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Evidence that an IRES within the Notch2 Coding Region Can Direct Expression of a Nuclear Form of the Protein

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Summary

We previously reported the isolation from a thymic tumor of a feline leukemia virus that had transduced a fragment of the *Notch2* gene. Here we present evidence that a nuclear form of Notch2 corresponding to the biologically active intracellular domain ($N2^{ICD}$) is expressed from this recombinant retrovirus through internal ribosome entry. Internal ribosome entry sites (IRESs) are RNA structural motifs that allow 5′ cap–independent recruitment of ribosomal subunits to mRNAs. The Notch2 IRES maps exclusively to *Notch2* sequences that correspond to the coding region of the cellular gene. Therefore, these studies not only provide insights into aberrant Notch2 expression in tumors, but they may also inform our understanding of $N2^{ICD}$ generation in the cellular context.

Introduction

The strategies employed by transforming retroviruses to express cellular sequences in a foreign viral context have provided important insights into mechanisms of cellular protein expression and regulation. We previously reported the isolation of three recombinant proviral clones from the thymic tumors of two cats infected with feline leukemia virus (FeLV). Each clone had transduced a portion of *Notch2* lacking most of the extracellular domain, suggesting that there may be a novel mechanism for Notch2 expression in the context of these recombinant retroviruses (Rohn et al., 1996).

The *Notch* gene family, which includes four mammalian homologs (Notch1–4), encodes cell surface transmembrane receptor proteins important in cell fate decisions at multiple stages of development (Artavanis-Tsakonas et al., 1999). The extracellular domain mediates ligand binding and receptor activation, while the intracellular domain has several subdomains important for specific protein–protein interactions. Surprisingly, the intracellular domain also contains functional nuclear localization signals, and nuclear forms of Notch have been detected in vivo (Kidd et al., 1998; Lecourtois and Schweisguth, 1998; Struhl and Adachi, 1998). In the case of Notch1, recent studies indicate that ligand binding triggers two sequential proteolytic events that release the intracellular domain (N^{ICD}) from its membrane anchor (Kopan et al., 1996; Schroeter et al., 1998; Brou et al., 2000; Mumm et al., 2000). However, there is little data on how the N^{ICD} is produced in other Notch proteins, and at least one study has indicated that nuclear forms of Notch may be generated in the absence of ligand (Struhl and Adachi, 1998).

Overexpression of the N^{ICD} results in a constitutive, ligand-independent Notch signal and a dominant phenotype. Several lines of evidence also implicate the N^{ICD} and similarly truncated forms of Notch in oncogenesis. For example, aberrant expression of the N^{ICD} is transforming in vitro (Capobianco et al., 1997), and tumor-derived Notch alleles (*TAN1*) are predicted to express proteins lacking most or all of the extracellular domain (Ellisen et al., 1991). The structural rearrangement in FeLV/Notch2 is quite similar to the chromosomal translocation in human *Notch1/TAN1*. We have shown that the $N2^{ICD}$ is expressed from the recombinant FeLV provirus, supporting a transforming role for FeLV/Notch2 (Rohn et al., 1996). Examination of the genetic organization of this recombinant provirus does not reveal any clear insights into how expression may be regulated. However, our previous data indicate that translational rather than transcriptional regulation may be important (Rohn et al., 1996).

Most translational regulation of gene expression is thought to occur at the level of initiation (Sonenberg, 1996). In the scanning model of initiation, ribosomes are recruited to the 5′ cap of mRNAs and scan the 5′ leader linearly until reaching the start codon, which is typically an AUG triplet (Sachs et al., 1997). This cap-dependent, scanning model does not account for the translation of picornaviral RNAs, which lack a 5′ cap structure. These viral RNAs are translated through internal initiation directed by an internal ribosome entry site (IRES; Pelletier and Sonenberg, 1988; Jang et al., 1989). Several cellular mRNAs also contain IRESs in their 5′ leader sequences (Macejak and Sarnow, 1991), and a recent study described an IRES within the coding region of a cellular mRNA (Cornelis et al., 2000).

Here we exploited our previous finding that a nuclear form of Notch2 is expressed from a tumor-derived FeLV provirus to examine the mechanism of ligand-independent $N2^{ICD}$ expression. We provide evidence for IRES-mediated expression of a truncated protein corresponding to the intracellular domain. Interestingly, the IRES maps exclusively to transduced *Notch2* sequence, suggesting that an IRES could potentially direct expression of the $N2^{ICD}$ from the cellular message.

Results

v-Notch2 Is Expressed from the Spliced Envelope mRNA

The FeLV/Notch2 provirus has *Notch2* sequences coding for the conserved cysteines, transmembrane domain, ankyrin repeats, and two nuclear localization

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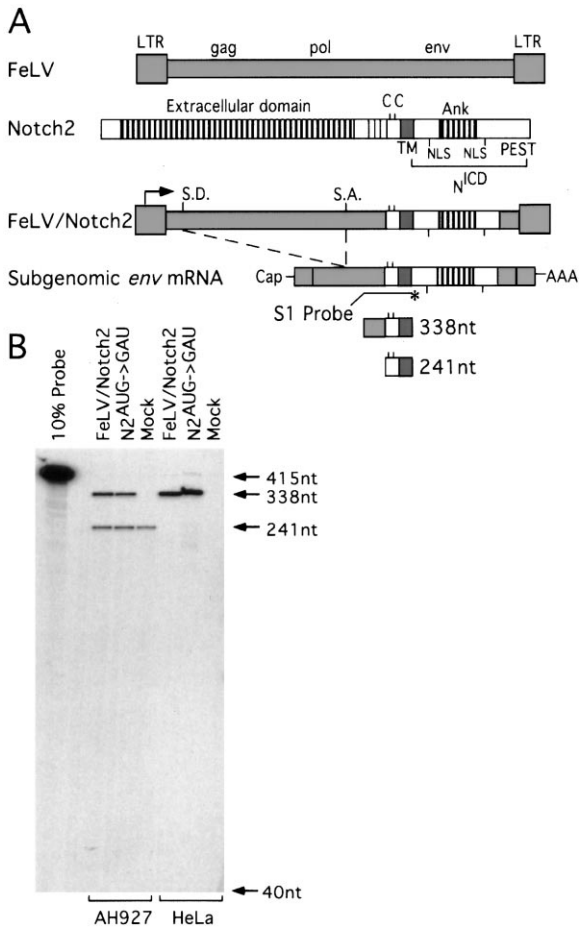

Figure 1. S1 Analysis of Viral Messages

(A) Schematic diagram of the recombinant FeLV/Notch2 provirus and parental sequences. The conserved cysteine residues ("CC"), transmembrane domain ("TM"), ankyrin repeats ("Ank"), nuclear localization signals ("NLS"), and PEST sequences are indicated. The structure of the FeLV/Notch2 recombinant is shown, and below it are the predicted subgenomic message and the S1 probe. S.D., splice donor; S.A., splice acceptor; Cap, m^7G cap structure.

(B) Total RNA was isolated from cells transfected with the indicated constructs and subjected to S1 nuclease protection analysis. $N2^{AUG\rightarrow GAU}$ is described in Figure 2A. The fragment sizes are indicated on the right, and the bottom of the gel corresponds to 40 nt. The far left lane represents 10% of the amount of probe that was used in each hybridization. The cell types used in the assay are indicated at the bottom of the figure.

signals (Figure 1A). FeLV expresses a genomic RNA that codes for the gag-pol polyprotein and a spliced message that codes for the envelope protein. Because the *Notch2* sequences replace much of the envelope open reading frame (ORF), we hypothesized that the v-Notch2 protein was expressed from the subgenomic envelope mRNA, and RT-PCR analyses supported this model (Rohn et al., 1996). In order to detect other potential messages generated by alternative splicing or cryptic promoter activity, we analyzed viral mRNAs using an S1 nuclease protection assay. In HeLa cells transfected with the FeLV/Notch2 proviral clone, a 415 base probe spanning the envelope start site and the first 240 nucleotides of Notch2 sequences protected only the expected 338 nucleotide fragment from the subgenomic viral message (Figure 1B). In feline fibroblasts, we also detected a smaller 241 nucleotide fragment corresponding to the endogenous feline Notch2 message. Thus, we found no evidence for an alternative message expressed from FeLV/Notch2 either by S1 nuclease protection, RT-PCR (Rohn et al., 1996), or Northern blot (not shown).

v-Notch2 Expression Is Regulated by a Novel Translational Mechanism

The FeLV/Notch2 proviral clone expresses a 65–70 kDa nuclear protein corresponding to the $N2^{ICD}$ (Rohn et al., 1996). The first initiation codon with a favorable context in the subgenomic message is the start site for the envelope protein, but the envelope ORF terminates just after the *Notch2* transduction junction (Figure 2A). There is a potential translation start site within an optimal Kozak consensus (AUC*AUG*G) 236 nucleotides downstream from the transduction junction, which codes for a methionine residue within the cellular Notch2 transmembrane domain (Weinmaster et al., 1992). We examined whether this AUG codon served as the v-Notch2 start site by changing it from an AUG to a GAU in the context of the proviral clone ($N2^{AUG\rightarrow GAU}$). We observed normal expression of the genomic and spliced messages from this provirus (Figure 1B) but did not detect v-Notch2 expression in transfected cells (Figure 2B). These data indicate that the v-Notch2 protein is translated from the internal Notch2 start codon rather than as an envelope fusion protein.

According to the scanning model of translation, most ribosomes traversing the subgenomic envelope message should initiate at the envelope start codon and therefore would not reach the internal Notch2 start site (Figure 2A). Although the envelope start codon lies within a near optimal context (GAG*AUG*G), a guanine at position −3 has been shown to be slightly less optimal for initiation than an adenine (Kozak, 1986b). To test whether "leaky" scanning past this suboptimal envelope AUG is occurring, we created an optimal Kozak consensus (ACC*AUG*G) surrounding the envelope start codon in the FeLV/Notch2 proviral clone (optimal env^{AUG}). We observed no significant difference in v-Notch2 expression between cells transfected with FeLV/Notch2 and those transfected with the optimal env^{AUG} construct when the levels are normalized to a cotransfected reporter construct (CS2-pvLUC; Figure 2C). Similarly, there was also no significant difference in v-Notch2 expression when the envelope AUG was mutated to a nonfunctional CUC codon ($env^{AUG\rightarrow CUC}$). Because v-Notch2 levels are not affected by either optimization or removal of the upstream envelope start site, these data argue against a model in which v-Notch2 expression is dependent on leaky ribosomal scanning.

In certain cases, ribosomes can become competent to reinitiate translation at a downstream start site after completing translation of an upstream ORF (Geballe, 1996). We tested the reinitiation model by making a construct in which the *envelope* and *Notch2* sequences are in the same translational reading frame, thereby eliminating termination. In cells transfected with this

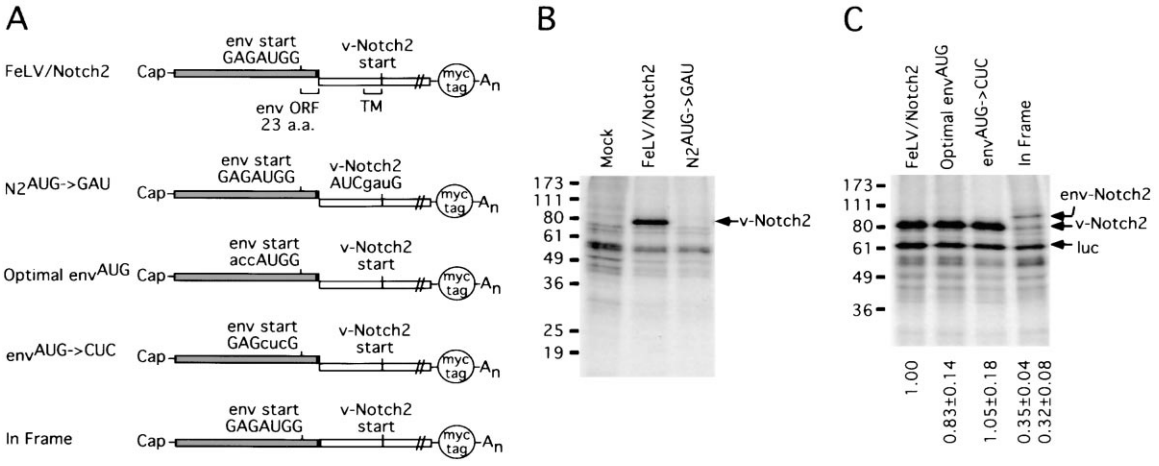

Figure 2. Analysis of v-Notch2 Translation from FeLV/Notch2

(A) Schematic of the subgenomic envelope mRNA expressed from the FeLV/Notch2 proviral clone and derivatives. The truncated envelope ORF, Notch2 transmembrane domain ("TM"), and the envelope and putative Notch2 start codons are indicated. Cap, m^{7}G cap structure; A_n, poly A tail, //, discontinuous sequence.

(B) Radioimmunoprecipitation analysis (RIPA) of HeLa cells transfected with the indicated constructs. Molecular mass markers (in kD) are indicated to the left of the gel and the v-Notch2 protein is indicated to the right.

(C) RIPA of HeLa cells cotransfected with the indicated constructs and a plasmid control for transfection and IP efficiency that expresses a myc-tagged version of luciferase (CS2-pvLUC). Molecular mass markers (in kD) are indicated to the left of the gel and v-Notch2, luciferase, and the envelope-Notch2 fusion protein are indicated to the right. Values at the bottom of each lane represent the relative luciferase-normalized Notch2 level compared to FeLV/Notch2 (mean ± SD, n = 4). For the in-frame construct, relative levels of both envelope-Notch2 and v-Notch2 are given.

construct (in-frame), we observed that the overall expression of Notch2, either v-Notch2 or the envelope-Notch2 fusion protein, was reduced compared to cells transfected with FeLV/Notch2 (0.67; Figure 2C). We also detected equal expression of both v-Notch2 (0.32 versus FeLV/Notch2) and the envelope-Notch2 fusion protein (0.35 versus FeLV/Notch2) from this clone. While the 2- to 3-fold reduction in v-Notch2 levels suggests that a portion of v-Notch2 is translated by reinitiation, the remaining v-Notch2 that is translated from the in-frame construct cannot be explained by this mechanism.

v-Notch2 Can Be Expressed by Internal Ribosome Entry

The internal v-Notch2 start codon is 814 nucleotides from the 5′ cap of the subgenomic envelope mRNA and is preceded by a sequence that is predicted to have stable secondary structure (data not shown and Zuker et al., 1999). Because long, structured leaders are characteristic of messages containing IRESs, we examined whether v-Notch2 could be translated by internal ribosome entry. To assay for IRES activity, we employed a bicistronic reporter system in which the first ORF in the bicistronic mRNA, CAT, is translated in a 5′ cap–dependent manner, while the second, luciferase, should only be expressed if there is an IRES in the inserted sequence that can recruit ribosomes (Macejak and Sarnow, 1991).

When we inserted the complete FeLV/Notch2 leader (1–814) between the CAT and luciferase ORFs, we detected an IRES activity comparable in strength to the poliovirus IRES (Figure 3B). This activity was 10- to 20-fold higher than a nonfunctional, internally deleted IRES from a related picornavirus (mutEMCV; Chen and Sarnow, 1995). We detected no significant IRES activity in this assay with the construct (1–579) containing only FeLV-derived sequences. However, the 235 nucleotide segment just upstream of the v-Notch2 start site (580–814) was sufficient to confer IRES activity. We observed higher IRES activity in fragments with little or no FeLV sequences, which could reflect a modest inhibitory effect of these sequences on IRES structure. S1 nuclease analyses suggested that there are no cryptic promoters or splice sites in this 235 nucleotide sequence that could result in a monocistronic luciferase message, and these data were supported by Northern blot analyses of cells transfected with bicistronic constructs (see supplemental figure at www.molecule.org/cgi/content/full/6/4/939/DC1). Interestingly, the minimal IRES (580–814) is derived from *Notch2* sequence and contains no viral sequences, indicating that the IRES activity maps entirely to the coding region of cellular *Notch2*.

In principle, some portion of the luciferase expression seen in the bicistronic reporter assay could be due to leaky scanning, stop codon readthrough, or translational reinitiation. To examine whether these mechanisms could account for the increased luciferase expression from the bicistronic constructs, we inserted a stable RNA hairpin structure ($\Delta G = -58.5$ kcal/mol; Zuker et al., 1999) capable of blocking scanning ribosomes between the 5′ cap and the CAT ORF (Pelletier and Sonenberg, 1985; Kozak, 1986a). We observed a greater than 20-fold reduction in scanning-dependent CAT expression from all of the constructs containing hairpins when compared to those lacking these structures (Figure 3C). In constructs containing the Notch2 IRES activity, we found

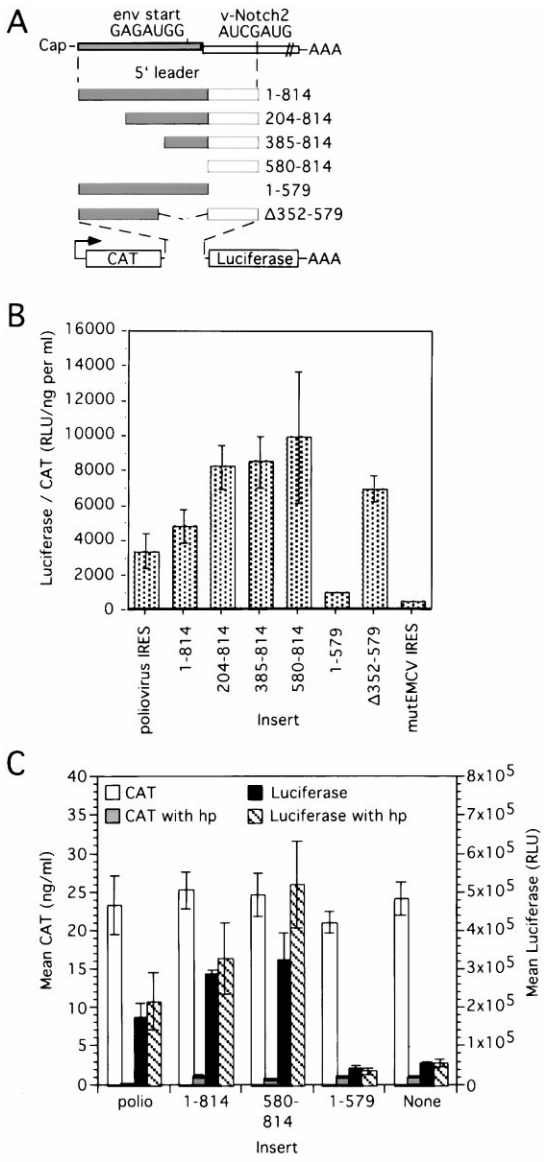

Figure 3. Bicistronic Reporter Assay

(A) Schematic of the v-Notch2 transcript leader. Viral sequences are shown as shaded boxes and Notch2 sequences are shown as open boxes. The FeLV/Notch2 fragments shown were inserted between the CAT and luciferase genes in pSV-CAT/ICS/LUC.

(B) HeLa cells were transfected in triplicate with bicistronic constructs containing the indicated inserts. Constructs containing the poliovirus IRES and an internally deleted EMCV IRES (mutEMCV) served as positive and negative controls, respectively. CAT and luciferase levels were determined from the same cell lysates. Values are expressed as the mean ± SD of triplicate transfections. Similar results were obtained from five independent experiments.

(C) Sequences capable of forming a 30 bp stable RNA hairpin were inserted between the promoter and the upstream CAT ORF, and the levels of protein expression were determined as in (B).

no inhibition of luciferase expression with hairpin insertion, similar to the construct containing the poliovirus IRES. Thus, the luciferase expression from the constructs with *Notch2* sequences is not due to leaky scanning, stop codon readthrough, or translational reinitiation promoted by these inserts.

v-Notch2 Is Expressed from the FeLV/Notch2 Provirus Even When Scanning Is Inhibited

We next asked whether this *Notch2* IRES activity could also direct expression of v-Notch2 from the viral RNA. A hairpin ($\Delta G = -62.1$ kcal/mol, Zuker et al., 1999) was inserted into the FeLV/Notch2 5′ long terminal repeat, such that both genomic and spliced mRNAs would have the hairpin just 55 bases from the 5′ cap. As a control, we also inserted an identical hairpin in a wild-type FeLV genome (61E). In cells transfected with the 5′ hp-FeLV construct, we observed a 10-fold reduction in expression of all viral proteins compared to immunoprecipitates from cells transfected with wild-type FeLV (Figure 4A). This suggests that the hairpin inhibits 5′ cap–dependent translation of both the genomic and subgenomic viral mRNAs. FeLV/Notch2 is predicted to express a 27 kDa truncated gag protein, and the 5′ hairpin also inhibited expression of this gene product from the genomic message. However, we found that hairpin insertion resulted in only a 2-fold reduction in v-Notch2 expression (Figure 4B). Thus, the effect of the hairpin on v-Notch2 expression was much more modest than its effect on expression of all other viral proteins in both wild-type FeLV and FeLV/Notch2.

To control for positional effects of the hairpin on global or local RNA structure, we inserted an identical hairpin just upstream of the envelope start site in the FeLV/Notch2 proviral clone. When we compared v-Notch2 levels in cells transfected with this 3′ hairpin construct to those transfected with FeLV/Notch2, we observed the same 2- to 3-fold inhibition of v-Notch2 expression (Figure 4B). The reduction in v-Notch2 levels is consistent with a model in which approximately half of v-Notch2 expression is due to a 5′ cap–dependent mechanism, and our analysis of expression from the in-frame proviral clone (Figure 2C) suggests that this mechanism is translational reinitiation. Based on analyses of the bicistronic reporter constructs and the hairpin proviral clones, we attribute the remaining v-Notch2 expression to internal ribosome entry mediated by the 235 nucleotide Notch2 IRES.

Discussion

We have taken advantage of the rearrangement of *Notch2* sequences in a tumor-derived FeLV provirus to better understand the origin and function of the $N2^{ICD}$. This form of Notch is not only important in aspects of development but has also been shown to be transforming. Here we show that a nuclear form of Notch2 expressed from the proviral clone is translated through internal ribosome entry. In addition, a portion of the $N2^{ICD}$ expressed from the provirus may be due to translational reinitiation. The Notch2 IRES is interesting because it is contained within what would normally be the coding region of the cellular *Notch2* gene. Therefore, our study of FeLV/Notch2 has revealed a novel mechanism of $N2^{ICD}$ expression that may also be relevant to the cellular gene.

In the FeLV/Notch2 provirus, v-Notch2 is translated from the spliced envelope message from an internal start codon located within the transduced *Notch2* sequence. Our data argue against a model in which the $N2^{ICD}$ is generated by proteolytic cleavage of an envelope-Notch2 fusion protein because we could not detect any

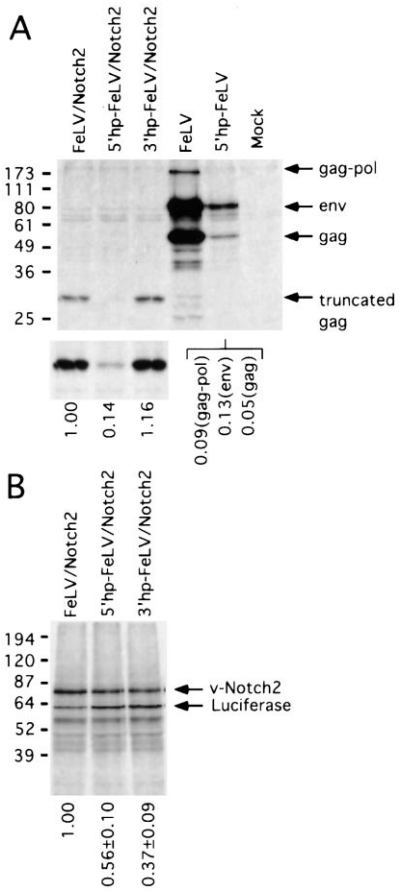

Figure 4. Analysis of v-Notch2 Expression from Viral RNAs Containing Hairpin Structures

(A) RIPA of HeLa cells transfected with the indicated proviral clones. Lysates were immunoprecipitated with a polyclonal anti-FeLV sera, and the immunoprecipitates were resolved by 10% SDS–PAGE. Molecular mass markers (in kD) are indicated to the left of the gel, and the positions of the FeLV proteins are shown on the right. The inset represents a prolonged exposure of the first three lanes of the same gel. Values below each lane represent the relative levels of the viral proteins compared to the respective clones lacking hairpins.

(B) HeLa cells were cotransfected with the indicated proviral clones and CS2-pvLUC. RIPA was performed as described in the legend for Figure 2. Molecular mass markers (in kD) are indicated to the left of the gel, and the v-Notch2 and luciferase are indicated to the right. Values below each lane represent the relative luciferase-normalized v-Notch2 level compared to FeLV/Notch2 (mean ± SD, n = 3).

evidence for such a precursor in pulse-labeled cells. Moreover, the fact that mutation of the Notch2 AUG codon abolishes v-Notch2 expression strongly suggests that v-Notch2 is initiated at this internal start site at the boundary of the transmembrane and intracellular domains. The v-Notch2 start codon is 814 nucleotides from the 5′ cap of the spliced envelope message and 300 nucleotides downstream from the viral envelope start site. While our mutational analyses of the envelope start site indicate that v-Notch2 translation does not occur by a leaky scanning mechanism, as much as half of v-Notch2 expression may be due to translational reinitiation.

IRESs are most often defined functionally using a bicistronic reporter assay because there are no consensus motifs that can reliably identify an IRES. We have used this assay to demonstrate an IRES activity within the v-Notch2 leader that maps to a 235 nucleotide region of *Notch2* sequence within the coding region of the cellular gene. These data from the bicistronic reporter assay were supported by direct analysis of v-Notch2 expression from the proviral clone, in which we used RNA hairpin structures to selectively inhibit cap-dependent scanning. Translation of v-Notch2 by internal ribosome entry represents a novel mechanism for expression of a retroviral oncogene. The *Notch2* IRES may also be representative of a novel class of IRESs that are present within open reading frames, the other example of which is PITSRLE (Cornelis et al., 2000). However, we interpret our observation of an IRES within the *Notch2* coding region with caution because surrounding sequences may play a role in facilitating internal ribosome entry by modulating the local RNA structure of the Notch2 IRES.

We hypothesize that IRES-mediated expression of a transduced oncogene may be particularly advantageous for a transforming retrovirus. Several studies have indicated that 5′ cap–dependent translation may be reduced during mitosis or times of cellular stress such as heat shock and hypoxia (Bonneau and Sonenberg, 1987), and there is evidence for IRES-directed expression of specific messages under these conditions (Stein et al., 1998; Cornelis et al., 2000; Pyronnet et al., 2000). In the case of FeLV/Notch2, translation of v-Notch2 by internal ribosome entry may allow for maintenance of v-Notch2 expression in the context of a retrovirally induced tumor where there is likely to be a high mitotic index and conditions of cellular stress such as hypoxia.

FeLV/Notch2 expresses a nuclear form of Notch2 corresponding to the intracellular domain. While the N^{ICD} is believed to represent the activated form of the protein, the molecular mechanisms involved in N^{ICD} generation for many Notch family members remain unknown. In the case of Notch1, there is evidence that the full-length transmembrane protein undergoes a series of three proteolytic events, the last of which liberates the N^{ICD} in a ligand-dependent manner (Kopan et al., 1996; Schroeter et al., 1998). It is noteworthy that the functional Notch2 start site we have identified is at a position in the protein analogous to the third and final Notch1 cleavage site at the end of the transmembrane domain. Therefore, translation initiation at this start codon would result in expression of a $N2^{ICD}$ identical in structure to the proteolytically generated $N1^{ICD}$.

There is evidence for both ligand-dependent and ligand-independent expression of the N^{ICD} in *Drosophila* (Struhl and Adachi, 1998). If the internal Notch2 start codon and upstream IRES are functional within the context of the cellular Notch2 mRNA, this single message could give rise to at least two protein species with different activities and subcellular localization. The first and likely most abundant would be a surface transmembrane protein that could give rise to a ligand-dependent $N2^{ICD}$ through proteolysis. The second would be predicted to be an activated nuclear protein that is presumably subject to regulation by proteins other than extracellular ligands. Internal initiation may only occur when 5′ cap–

dependent translation is inhibited or when specific *trans*-acting factors that allow for IRES-driven expression are present. Thus, the activity of a Notch2 IRES could potentially be regulated in a cell type– or cell cycle–specific manner as has been shown for certain cellular IRESs (Bernstein et al., 1997; Ye et al., 1997; Cornelis et al., 2000; Pyronnet et al., 2000). Intriguingly, the recently described PITSRLE IRES also maps to the coding region of its mRNA, and IRES-driven expression of a smaller form of the protein is regulated, occurring specifically during the G2/M phase of the cell cycle (Cornelis et al., 2000).

At this point, we can only speculate on whether other Notch family members contain sequences capable of directing internal initiation. In this respect, it is noteworthy that a functional translational start site was identified in the transmembrane domain of Notch1 in the context of artificially truncated alleles (Kopan et al., 1996). Given the importance of the Notch proteins in directing cell fate decisions in many lineages at multiple stages of development, an alternative means of generating the N^{ICD} in a ligand-independent, cell-specific manner would provide another layer of regulation for this critical pathway.

Experimental Procedures

Plasmid Construction and Transfections

FeLV-61E (GenBank accession number M18247), FeLV/Notch2-B (GenBank accession number U47644), and FeLV/Notch2-Bmyc (here called FeLV/Notch2) have been described previously (Rohn et al., 1996). $N2^{AUG\rightarrow GAU}$ was generated by an overlap PCR strategy. All other mutants were generated using the Quickchange protocol (Stratagene). The hairpins for 5′ and 3′ FeLV/Notch2 constructs were inserted at positions 394 and 5818 of FeLV, respectively. pSV-CAT/ICS/LUC, pSV-CAT/polio/LUC, and pSV-CAT/mutEMCV/LUC were gifts of P. Sarnow. All bicistronic plasmids are referred to here by the portion of the viral mRNA inserted into the construct, and these inserts were generated by PCR or RT-PCR of FeLV/Notch2 sequences. CS2-pvLUC contains a truncated version of the luciferase gene with an in-frame, C-terminal myc tag. Details of cloning procedures are included in the supplemental data (at www.molecule.org/cgi/content/full/6/4/939/DC1). All transfections were carried out using Effectene (Qiagen).

S1 Nuclease Protection Assay

Total cellular RNA was isolated using the RNEasy system (Qiagen). Probe fragments were generated by PCR amplification using a biotinylated T3 primer to prime the sense strand and a FeLV/Notch2 primer to prime the antisense strand. PCR products were end-labeled with ^{32}P using T4 polynucleotide kinase. The labeled fragments were bound to streptavidin-coated magnetic beads (Dynal), and the probe was eluted by denaturation in weak base. The 415 base probe contained 338 nucleotides of gene-specific sequence (from position 5950 in FeLV-61E to position 241 in FeLV/Notch2) and 77 nucleotides of vector sequence. S1 analysis was performed according to standard protocols with 10^5 counts of probe and 15 μg total RNA per sample (Ausubel et al., 1987).

Radioimmunoprecipitation Analysis

Transfected HeLa cells were starved and then labeled for 1 hr in DMEM containing 100 μCi/ml [^{35}S]methionine, and [^{35}S]cysteine (NEN). Cells were lysed on ice in RIPA buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% NP-40, 1% Deoxycholic acid, 0.1% SDS, 100 μg/ml PMSF) and clarified by centrifugation. Supernatants were precleared with protein G Sepharose for 1.5 hr at 4°C. Lysates were then immunoprecipitated with 2.5 μg anti-myc epitope mAb 9E10 (ascites provided by B. Lauring) or 2.5 μl anti-FeLV polyclonal antisera (Quality Biotech Inc.), that were prebound to protein A Sepharose. Immunoprecipitates were washed four times in a high salt wash buffer (50 mM Tris [pH 7.5], 2 M KCl, 100 mM NaCl, 5 mM EDTA, 0.5% NP-40) followed by one wash in PBS. Proteins were eluted from the beads in sample buffer and one half of the immunoprecipitate was resolved by SDS–PAGE.

Bicistronic Reporter Assay

Transfected HeLa cultures were lysed on ice 40–48 hr posttransfection in 300 μl lysis buffer (1% Triton X-100, 25 mM glycylglycine [pH 7.8], 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT). Luciferase assays were performed according to a standard protocol using 100 μl of this lysate, corresponding to approximately 1.5×10^5 cells (Ausubel et al., 1987). CAT protein levels were determined from 10 μl of the same lysates using an ELISA system (Roche).

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