocation is a rare event. Interestingly, we observed that in a human T-ALL cell line that harbors an Ik mutation, there was constitutively nuclear N^{ic}, suggesting that the role of Notch in leukemia should be addressed independently from the translocation. Future clinical studies will be useful in determining the precise link between mutations in Ik and activated Notch in human leukemia.

2002; Ellisen et al., 1991; Pear et al., 1996; Rohn et al., 1996). The Notch1 locus was found to be involved in the chromosomal translocation t(7;9)(q34;q34.3) in cells (SUP-T1) derived from a human T cell acute lymphoblastic leukemia (T-ALL) (Ellisen et al., 1991). In this translocation, the sequences encoding most of the extracellular domain of Notch1 are replaced with sequences derived from the T cell receptor β locus. This results in the aberrant expression of Notch1 proteins truncated of almost the entire extracellular domain and, therefore, constitutively active signaling by N^{ic}-like molecules. Reconstitution of lethally irradiated mice with BM cells expressing analogous N^{ic} constructs resulted in the development of T cell leukemia, indicating that aberrant N^{ic} expression predisposes mice to T cell leukemia, and therefore recapitulates some aspects of the human disease (Aster et al., 2000; Pear et al., 1996). Aberrant expression of other Notch family members has also been implicated in leukemogenesis. For example, infection of cats with replication competent feline leukemia virus (FeLV) yielded T cell lymphoma that harbored recombinant FeLV that had transduced a portion of the Notch2 gene (Rohn et al., 1996). Furthermore, forced expression of $Notch3^{ic}$ in T cells under control of the *lck* promoter resulted in aggressive T cell leukemia/lymphoma in transgenic mice (Bellavia et al., 2002).

Notch signaling is initiated upon direct interaction between Notch and a DSL ligand presented on an adjacent cell (Kidd et al., 1989; Muskavitch, 1994; Nye and Kopan, 1995; Tax et al., 1994; Wharton et al., 1985; Bray and Furriols, 2001; Mumm and Kopan, 2000). This interaction results in proteolytic processing that releases N^{ic} from the plasma membrane. Subsequently, N^{ic} translocates to the nucleus, where it interacts directly with the DNA binding protein CSL and the coactivator protein Mastermind-like1 (maml-1) to activate transcription of target genes (Jeffries et al., 2002; Kitagawa et al., 2001; Wu et al., 2000). In addition, there is evidence that suggests that other proteins might be associated with Notch, CSL, and maml-1 in this activator complex (Jeffries et al., 2002; Oswald et al., 2001; Wallberg et al., 2002). Although the exact mechanism by which N^{ic} drives tumorigenesis is not entirely understood, it is clear that Notch must accumulate in the nucleus and incorporate into the activator complex (Jeffries and Capobianco, 2000; Jeffries et al., 2002; Ronchini and Capobianco, 2000). Mutations in N^{ic} that disrupt complex formation also fail to activate transcription of targets genes, such as cyclin D1 (Ronchini and Capobianco, 2001). Furthermore, growth of SUP-T1 cells is inhibited by disrupting the complex using dominant negative alleles of maml-1 encoding only the sequences encompassing the Notch binding domain (Weng et al., 2003). Taken together, this data indicates that the integrity of this complex is absolutely critical for N^{ic} transforming activity.

Ikaros (Ik) is a transcriptional regulator that is critically important in the development of all lymphoid-derived cells (Georgopoulos et al., 1994; Wang et al., 1996). Ik is expressed as multiple isoforms that arise from differential splicing. All reported isoforms contain two protein dimerization zinc fingers, but have a variable number of DNA binding zinc fingers (Hahm et al., 1994; Molnar and Georgopoulos, 1994). Ik isoforms that do not contain the internal DNA binding zinc fingers are able to dimerize with DNA binding isoforms of Ik and behave as dominant inhibitors (DI) of Ik DNA binding activity (Sun et al., 1996). A role for DI isoforms in tumorigenesis has been established in a mouse model by using targeted disruption of the Ik exons encoding zinc fingers critical for DNA binding. Mice that are heterozygous for a DI isoform of Ik develop aggressive T cell leukemia (Winandy et al., 1995). Moreover, in a majority of human pediatric T- and B-ALL patients, DI isoforms are overexpressed (Sun et al., 1999a, 1999b, 1999c). The spontaneous appearance of DI isoforms in leukemia patients in conjunction with the DI transgenic mouse data suggests that inhibition of normal Ik function may be a common event in the progression of human leukemia/lymphoma.

Using proviral insertional mutagenesis we have identified the Ik locus as a common target of proviral integration in 40% of tumors arising in N^{ic} transgenic mice that were infected with Moloney murine leukemia virus. We demonstrate that Ik and the Notch effector protein CSL are able to bind the same consensus core DNA sequence in vitro. Using a reporter containing multiple copies of this consensus core sequence, we also demonstrate that Ik can inhibit the CSL-dependent transcriptional activity of N^{ic}. We propose a model in which regulatory sequences normally occupied by Ik become unoccupied through loss of Ik DNA binding activity, therefore allowing access to CSL. As a consequence, in cells that have constitutive N^{ic} and altered Ik isoform expression, genes normally repressed by Ik can be activated by N^{ic}/CSL. Furthermore, we demonstrate that a cell line derived from a patient with DI Ik isoforms expresses activated Notch protein in the nucleus, indicating that mutation of these two genes may be a common event in leukemogenesis.

Results

Infection of neonatal *lck*-N^{ic} mice with MLV accelerates the onset of T cell leukemia

Transgenic mice that constitutively express N^{ic} under the T cell-specific *lck* proximal promoter were used as a model for N^{ic} induced T cell leukemia (Robey et al., 1996). *lck*-N^{ic} transgenic mice develop T cell leukemia at a frequency of approximately 20% by five months of age (see Supplemental Data at http://www.cancercell.org/cgi/content/full/3/6/551/DC1). To identify genes important in the development of N^{ic}-induced leukemia, we used proviral insertional mutagenesis. Neonatal N^{ic} transgenic mice and nontransgenic littermates were infected intraperitoneally with tissue culture supernatants containing Moloney murine leukemia virus (MLV). Nontransgenic littermate control mice that were infected with MLV developed leukemia at an approximate frequency of 50% by 6 months of age. In contrast, infection of N^{ic} transgenic mice with MLV (N/MLV) increased the penetrance of leukemia to 100% and decreased the latency to 2 months of age (see Supplemental Data). These data indicate that proviral integration is acting as a cooperative event for leukemogenesis in N^{ic} transgenic mice. To demonstrate that N/MLV tumors harbored novel ectopic proviral integrations, DNA was extracted from tumors and analyzed by Southern blot analysis using a portion of the env and LTR proviral sequences (Figure 1A; probe: E/S). In all samples, including the noninfected, nontransgenic normal spleen (ni), the E/S probe detected a band at approximately 3 kb that corresponds to endogenous proviral sequences. However, the tumor samples harbored multiple novel integrations of the ectopic MLV provirus in the genome. To assess if the tumor samples were clonal in origin, Southern blots were probed using a portion of the TCRβ locus (Figure 1B).

Previous work by others has shown that the Notch gene locus is a common site of proviral integration in transgenic mice infected with MLV (Feldman et al., 2000; Girard et al., 1996;

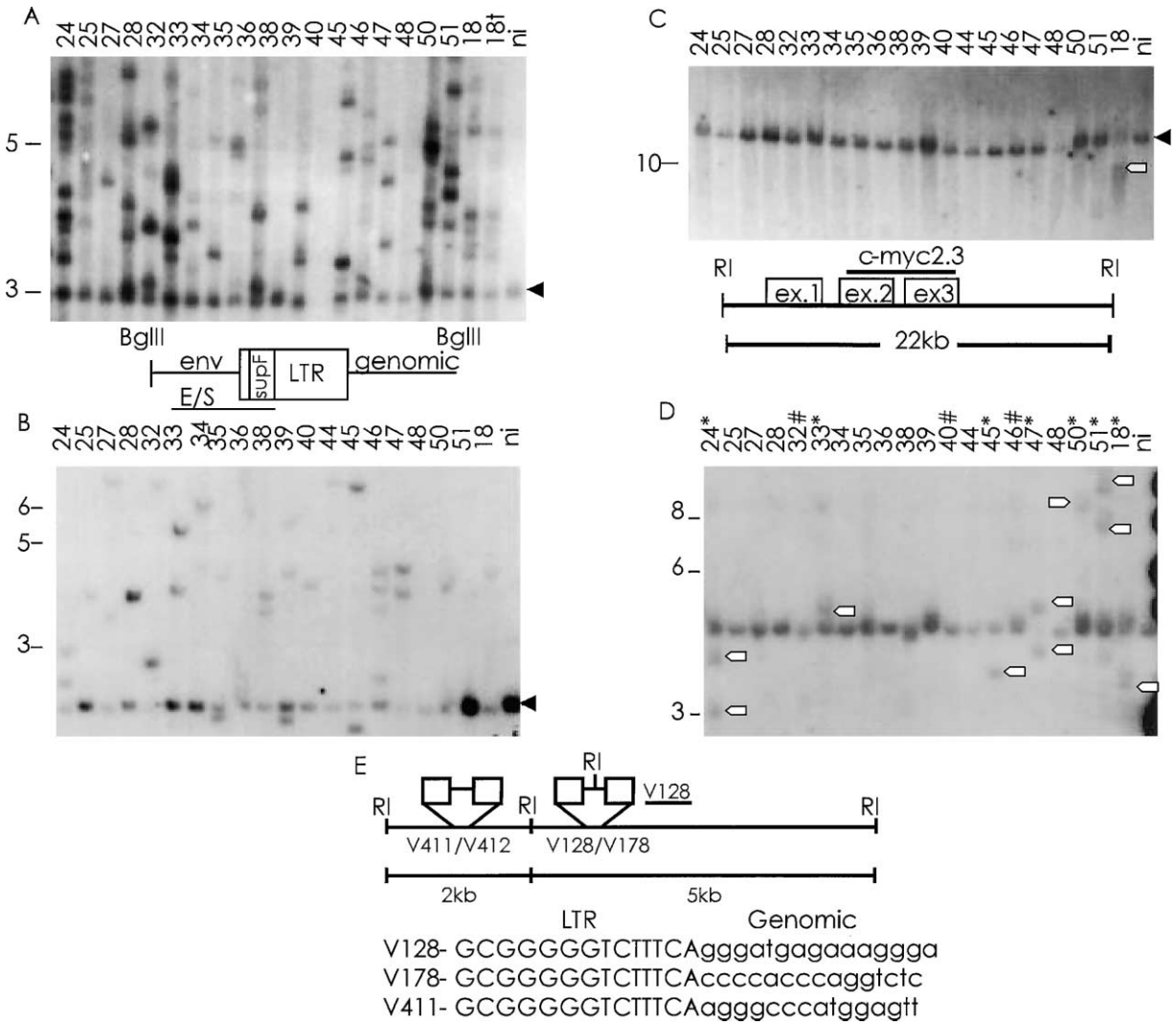

Figure 1. Southern blot analysis of N/MLV accelerated lymphomas

A: Genomic DNA was extracted from spleen samples of leukemic N/MLV mice and one nontransgenic, noninfected mouse (ni). Genomic DNA was digested with BglII and subjected to Southern blot analysis using the E/S probe (top). The arrow indicates the endogenous proviral sequences detected by the E/S probe. Molecular weight markers are indicated at the left in kilobase pairs (kb). The bottom panel illustrates the location of the BglII restriction site within the proviral env sequences, as well as the region of the provirus used for the probe (E/S).

B: Genomic DNA from the samples was digested with EcoRI and subjected to Southern blot analysis using a portion of the TCRβ locus. The arrow indicates the unrearranged TCRβ locus.

C: Genomic DNA was digested with EcoRI and Southern blot analysis was performed using c-myc2.3 probe (top). The open arrow indicates the potential integration in the c-*myc* locus. The solid arrow indicates the normal c-*myc* allele.

D: Genomic DNA was digested with EcoRI and Southern blot analysis was performed using the IPCR clone V128 as a probe. A band corresponding to the endogenous locus migrates just below the 5 kb marker. Open arrows indicate loci rearranged due to introduction of proviral sequences. *, integrations identified by Southern blot, #, integration identified by directed PCR.

E: A schematic representation of the 5 kb EcoRI fragment is shown illustrating integrations and viral LTR/genomic junctions.

Hoemann et al., 2000; Kim et al., 2003; Lund et al., 2002; Mikkers et al., 2002). In fact, it has been reported that the Notch locus is targeted by MLV integration in more than 50% of c-*myc*-induced MLV leukemias (Girard et al., 1996). This data indicates that misregulation of the Notch gene may be an important event in leukemogenesis with overexpressed c-*myc* as the first genetic alteration. To determine if the c-*myc* locus is a common target of proviral integration in N/MLV tumors, we performed Southern blot analysis on tumors using a probe specific for exons 2 and 3 of c-*myc*. In contrast to the finding that the Notch locus is commonly targeted by proviral integration in c-*myc*/MLV tumors, only one of the N/MLV tumors appeared to harbor an integration in the c-*myc* locus (Figure 1C). These results indicate that, although MLV infection of N^{ic} transgenic mice is a cooperative event that accelerates leukemogenesis, it is not likely due to integration and disruption of the c-*myc* locus.

Identification of a common site of proviral integration

In order to determine what gene(s) might be important for the rapid onset of leukemia in N/MLV mice, we sought to identify

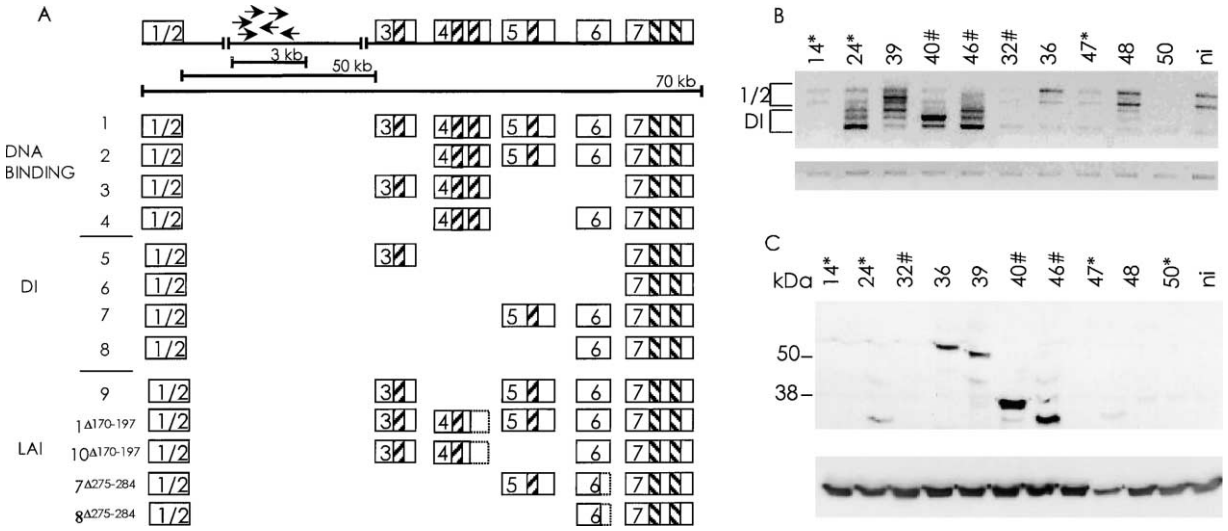

Figure 2. MLV integration into the Ik locus disrupts normal Ik expression

A. The Ik locus is illustrated to show the relative positions of the exons and the size of the locus. Arrows above the diagram represent the cluster of integrations identified in the intron between exon 2 and exon 3. DNA binding, isoforms capable of binding DNA. Non-DNA binding, naturally occurring isoforms not capable of binding DNA. LAI, leukemia associated isoforms identified herein. Deletions in exon 4 and 6 are illustrated by the dashed lines, amino acid numbering corresponds to Ik-1.

B: rt-PCR analysis was performed on RNA from a panel of tumor samples using primers specific for exons 1 and exon 7 of Ik. *, integration found by Southern blot, #, integrations identified by directed PCR. rt-PCR using β-actin primers is shown on the bottom for loading control (actin).

C: Western blot analysis on whole cell lysates from the same panel of tumor samples (**B**) shows the overexpression of DI Ik isoforms in samples with a skew in isoform expression (**B**). A Western blot for tubulin is shown from the same lysates as a loading control (Tub).

common sites of proviral integration. We used inverse PCR (IPCR) as a high-throughput method for cloning *pro*viral *i*ntegration *t*ags (pvits). IPCR-derived clones were sequenced and subsequently analyzed using the NCBI blast database. Individual pvits were then used to screen slot blots of BAC clones obtained from the BAC library screen. DNA probes made from pvits V128 and V178, obtained from tumor samples 18 and 47 respectively, hybridized to the same BAC clone (1653 443-L7). To determine the frequency of proviral integration into this locus, we performed Southern blot analysis on tumor samples using the V128 probe (Figure 1D). The V128 probe detected a band that migrated at approximately 5 kb in all samples, including the sample obtained from a noninfected, nontransgenic mouse (ni). As expected, the V128 probe detected bands in samples 18 and 47 that migrated with altered mobility due to the introduction of an EcoRI restriction site that is present in the provirus (Figures 1D and 1E). Interestingly, five other samples also contained bands that migrated with altered mobilities. Directed PCR cloning was performed using MLV-specific primers and primers designed from the 5 kb EcoRI locus to determine LTR/genomic DNA boundaries of the proviral integrations (Figure 1E). Using this technique, we demonstrated that samples 32, 40, and 46 also harbored proviral integrations that were located just upstream of the EcoRI restriction fragment (V411 and V412). Therefore, at least 40% of tumors analyzed contain proviral integration into the V128 locus. Sequence analysis of the locus using the Celera database showed that the V128 locus is contained within a 50 kb intron between exon 2 and exon 3 of the Ik gene (Figure 2A). This is the first demonstration that Ik is a common target of proviral integration, and indicates that integration into the Ik gene is a specific cooperative event with N^{ic}.

Proviral integration into the Ik locus leads to aberrant isoform selection

Since previous work has demonstrated that DI isoforms of Ik can induce T cell leukemia, we were interested in determining which Ik isoforms were produced in N/MLV tumors (Winandy et al., 1995). RNA from a panel of N/MLV tumors was analyzed for expression of Ik mRNA by rt-PCR (Figure 2B). Spleen tissue from a noninfected, nontransgenic (ni) mouse contained predominantly Ik-1 and Ik-2 (Ik-1/2) message. Samples from MLV infected N^{ic} transgenic mice that do not harbor a proviral integration in the Ik locus also predominantly express Ik-1/2. However, tumor samples from MLV infected N^{ic} mice that harbor proviral integration in the Ik locus displayed Ik mRNA expression that was altered in one of two ways. The first consequence of integration in the Ik locus was a dramatic reduction in total Ik-1/2 message (Figure 2B, lanes 14, 32, 47, 50). The second is that some of the tumors with integrations in the Ik locus had a skew in isoform selection, which led to the expression of the smaller DI isoforms of Ik (Figure 2B, lanes 24, 40, 46). We cloned the rt-PCR products from these samples (as described in Experimental Procedures) and sequenced the resulting clones to determine if mRNA products corresponded to previously identified isoforms. A number of previously identified DI isoforms were cloned from the N/MLV tumors, as well as a number of uncharacterized isoforms, which we collectively termed *l*eukemia *a*ssociated *i*soforms (LAI) (Figure 2A). Three of these isoforms have exon configurations that have not been described previously (9, $10^{\Delta 170-197}$, $1^{\Delta 170-197}$). Ik-9 encodes all exons except exon 4, which encodes the critical DNA binding zinc fingers. Ik-$10^{\Delta 170-197}$ does not encode exon 5 and has a deletion in exon 4 (amino acids 170–194) that results in the loss of the second DNA binding zinc finger.

This deletion was also identified in the context of full length Ik-1 ($1^{\Delta 170-197}$). Two other LAI cloned from the N/MLV tumors have been previously described and cloned from pediatric T-ALL patients (Sun et al., 1999a, 1999b). These isoforms contain a 10 amino acid deletion corresponding to the last 30 base pairs of exon 6 (Ik-$7^{\Delta 275-284}$ and Ik-$8^{\Delta 275-284}$). All LAI are predicted not to bind DNA, and therefore should behave as DI isoforms, due to disruption or lack of residues critical for DNA binding. Tumor 39 displayed altered Ik isoform expression; however, we could not conclusively determine that there was an integration in the Ik locus by Southern blot analysis or directed PCR. It is possible that we were unable to detect an integration because it is out of the relatively small region that we are examining (~3 kb) within the 50 kb intron. To determine if the Ik mRNA profile of the N/MLV tumors correlated with protein expression, we performed Western blot analysis on the same panel of tumor samples using a polyclonal antibody (anti-IK) generated against Ik-6 that recognizes exons 1, 2, and 7 (found in all known isoforms of Ik) (Figure 2C). Samples that had an increase in mRNA levels of the DI isoforms by rt-PCR analysis also displayed increased levels of DI Ik proteins. In contrast, there were no detectable Ik proteins in either noninfected, nontransgenic (ni) spleen tissue or the tumor samples that display a decrease in Ik message. We observed a band approximately the size of Ik-1 in tumor 36, however, as in tumor 39 we have not been able to conclusively determine that there is an integration into the Ik locus in this tumor. Since this sample has a relatively normal Ik mRNA expression pattern, compared to the ni sample, we predict that this sample might also contain the LAI Ik-$1^{\Delta 170-197}$. These data indicate that MLV integration into the Ik locus results in a perturbation of Ik expression and isoform selection.

To determine that the effects seen on the expression pattern of the Ik gene are due specifically to MLV integration in the Ik locus, we generated a number of tumors from non-N^{ic} transgenic mice that were infected with MLV (MLV tumors). These tumors displayed multiple ectopic proviral integrations, similar to the N/MLV tumors (Figure 3A). However, unlike the N/MLV tumors, none of the MLV tumors harbored integration in the Ik locus (Figure 3B, non-N^{ic} samples 1–13 compared to N^{ic} samples). Furthermore, analysis of Ik mRNA from MLV tumors revealed that expression from the Ik locus was normal compared to noninfected (ni) spleen tissue (Figure 3C). These data indicate that proviral integration into the Ik locus is a specific event for the N^{ic} transgenic background and integration into the Ik locus causes an alteration in Ik expression. Futhermore, these data support the prediction that tumors 36 and 39 likely contain integrations into the Ik locus.

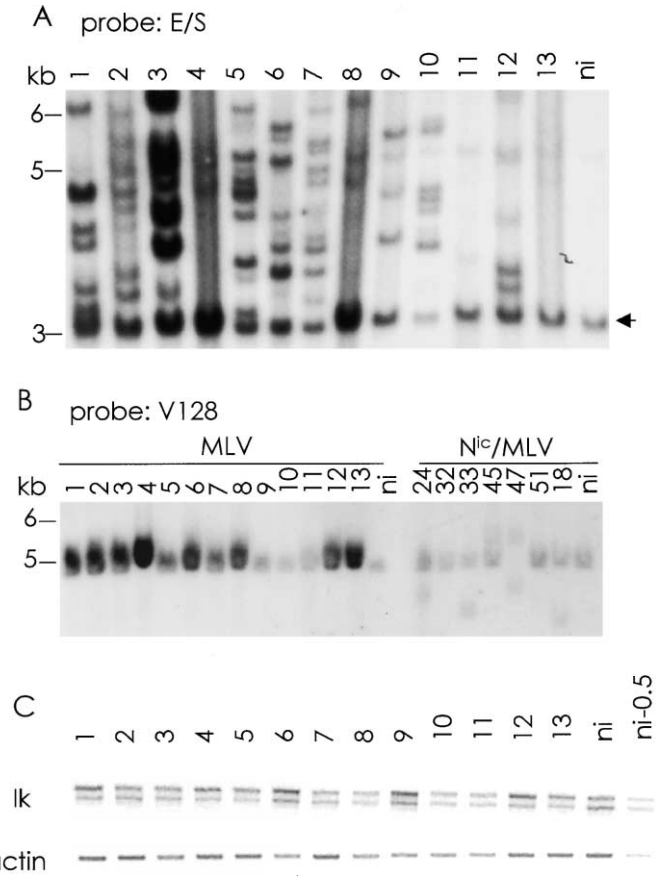

Figure 3. MLV integration into the Ik locus is specific for the N^{ic} transgenic background

A: Southern blot analysis was performed using the E/S probe on genomic DNA obtained from non-N^{ic} transgenic mice infected with MLV (samples 1–13). The arrow indicates the endogenous proviral sequences recognized by the E/S probe.

B: The V128 probe was used for Southern blot analysis on the MLV tumor samples (samples 1–13). Samples from both the MLV tumors and N/MLV tumors were analyzed with the V128 probe to demonstrate that non-N^{ic} transgenic mice do not harbor integration into the Ik locus.

C: rt-PCR analysis of RNA extracted from the MLV tumors was performed as described for **B**. To demonstrate the analysis was performed in the linear range, the ni cDNA was diluted in half (ni-0.5) before rt-PCR was carried out. All samples contain comparable levels and isoforms of Ik (Ik). β-actin was used to normalize the amounts of cDNA used in each reaction (actin).

Ik and CSL recognize a similar core DNA consensus sequence, TGGGAA

Examination of the consensus DNA binding sequence for Ik and CSL revealed that both proteins recognize the core sequence TGGGAA (Figure 4A). Therefore, we formulated the hypothesis that Ik and CSL might compete for the same regulatory elements within promoters of certain genes and differentially regulate transcription. To demonstrate that both Ik and CSL are capable of binding the same consensus DNA sequences, we performed electrophoretic mobility shift assays (EMSA) using an oligo that contains the consensus core sequence TGGGAA (IK-BS2) and has been previously shown to be a binding site for Ik (Molnar and Georgopoulos, 1994). Glutathione-agarose purified GST fusion proteins of CSL, Ik-1, and Ik-6 were assayed for the ability to bind the IK-BS2 oligo (Figure 4B). Ik-1 and CSL were capable of binding to the IK-BS2 oligo with similar affinity. Ik-6, however, did not bind the IK-BS2 oligo. The interactions of Ik-1 and CSL with the IK-BS2 oligo were specific, as they were able to be competed with 100-fold molar excess of the unlabeled wt oligo (TGGGAA), but not with 100-fold molar excess of unlabeled mutant oligo (TGAGAA for Ik-1 and CTGGAA for CSL). Moreover, a single base pair mutation in the consensus sequence of the IK-BS2 oligo (TGAGAA) abrogated Ik-1 binding but only moderately affected the binding of CSL to the oligo. The ability of CSL to bind the mutant oligo indicates that contact sites for CSL and Ik may differ and that there might be sites that can be bound exclusively by CSL. A similar experiment was performed to determine if any of the Ik LAI were capable of binding

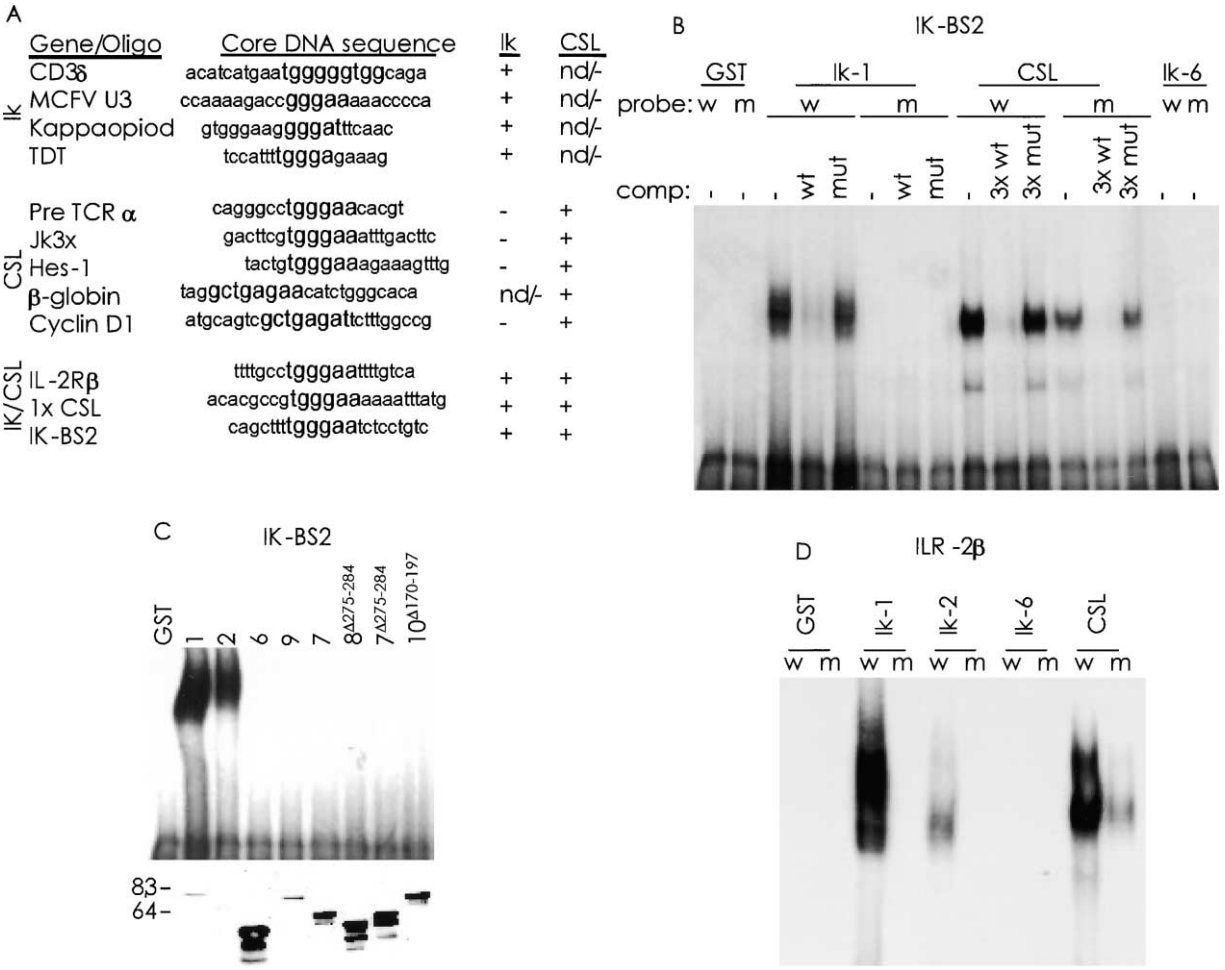

Figure 4. Ik and CSL bind the same consensus DNA sequence, TGGGAA

A: Sequences previously described that bind either Ik or CSL. Ik, sequences that Ik has been shown to specifically bind; CSL, sequences that only CSL has been shown to bind; Ik/CSL, sequences that we found both proteins are capable of binding. The column to the right of the sequences summarizes the experimental binding data. +, binding; −, no binding; nd/−, binding was not examined experimentally, but is predicted not to bind. Abbreviations for genes are as follows: CD3δ, CD3-T cell receptor complex component; MCFV U3, mink cell focus virus U3 portion of the LTR; TDT, terminal deoxynucleotidyltransferase gene; pre TCRα, component of the pre-T cell receptor complex; IL-2Rβ, interlukin-2 receptor subunit.

B: DNA binding experiments were performed using the oligo IK-BS2, originally described as an Ik binding site containing the consensus sequence TGGGAA. GST fusion proteins of Ik-1 (Ik-1), Ik-6 (Ik-6), and CSL were produced and purified as described in the Experimental Procedures. Probes correspond to: w, wt oligo containing the consensus TGGGAA; m, mutant oligo containing single base mutation to TGAGAA. For competition experiments (comp), 100-fold molar excess of cold oligo was used. Since CSL is capable of binding both the mutant and wild-type IK-BS2 oligo, competition was carried out with oligos that contain either three copies of the the wild-type TGGGAA (3× wt) or mutant site CTGGAA (3× mut).

C: EMSA analysis was performed using the IK-BS2 wt oligo with the LAI cloned from the N/MLV tumors. Lane numbers correspond to the Ik isoforms illustrated in Figure 2A. The bottom panel displays the relative amount of each protein used in each binding reaction as determined by Western blot analysis using an anti-Ik antibody.

D: EMSA experiments were performed as described for Figure 5B using an oligo designed from the IL2r-β promoter sequence. Probes: w, oligo derived from IL2r-β promoter; m, single base pair mutation to TGAGAA.

to IK-BS2 under the same conditions (Figure 4C, upper panel). Ik-1 and Ik-2 bound the oligo with similar affinity. However, no protein/DNA complexes were observed with either GST or any of the LAI tested. Western blot analysis was performed on proteins to show relative amounts of each isoform used for the DNA binding experiments (Figure 4C, lower panel). This data indicates that the isoforms produced in the N/MLV tumors are not capable of binding DNA and are therefore likely to behave as DI isoforms. To further investigate if there are any sites in physiological promoters that Ik and CSL might be able to bind and regulate, we scanned promoter sequences of T cell specific genes for exact matches to the six base pair consensus core. We found that the IL-2 receptor β (IL2R-β) contains a perfect match to the consensus sequence TGGGAA located 1512 nucleotides upstream from the start site of transcription (Codias et al., 2000). Oligos that encompass this sequence, including ten nucleotides specific to the IL2R-β promoter on either side, were designed. As before, a single base pair mutation of the core sequence to TGAGAA was also designed. EMSA analysis using these oligos demonstrated that Ik-1, Ik-2, and CSL bound the sequence derived from the IL2R-β promoter (Figure 4D). Only CSL exhibited the ability to bind the oligo with the single

base pair mutation, but with a decreased affinity. No DNA/protein complexes were detected in the GST or Ik-6 lanes, supporting the model that Ik-6 does not bind DNA and could act as a DI Ik isoform sequestering the DNA binding isoforms through heterodimer formation. We also identified a number of promoters in the literature that either CSL or Ik have been reported to bind individually, as well as the sequences used herein that both proteins are capable of binding (Figure 4A) (DiFronzo et al., 2002; Georgopoulos et al., 1992; Hu et al., 2001; Jarriault et al., 1995; Lam and Bresnick, 1998; Reizis and Leder, 2002; Ronchini and Capobianco, 2001; Trinh et al., 2001). We performed EMSA analysis with a number of oligos designed on the basis of these sequences to test if Ik and/or CSL were capable of binding them in vitro. We found that although the core TGGGAA may be the preferred binding sequence for each of these proteins, it may not, in some cases, be sufficient. Moreover, Ik appears to have a more stringent requirement for the base pairs that flank the core sequences, as displayed by its inability to bind some of the indicated oligos, consistent with a previous report by the Georgopoulos group (Molnar et al., 1996). These data indicate that Ik and CSL are capable of binding similar core DNA consensus sequences and, therefore, may differentially regulate the transcription of target genes containing these overlapping regulatory elements in their promoters.

Ik-1 blocks CSL-dependent N^{ic} transcriptional activation

Ik and CSL have both been shown to function as sequence-specific DNA binding transcriptional regulators. Since we have demonstrated that both proteins are capable of binding the same DNA sequence, we wanted to determine if Ik had any effect on N^{ic}-induced transcriptional activation. To determine the functional consequence of the competition between Ik-1 and CSL, we performed luciferase reporter assays using a reporter construct that contains eight tandem copies of a sequence containing the core consensus TGGGAA. This reporter has previously been used to measure CSL-dependent N^{ic} transcriptional activity (Fuentes-Panana and Ling, 1998; Jeffries and Capobianco, 2000). N^{ic} activated the reporter construct twelve-fold compared to vector, whereas expression of Ik-1 or Ik-6 did not activate the reporter (Figure 5A). However, when increasing amounts of Ik-1 were cotransfected with N^{ic}, the reporter activity decreased in a dose-dependent manner to levels comparable to vector alone. In contrast, cotransfection of N^{ic} with increasing amounts of Ik-6 had no effect on activation of the reporter by N^{ic}. These results indicate that Ik competes for binding to the CSL sites and blocks transcriptional activation by N^{ic}.

Several of the N/MLV tumor samples displayed a skew in Ik isoform expression that led to an increased level of the DI isoforms expression. To determine if overexpression of DI Ik-6 is able to inhibit Ik-1 repressive activity, we cotransfected increasing amounts of Ik-6 in the presence of constant N^{ic} and Ik-1. As before, N^{ic} alone activated the reporter and cotransfection of N^{ic} with an equal amount of Ik-1 blocked this activity. However, when Ik-6 was cotransfected with N^{ic} and Ik-1, we observed a dose-dependent relief of Ik-1 repression of the reporter, whereas increasing amounts of GFP had no effect (Figure 5B). These experiments demonstrate that Ik-1 is able to block the transcriptional activity of N^{ic} on a CSL-dependent reporter, and this transcriptional block can be relieved by expression of DI Ik (Ik-6). Therefore, the loss of Ik-1 DNA binding activity allows CSL-dependent N^{ic} transcriptional activation at sequences that were previously occupied and repressed by Ik-1.

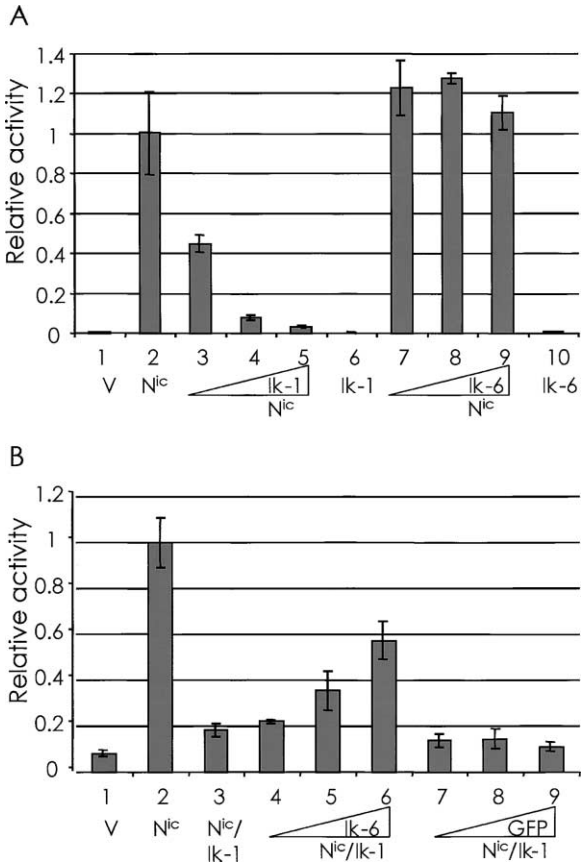

Figure 5. Ik-1 represses N^{ic} activity in vitro and coexpression of Ik-6 relieves this repression

A: A luciferase reporter assay was performed using a reporter that contains eight copies of the consensus sequence TGGGAA. Relative activity is based on activation of the reporter by N^{ic} and is set to 1 (lane 2). Empty vector (v, lane 1) shows basal level of activation from the reporter. Increasing amounts of Ik-1 represses the transcriptional activation in the presence of constant N^{ic} (lanes 3–5), while increasing amounts of Ik-6 do not repress N^{ic} activity (lanes 7–9). Ik-1 (lane 6) or Ik-6 (lane 10) alone do not activate the reporter over the level of empty vector. The total amount of DNA in each lane is held constant by addition of empty vector. Experiments were performed at least twice. Error bars represent the standard deviation of a representative experiment performed in triplicate.

B: Luciferase reporter assays were performed using the same reporter used in Figure 6A. N^{ic} activity is repressed by Ik-1 (lane 3). Cells were transfected with 100 ng of N^{ic} and Ik-1 with an increasing amount of Ik-6 (100 ng–600 ng, lanes 4–6) or GFP (100 ng–600 ng, lanes 7–9).

Human leukemia cells with DI Ik and activated N^{ic}

We have previously shown that activated N^{ic} is localized to the nucleus and is part of a high molecular weight multiprotein complex in both RKE cells and the T-ALL derived cell line, SUP-T1 (Jeffries et al., 2002). Both nuclear localization and complex formation appear to be strict requirements for N^{ic} transforming activity (Jeffries and Capobianco, 2000; Jeffries et al., 2002; Ronchini and Capobianco, 2000). To determine if there is a link in human leukemia between DI Ik and activated Notch, we examined several leukemic cell lines for evidence of activated Notch. The Molt-3 cell line has been reported to express DI Ik,

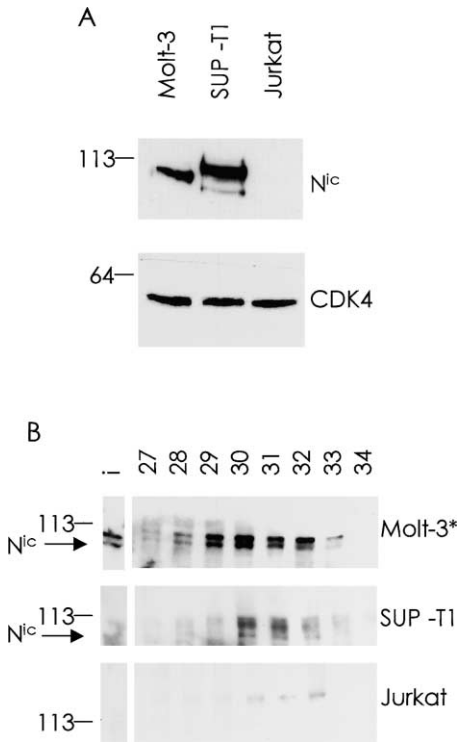

Figure 6. A cell line derived from human T cell leukemia expresses Dl Ik and activated Notch

A: Nuclear extracts from MOLT-3 and SUP-T1 cells contain large amounts of N^{ic}. Nuclear lysate from each cell line was immunoprecipitated using the Notch polyclonal antibody, 927, followed by Western blot analysis using the Notch monoclonal antibody 15A (top panel). Western blot analysis nuclear lysate from each cell line was performed with an anti-CDK4 antibody to show equal amount of protein in the lysates.

B: 2 mg of nuclear lysate from each cell line was separated on a superpose 6 gel filtration column using FPLC. Column fractions were TCA precipitated and analyzed by Western blot using anti-Notch 927 antibody. i represents 5% of the total input loaded onto the column, and the lane numbers correspond to the fractions collected. * indicates the Molt-3 profile Western blot was exposed half the time as compared to the SUP-T1 or Jurkat Western blot. Approximate molecular weight is indicated on the right in kDa and arrows indicate the size at which N^{ic} migrates. The arrowhead above fraction 34 indicates the migration of the size standard, thyroglobulin (669 kDa).

but has no known mutation in Notch (Sun et al., 1999a, 1999b). SUP-T1 cells harbor the t(7;9)(q34;q34.3) translocation involving Notch1, but have unknown Ik status (Ellisen et al., 1991). Jurkat cells have been shown to express apparently normal Notch and Ik (Aster et al., 1994; Sun et al., 1999a). To determine if these cell lines expressed activated Notch in the nucleus, we performed subcellular fractionation. Equal amounts of nuclear proteins from each cell line were immunoprecipitated with an affinity purified Notch polyclonal antibody (927) followed by Western blot analysis using the Notch monoclonal antibody 15A (Figure 6A, top panel). Similar amounts of N^{ic} were detected in the nucleus of both Molt-3 and SUP-T1 cells, indicating that Molt-3 cells have constitutively activated Notch in the nucleus. In contrast, no detectable Notch protein was immunoprecipitated from the Jurkat nuclear fractions. For normalization of the nuclear lysates, we determined the expression of CDK4 and found that all three cell lines expressed equal levels in the nuclear lysates (Figure 6A, bottom panel). To assess if the nuclear Notch detected in the Molt-3 cells is associated in a large molecular weight complex, 2 mg of each nuclear extract was separated on a Superose-6 gel filtration column (Figure 6B). N^{ic} migrated with a peak of immunoreactivity at fraction 30, similar to that observed for SUP-T1 cells (Figure 6B and Jeffries et al., 2002). However, Jurkat cells contained only very faint bands that peaked in fraction 31, but migrate at a size larger than that of N^{ic} and may represent a precursor form of Notch, probably due to membrane contamination of the nuclear fraction. Nevertheless, these data provide evidence that disruption of normal Ik gene expression, in conjunction with activated Notch, is a common event in leukemogenesis in N/MLV induced mouse tumors, and might also be in spontaneous human neoplasms.

Expression N^{ic} and Ik-6 in bone marrow cells leads to more aggressive T cell lymphoma

To directly assess if coexpression of N^{ic} and Ik-6 cooperates in T cell leukemogenesis, we performed bone marrow reconstitution assays. Bone marrow (BM) cells were isolated from pretreated 5-Flourouracil Top-Notch transgenic mice. Top-Notch mice express N^{ic} under the control of the tetracycline operator sequences. Isolated BM was either infected with bicistronic retroviruses expressing the tetracycline transactivator (tTA) and Ik-6 (MIT-I6) or the tTA alone (MIT-RX). Infection with these viruses leads to inducible expression of the N^{ic} transgene in the absence of doxycycline (dox), whereas addition of dox in the drinking water silences expression of N^{ic}. Following infection, BM was transplanted into lethally irradiated syngeneic mice. Cohorts of mice were either treated with doxycycline to inhibit activation of the N^{ic} transgene or not treated. Eight weeks following transplantation, mice that were reconstituted with BM expressing N^{ic} and Ik-6 were moribund with visible signs of lymphoma, whereas mice reconstituted with BM expressing N^{ic} alone displayed only mild signs of disease and were diagnosed with lymphoma due to a palpable spleen. Control mice displayed no visible signs of lymphoma. Mice were euthanized and visually examined for internal distress caused by lymphoma. Single cell suspensions were prepared from the spleens of all animals and analyzed by FACS analysis for CD4 and CD8. Control mice expressing neither N^{ic} nor Ik-6, as well as mice expressing only Ik-6, appeared normal by both FACS analysis and visual autopsy (Figure 7A). Mice expressing N^{ic} had a grossly enlarged spleen that was populated by immature CD4+/CD8+ double positive T cells, characteristic of leukemia. In contrast, mice expressing N^{ic} and Ik-6 appeared to have lymphoma that was much more aggressive by both FACS analysis and autopsy (Figure 7A). These mice displayed involvement of lymph nodes, thymus, spleen, and liver. Cells isolated from the spleen were almost exclusively CD4+/CD8+ double positive (Figure 7A). Primary leukemic cells (5×10^5) were then transplanted from these mice into the tail vein of syngeneic FVB/n mice and monitored until moribund. Leukemic cells that express both N^{ic} and Ik-6 were much more aggressive in the development of leukemia, with nearly 3-fold decrease in the mean latency of terminal disease compared to cells that express N^{ic} alone, 24 days compared to 64 days (Figure 7B). Cells that did not express N^{ic} did not lead to the development of leukemia. These data demonstrate that N^{ic} and Ik-6 cooperate in leukemogenesis. Furthermore, they demonstrate that sustained Notch activity is required for maintenance of the leukemic phenotype even in the presence of a cooperating mutation.

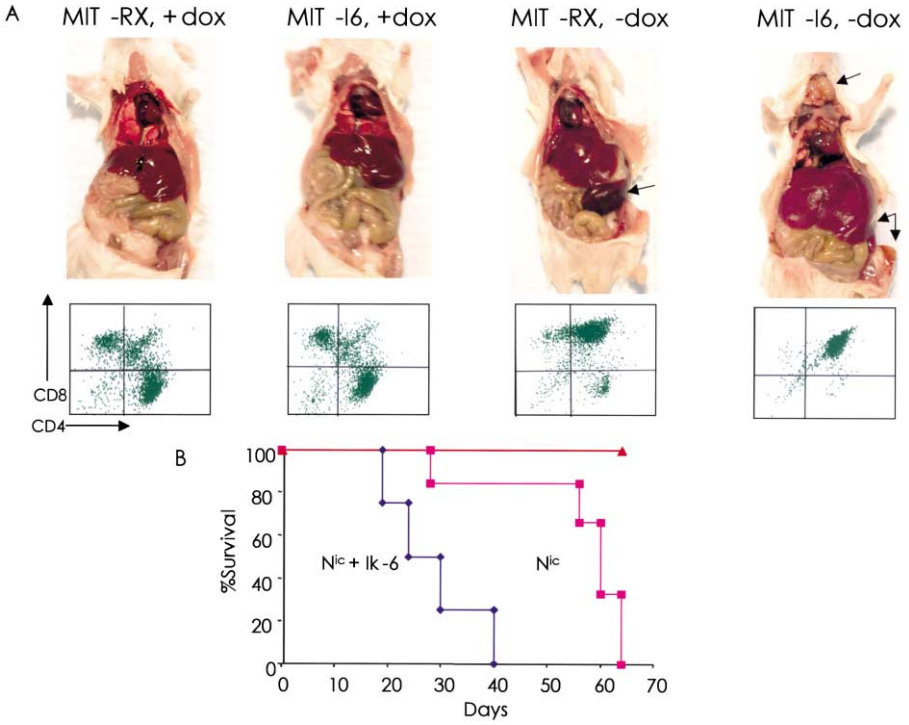

Figure 7. Nic and Ik-6 directly cooperate in T cell leukemia

A: Visual autopsy and FACS analysis of mice eight weeks following bone marrow reconstitution. Mice were reconstituted with BM expressing no protein (MIT-RX, +dox), Ik-6 alone (MIT-I6, +dox), N^{ic} alone (MIT-RX, −dox), or both Ik-6 and Nic (MIT-I6, −dox). Mice receiving BM expressing N^{ic} alone display early signs of leukemia, including enlarged spleen and an increase in CD4+/CD8+ T cells in the spleen. Mice receiving cells expressing both Ik-6 and N^{ic} display signs of a much more aggressive leukemia, including enlargement of spleen, lymph nodes, liver, and thymus, as well as complete effacement of the spleen by immature CD4+/CD8+ T cells.

B: Transplantation of primary leukemias expressing N^{ic} and Ik-6 leads to a marked decrease in survival of transplant recipients. Single cell suspensions of leukemia were prepared from the spleen of MIT-RX, −dox mice or MIT-I6, −dox mice. Cells were then transplanted into the tail vein of syngeneic mice and assayed for the development of leukemia. Mice receiving cells expressing N^{ic} alone became moribund with leukemia with a mean latency of 64 days, while mice receiving cells expressing both Ik-6 and N^{ic} succumb to leukemia with a mean latency of 24 days. Mice that received cells expressing neither N^{ic} nor Ik-6 did not develop any signs of disease during the course of the experiment.

Discussion

In this report, we provide evidence that loss of Ik DNA binding activity is a common step in the progression of N^{ic}-induced T cell leukemia. Consistent with current multistep models for tumorigenesis, N^{ic} transgenic mice succumb to T cell leukemia after a lengthened latency, suggesting that other genetic mutations must accumulate in these mice in order for leukemia to develop. By using proviral insertional mutagenesis as a genetic tool to introduce somatic mutations in N^{ic} transgenic mice, the onset of leukemia was greatly accelerated. Since integration of the provirus is a random event, common sites of integration found among different leukemia samples likely represent a gene(s) that, when disrupted by the provirus, cooperates with the transgene to accelerate leukemogenesis. Here we identify that the Ik gene is a common target of proviral integration in N^{ic} transgenic mice (Figure 1D).

We demonstrated that MLV specifically integrates into a 50 kb intron in the Ik gene in N^{ic} transgenic mice. Integration into this locus effects Ik expression in two different manners: either the normal process of isoform selection is skewed, such that there is an increase in expression of dominant inhibitory (DI) isoforms, or there is a dramatic loss in detectable message encoding DNA binding Ik isoforms (Figures 2B and 2C). We cloned Ik mRNAs from tumors that expressed DI isoforms and identified a number of previously characterized DI Ik isoforms, as well as isoforms that we termed *l*eukemia *a*ssociated *i*soforms (LAI). All these isoforms lack the critical DNA binding zinc finger motifs encoded by exon four, and we demonstrated that they could not bind DNA in vitro by EMSA analysis (Figure 4C). The other N/MLV tumors that had integrations into the Ik locus displayed a loss of Ik-1/2 expression when analyzed by rt-PCR. A simple explanation for this observation is that transcription is being initiated from the proviral LTR, resulting in Ik message that does not contain sequences derived from exon 1 or 2 and, therefore, would not be amplified by the PCR primers used. To address this issue, we performed rt-PCR analysis using primers located in the LTR of the provirus and combinations of primers in the Ik gene. However, no hybrid messages were detected. Furthermore, we did not detect any Ik proteins in these tumor samples by Western blot analysis. The Ik antibody that we used recognizes residues encoded by exon 7 that are essential for heterodimerization and are found in all DI Isoforms. Since we did not detect any isoforms in these samples, we conclude that in these tumors, the effect of proviral integration is the loss of Ik-1/2 expression. In support of our observations, it was recently reported that loss of Ik activity correlates with progression of chronic mylogenous leukemia from the chronic phase of the disease to blast crisis (Nakayama et al., 1999). In this study, the Ik expression status was determined by rt-PCR and Western blot analysis in a small set of patients that were either in chronic phase or blast crisis. Patients in the chronic phase of the disease had normal Ik expression patterns. In contrast, a majority of patients in blast crisis had altered Ik expression. Similar to the observations reported herein, there were essentially two effects on Ik expression, either a loss of Ik-1/2 mRNA expression or overexpression of DI isoforms. Therefore, we conclude that the effect of proviral integration into the 50 kb intron separating exons 2 and 3 results in the perturbation of normal Ik expression and regulation of isoform selection. Interestingly, Southern blot analysis of the DNA derived from the blast crisis patients indicated that there might be small deletions and/or insertions in the Ik locus. These mutations were not finely mapped within the locus; however, our prediction is that these mutations are

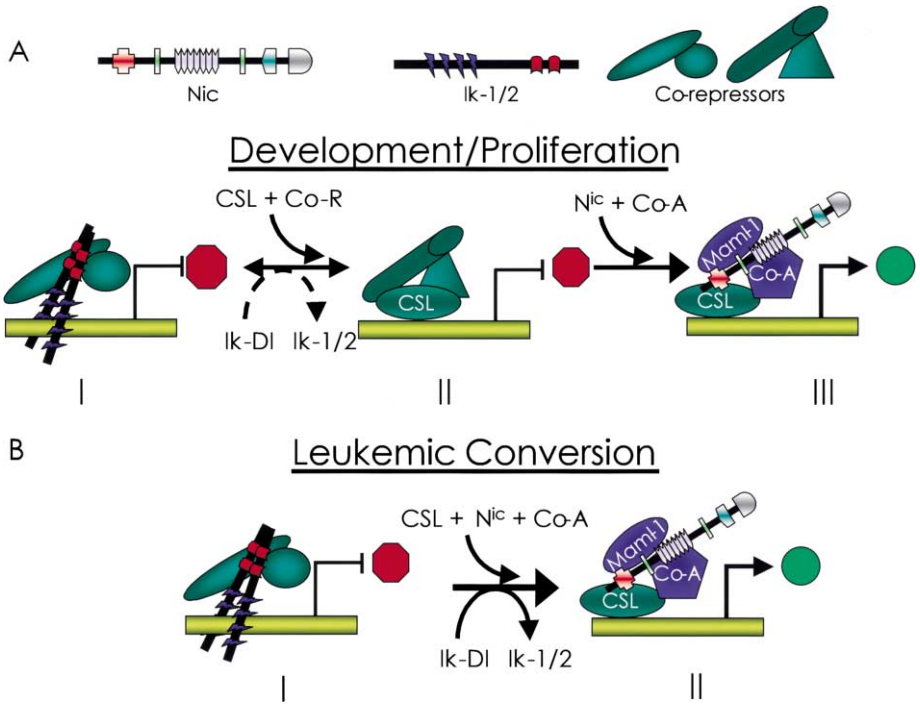

Figure 8. Reduced transcriptional repression by Ik cooperates with N^{ic} to accelerate T cell leukemogenesis

A: Regulation of genes during proliferation/differentiation is a tightly coordinated process. See text for details.

B: Leukemic conversion in Nic transgenic mice through loss of Ik repressor activity. See text for details.

likely located in a similar location, as we have described for proviral integration. Furthermore, we suggest that sequences in the intron located near the site of integration might play a vital role in regulating isoform selection.

What is the biochemical basis for cooperation between Notch and Ik in leukemogenesis? We have provided compelling evidence that indicates that cooperation is due to derepression of Ik-regulated genes that can consequently be activated by the Notch pathway. The first piece of data that led us to this model of cooperation is that Ik and the Notch effector protein, CSL, are capable of binding the same core DNA consensus sequence, TGGGAA (Figure 4). We reasoned that if Ik is a repressor of transcription and can bind to the same regulatory element as CSL, then Ik expression might block N^{ic} activation of the reporter. Investigating the consequence of Ik expression on N^{ic}-induced CSL-dependent transcriptional activation, we found that this was the case. Coexpression of Ik in the N^{ic} reporter assay resulted in a dose-dependent decrease in reporter activity (Figure 5A). Furthermore, the DI isoform Ik-6 was able to relieve this repression in a dose-dependent manner (Figure 5B). These results demonstrate that the loss of Ik DNA binding activity, either by reduced expression of DNA binding isoforms or overexpression of DI isoforms, can have dramatic effects on transcription of N^{ic}/CSL regulated genes.

In Figure 8 we illustrate a model of a gene that can be regulated by both Ik and CSL through a common regulatory element. Under physiological conditions there is likely a dynamic balance between scenarios I, II, and III, depending on the developmental stage of the cell. Ik is thought to silence gene expression through chromatin remodeling and localization of genes to regions of heterochromatin (Cobb et al., 2000; Kim et al., 1999; Koipally and Georgopoulos, 2000, 2002; Trinh et al., 2001). How would this gene become activated if transcriptionally silenced by Ik? During certain stages of hematopoietic development, DI Ik isoforms are expressed through alternative splicing (Hahm et al., 1994; Molnar and Georgopoulos, 1994). This would result in the removal of Ik1/2 from the regulatory element, and this unoccupied Ik/CSL site can then be bound by CSL (Figure 8A, II). However, in the absence of activated Notch, CSL will recruit additional corepressors (Co-R) and keep the gene in a repressed state (Honjo, 1996; Hsieh et al., 1999; Kao et al., 1998; Zhou et al., 2000; Zhou and Hayward, 2001). Following ligand activation, Notch translocates to the nucleus and converts the CSL repressor complex into an activator complex by displacing the Co-R with coactivators (Co-A) (Figure 8A, III) (Jeffries et al., 2002; Kitagawa et al., 2001; Oswald et al., 2001; Wallberg et al., 2002; Wu et al., 2002). Therefore, the transition from scenario I to II would maintain the repressive state of the gene until instructive signaling, i.e., activation of Notch in this case.

It is not currently clear what signals control Ik isoform selection during development, nor is it clear if under physiological conditions Ik can ever be converted into an activator of transcription by a mechanism other than sequestration by DI isoforms. For example, mature hematopoietic cells do not express DI Ik isoforms, indicating that Ik-regulated genes are terminally silenced. However, it is clear that maintaining Ik activity and silencing certain genes is very important, since aberrant expression of DI isoforms leads to leukemia in mice and humans (Nakayama et al., 1999; Sun et al., 1999a; Winandy et al., 1995). Recently, the Smale group provided evidence that demonstrates that Ik and the Ets family member Elf-1 directly compete for DNA binding sites on the TDT promoter (Trinh et al., 2001). Following stimulation of double positive T cells, Ik displaces the activator protein Elf-1 at a specific site in the TDT promoter to repress transcription. Downregulation of TDT expression was also correlated with localization of the gene to pericentrimeric chromatin, indicating that Ik rendered the TDT gene transcriptionally silent. In the developing *Drosophila* eye, Pointed (pnt)

blocks Notch-dependent induction of yan expression by blocking access of su(H), the *Drosophila* homolog of CSL, to a regulatory element by binding to an overlapping regulatory element (Rohrbaugh et al., 2002). Therefore, pnt and su(H) compete for a binding site in the enhancer of yan, such that pnt occupation of the sequences inhibits Notch signaling from activating transcription of the gene. pnt is a component of the receptor tyrosine kinase (RTK) signaling cascade and provides a means by which RTK signaling can directly oppose the effects of Notch signaling. These examples clearly demonstrate that multiple transcription factors can coordinate the regulation of specific genes through common regulatory elements regulating processes such as differentiation and proliferation during development.

We propose that the transition from a normal (or preneoplastic) cell to a leukemic cell in N^{ic} transgenic mice occurs through the loss of Ik DNA binding activity either by decreasing expression of Ik1/2 or skewing isoform selection toward DI Ik isoforms (Figure 8B). This event leads to Ik/CSL-regulated genes that were previously occupied by Ik to become unoccupied and therefore bound by CSL/N^{ic}, resulting in constitutive unregulated activation of this subset of genes. Since there are regulatory elements that are Ik- and CSL-specific, how do we know that it is the Ik/CSL-regulated genes that are important for leukemogenesis? Two lines of evidence indicate that this might be the case. The first is that the Molt3 cell line, which has mutant Ik isoforms, also has constitutively active N^{ic}. The second line of evidence is that integration of MLV into the Ik locus was not a selective advantage in general, since we observed no integrations into the locus unless we used the N^{ic} transgenic background. Although this evidence is compelling, we cannot rule out the possibility that specific Ik regulated genes that are not regulated by CSL are contributing to the process of leukemogenesis.

What drives the selective process for additional mutations in leukemogenesis? In a recent review, Hanahan and Weinberg suggest that the driving force for the selection of multiple mutations in tumorigenesis is the acquisition of mutations that subvert six distinct physiological processes, including dependence on proliferative signals and evasion of apoptosis (Hanahan and Weinberg, 2000). Several recent studies have demonstrated that the Notch locus is a common target of proviral integration (Feldman et al., 2000; Girard et al., 1996; Hoemann et al., 2000; Kim et al., 2003; Lund et al., 2002; Mikkers et al., 2002). In fact, in MMTV(D)/myc transgenic mice, the Notch locus is targeted in more that 50% of accelerated leukemias, indicating that mutation of Notch is an important event in c-*myc* induced leukemogenesis (Girard et al., 1996). Intuitively, one would assume that in Notch-induced leukemia, disruption of c-*myc* might also be a common event. For example, pim-1 is targeted by MLV integration in 35% of Eμ-myc-induced lymphomas, whereas myc (c- or n-myc) is targeted by MLV integration in more that 90% of Eμ-pim-1 induced lymphomas (van Lohuizen et al., 1989, 1991). However, we demonstrated that reciprocal cooperation between two genes is not necessarily the rule, since we observed that the c-*myc* locus is not commonly disrupted in N^{ic} transgenic mice. This suggests that the initiating oncogenic event might dictate the need for subsequent mutations. For example, Bmi-1 was originally identified as a common target of proviral integration in 35% of Eμ-myc induced lymphomas (van Lohuizen et al., 1991). It has since been demonstrated that Bmi-1 overexpression cooperates with c-*myc* by inhibiting the apoptotic pathway induced by myc through inhibition of INK4a/ARF expression, indicating that myc-induced tumorigenesis requires additional genetic mutations that are able to inhibit apoptotic functions (Jacobs et al., 1999). Interestingly, Notch has been demonstrated to play a role in inhibition of apoptosis by interaction with Nur-77, suggesting that the need for activation of Notch in MMTV(D)-myc induced leukemogenesis is for the inhibition of apoptosis (Jehn et al., 1999). In contrast, both Notch and c-*myc* directly upregulate components of the cell cycle and drive proliferation, independent of growth signals (Bouchard et al., 1999; Hermeking et al., 2000; Mateyak et al., 1999; Ronchini and Capobianco, 2001). Therefore, there might not be a selective advantage to overexpress c-*myc* in N^{ic}-induced leukemia. What is the selective advantage gained by disruption of Ik expression for N^{ic} induced leukemogenesis? At this point, it is not clear what cellular processes might be subverted by loss of Ik repressor activity. However, it appears that cooperation is occurring, at least in part, by making Notch a better oncogene by providing access to previously unavailable regulatory elements. Future studies should provide a physiological basis for this cooperation.

Experimental procedures

Detailed experimental procedures will be provided upon request and can be found in the Supplemental Data at http://www.cancercell.org/cgi/content/full/3/6/551/DC1.

N^{ic} transgenic mice and MLV infections

The *lck*-N^{ic} transgenic mouse line was obtained from Ellen Robey and has been previously described (Robey et al., 1996). Proviral insertional mutagenesis was performed by intraperitoneal injection of neonatal mice with viral supernatants containing MoMLV.

Southern blot analysis

Southern blot analysis was performed using standard techniques.

Isolation of proviral integration tags (pvits)

High molecular weight genomic DNA (2 μg) was digested overnight at 65°C using the restriction enzyme TaqI (NEBL). DNA was circularized by ligation at a final concentration of 1 ng/μl using T4 DNA ligase (NEBL). 5 ng of recircularized DNA was subjected to two rounds of inverse PCR (IPCR) using AGS gold taq polymerase (Hybaid). Following the second round of PCR, IPCR products were ligated directly into the PCR2.1 TA vector system (Invitrogen) using the manufacturer's recommended protocol.

RT-PCR analysis

RNA was extracted from tumor samples using Trizol (Gibco) following the manufacturer's protocol. Total RNA (2 μg) was used to produce cDNA in a 20 μl reaction using MLV reverse transcriptase (Promega) according to manufacturer's specifications.

Protein analysis

Frozen tumor samples were pulverized in liquid nitrogen using a mortar and pestle. Proteins extracts were prepared by lysis of cells in standard lysis buffer followed by standard immunoblotting techniques. Subcellular fractionation, size exclusion chromatography, TCA precipitations, and immunoprecipitations were performed as previously described, with the exceptions noted in the Supplemental Data.

Luciferase reporter gene assay

Luciferase reporter gene assays were performed as previously described (Jeffries and Capobianco, 2000).

Plasmid constructs and expression analysis

Ik cDNAs were cloned from RT-PCR reactions (described above). Briefly, cDNA was amplified for 30 cycles using Ik-specific primers and cDNAs were

purified using Qiagen quick spin column. cDNA was then digested using EcoRI and XhoI (NEBL) and ligated into pcDNA3.1 (Invitrogen) and pGEX-4T1 (Pharmacia). Cloned PCR products were verified by sequencing.

Electrophoretic mobility shift assays

GST fusion proteins were produced in *E. coli*. GST fusion proteins were purified by glutathione-agarose beads. 60 fmol of double-stranded oligo were labeled with 50 μCi of [γ-^{32}P]ATP using T4 polynucleotide kinase (NEBL). [^{32}P]-labeled oligos were purified through Sephadex G-50 spin column. DNA binding reactions were performed in HGED buffer with 0.6 fmol of probe and approximately 10 ng of GST protein at room temp for 30 min. DNA/protein complexes were separated on 4% polyacrylamide gel containing 5% glycerol in 1× TGE.

Bone marrow reconstitution assays

Mice transgenic for a tetracycline operator N^{ic} were injected i.p. with 150 μg of 5-flourouracil. Two days following treatment, the bone marrow cells (BM) from these mice were harvested using standard procedures. Red blood cells were lysed in RBC lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 10 μM EDTA). Cells were spinfected with the described viruses in the presence of 24 μg of polybrene. Spinfections were carried out at 3100 rpm at 30°C for 2.5 hr in a Sorvall RT7 swinging bucket centrifuge. Following spinfection, BM was resuspended in complete media and incubated for 30 min at 37°C. Transduced BM was used to reconstitute syngeneic FVB/n mice that had been lethally irradiated with 1100 rads given in two separate 550 rad doses four hours apart.

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