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Suppressor of Hairless-independent events in Notch signaling imply novel pathway elements

Kenji Matsuno[1], Masahiro J. Go, Xin Sun, Deborah S. Eastman and Spyros Artavanis-Tsakonas*

Howard Hughes Medical Institute, Departments of Cell Biology and Biology, Yale University, New Haven, Connecticut 06536-0812, USA

*Author for correspondence (e-mail: spyros.artavanis@yale.edu)

[1]Present address: Department of Neuroanatomy, Osaka University Medical School, 2-2 Tamadaoka, Sulta, Osaka 565, Japan

SUMMARY

The Notch (N) pathway defines an evolutionarily conserved cell signaling mechanism that governs cell fate choices through local cell interactions. The ankyrin repeat region of the Notch receptor is essential for signaling and has been implicated in the interactions between Notch and two intracellular elements of the pathway: Deltex (Dx) and Suppressor of Hairless (Su(H)). Here we examine directly the function of the Notch cdc10/ankyrin repeats (ANK repeats) by transgenic and biochemical analysis. We present evidence implicating the ANK repeats in the regulation of Notch signaling through homotypic interactions. In vivo expression of the Notch ANK repeats reveals a cell non-autonomous effect and elicits mutant phenotypes that indicate the existence of novel downstream events in Notch signaling. These signaling activities are independent of the known effector Su(H) and suggest the existence of yet unidentified Notch pathway components.

Key words: Notch, Suppressor of Hairless, ankyrin repeats, *Drosophila*

INTRODUCTION

The Notch (N) signaling pathway defines an evolutionary conserved cell interaction mechanism that controls the progression of precursor cells into a more differentiated state. Genetic and molecular analyses have identified components of this pathway, which include a transmembrane receptor N, two transmembrane ligands Delta (Dl) and Serrate (Ser), a cytoplasmic protein Deltex (Dx), and nuclear components including a transcription factor Suppressor of Hairless (Su(H)) and basic helix-loop-helix (bHLH) genes encoded by the *Enhancer of split* (*E(spl)*) locus (reviewed in Artavanis-Tsakonas et al., 1995).

Genetic analyses have indicated that Su(H) behaves as a downstream effector of Notch signaling, while biochemical studies have demonstrated that it directly interacts with the intracellular domain of Notch (Fortini and Artavanis-Tsakonas, 1994; Tamura et al., 1995). The expression of at least some of the bHLH proteins encoded by *E(spl)* has been shown to depend on Notch signaling and the binding of Su(H) on their regulatory sequences (Jennings et al., 1994; Lecourtois and Schweisguth, 1995; Bailey and Posakony, 1995). It is not known whether Notch uses effectors other than Su(H) since, so far, only Su(H)-dependent Notch activities have been identified. A possible exception is implied by the finding that, while the *Drosophila* mesoderm formation and the expression of the *single-minded* (*sim*) gene in the midline depends on Notch, Su(H) loss-of-function mutations do not affect *sim* expression or mesoderm formation (Lecourtois and Schweisguth, 1995). A biochemical study involving Notch signaling in mammalian tissue culture cells has also raised the possibility of additional effector molecules (Shawber et al., 1996).

Several independent studies have implicated the region of the Notch intracellular domain composed of six tandem cdc10/ankyrin repeats (ANK repeats) in Notch signaling (Rebay et al., 1993; Lieber et al., 1993; Shawber et al., 1996; Roehl et al., 1996). Protein-binding assays involved this Notch domain in interactions with both Su(H) as well as with Dx, which acts as a positive regulator of the pathway (Fortini and Artavanis-Tsakonas, 1994; Diederich et al., 1994; Matsuno et al., 1995). The existence of a tandem ANK repeat motif has been documented in a very large number of proteins, but its general biochemical and biological properties, as well as its role in Notch signaling, are not well understood (Bork, 1993).

Here we examine directly the biochemical and genetic activities of the Notch ANK repeats. These sequences are found to mediate homotypic interactions. Moreover, the analysis of a mutation affecting the ankyrin repeats implicates ANK-mediated interactions in modulation of Notch receptor activity. In vivo analysis unravels a hitherto unidentified biological activity associated with the ANK repeats. Molecular markers demonstrate that this activity affects Notch signaling through Su(H)-independent, non-autonomous events suggesting the possible existence of novel Notch pathway elements.

MATERIALS AND METHODS

Germline transformation of *Drosophila* and phenotypic analysis

A Notch cDNA encoding amino acids 1890-2108 (Wharton et al.,

1985) downstream of a translation initiation codon from the *actin 79B* gene was cloned into the *sev* promoter vector. A fragment including the 3′ untranslated portion of the *Drosophila alcohol dehydrogenase* gene was inserted downstream of the Notch cDNA. Germline transformation was performed using standard procedures (Rubin and Spradling, 1982). Two independent transformant lines showing dominant rough eye phenotypes were used for further analyses. Compared to *sev-N^{act}* or *sev-N^{nucl}* lines, *sev-ANK* lines showed weaker rough eye phenotypes (Fortini et al., 1993). Antibody staining was carried out as described previously (Fortini et al., 1993). To detect the ANK repeat peptide, mouse monoclonal antibody C479-2B, which recognizes an epitope within the Notch ANK repeats, was used (R. G. Fehon, I. Rebay and S. Artavanis-Tsakonas, unpublished data). Mouse monoclonal antibody, mAb174, a generous gift from Sarah Bray (Cambridge University), was used to detect E(spl) Mδ (Jennings et al., 1994). Scanning electron microscopy, sectioning and staining of eyes were performed as described previously (Fortini et al., 1993).

Yeast interaction trap assay

Constructs designed to produce fusion proteins with a DNA-binding domain of LexA and with an acidic transcription activation domain are referred to as pLEX and pJG, respectively (Zervos et al., 1993). The interaction trap and β-galactosidase assays were performed as described previously (Matsuno et al., 1995). Interaction was monitored by measurement of β-galactosidase activity in liquid cultures grown in galactose. Basal activity was measured when cells were grown in the equivalent glucose medium. Assays of β-galactosidase activity were performed on three or six independent transformants. pLEXICN1, pJGICN1, pJGICN2, pLEXANK1-5, pJGANK1-5, pLEXICN1DANK, pJGICN1DANK, pJGICN1827-2109, pJGICN1827-2076, pJGICN1827-1996, pJGICN1827-1963, pJGICN 1827-1921, pJGICN1846-2076, pJGICN1889-2076, pJGANK3-5, pJGANK5, pJGANK1, pJGSTOP and pJGdx are described previously (Matsuno et al., 1995). pLEX-Hairless and pJGH-Hairless contains an entire coding region of Hairless cDNA in *Eco*RI site of pEG202 and pJGSTOP, respectively. The Hairless clones were a generous gift of Anette Preiss (University of Freiburg). pJGhN-1ANK and pJGhN-2ANK contain cDNAs encoding ANK repeats regions of human Notch-1 (aa 1826-2147) and Notch-2 (aa 1772-2084), respectively, in the *Eco*RI site of pJGSTOP vector (Ellisen et al., 1991; Stifani et al., 1992). pLEXCactus and pJGCactus contain a cDNA encoding ANK repeats region of *Drosophila* Cactus protein (aa 173-500) in *Eco*RI sites of pEG202 and pJGSTOP vectors, respectively (Kidd, 1992). pLEXICN1Su42c contains a mutant ICN1 (aa1827-2259) cDNA which has a base substitution from C to A at 6920 (Diederich et al., 1994). pJGICN11769-2259, pJGSu(H)BS-2 and pJGANK1-6 contain parts of Notch cDNAs encoding aa 1796-1825, 1769-1825 and 1891-2113, respectively, in the *Eco*RI site of pJGSTOP. Fragments described above were generated by PCR or isolated by restriction digestion.

Co-localization assay in *Drosophila* tissue culture cells

NTMICN1Myc consists of Notch peptide amino acid 1-107 and 1702-2157 (amino acid numbers according to Wharton et al., 1985). DlTMICN1Myc encompasses Dl amino acids 1-33 and 580-633 as well as Notch amino acids 1791-2157 (amino acid numbers according to Kopczynski et al., 1988). NTMMyc encompasses Notch amino acids 1-107 and 1702-1792. DlTMMyc encompasses Delta amino acids 1-33 and 580-633. Co-localization assays were performed as described previously (Diederich et al., 1994; Fortini and Artavanis-Tsakonas, 1994; Matsuno et al., 1995). Transfected cells were double-labeled with anti-Myc mouse monoclonal antibody and rat anti-Notch extracellular domain polyclonal antibody, Rat-3 (R. G. Fehon, I. Rebay and S. Artavanis-Tsakonas, unpublished data).

Reporter assay

The CAT reporter construct and the conditions of the transcription assay (Fig. 4) were described in Eastman et al. (1997).

RESULTS

Truncations of the extracellular domain of Notch result in the constitutive activation of the Notch receptor, triggering the expression of downstream genes in the *E(spl)* complex (Jennings et al., 1994; Sun and Artavanis-Tsakonas, 1996). The expression of such constructs in the developing eye under the cell-specific promoter *sevenless* (*sev*) have proven to be particularly informative in the analysis of Notch signaling (Fortini et al., 1993; Verheyen et al., 1996). We sought to examine the biological activity of the Notch ANK repeats by a similar approach. To that end, *sev*-driven constructs carrying the ANK repeats were expressed in the developing eyes of transgenic flies and the phenotypes were compared to those induced by the expression of activation forms of Notch (Fig. 1).

The ankyrin repeats elicit distinct mutant phenotypes

The expression of the entire intracellular domain of Notch under the *sev* promoter (*sev-N^{act}* and *sev-N^{nucl}* in Fig. 1) in the developing eye disc results in specific cell fate changes. In a wild-type eye disc, each ommatidial cluster has four cone cells (Fig. 2D). In *sev-N^{act}* discs, clusters containing five cone cells are observed (Fig. 2E). This phenotype is consistent with the idea that this truncated form of Notch inhibits the R7 precursor (in which the *sev* promoter is active) that acquires R7 fate and, instead, it turns into the additional cone cell (Fortini et al., 1993). In the same disc, the earlier and transient expression of *sev-N^{act}* in R3 and R4 precursors results in the transformation of these cells into two R7 cells (Fig. 2K and Fortini et al., 1993). As a result, a rough eye phenotype is observed (Fig. 2 and Fortini et al., 1993).

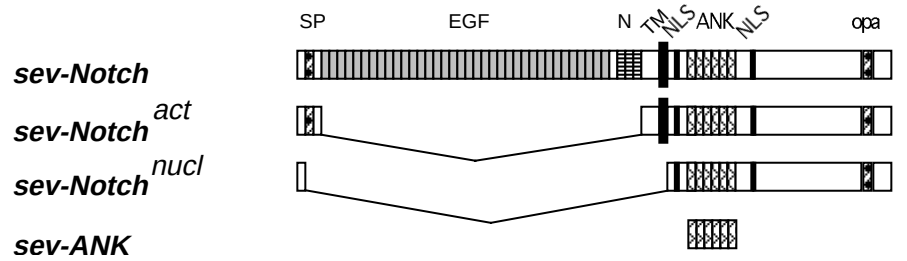


Fig. 1. Schematic diagram of Notch proteins expressed under the control of the *sev* promoter. Protein motifs include the signal peptide (SP), 36 epidermal growth factor-like repeats (EGF), 3 Notch/Lin-12 repeats (N), the transmembrane domain (TM), two putative nuclear localization signals (NLS), 6 Cdc10/Ankyrin repeats (ANK) and the polyglutamine repeat (opa). *sev-Notch* expresses full-length Notch (Fortini et al., 1993). *sev-N^{act}* and *sev-N^{nucl}* express, respectively, membrane-bound and nuclear forms of the constitutively activated forms of the receptor. Both were shown to induce identical phenotypes (Fortini et al., 1993). *sev-ANK* expresses a Notch polypeptide consisting of the ANK repeats (aa 1890-2108: Wharton et al., 1985).

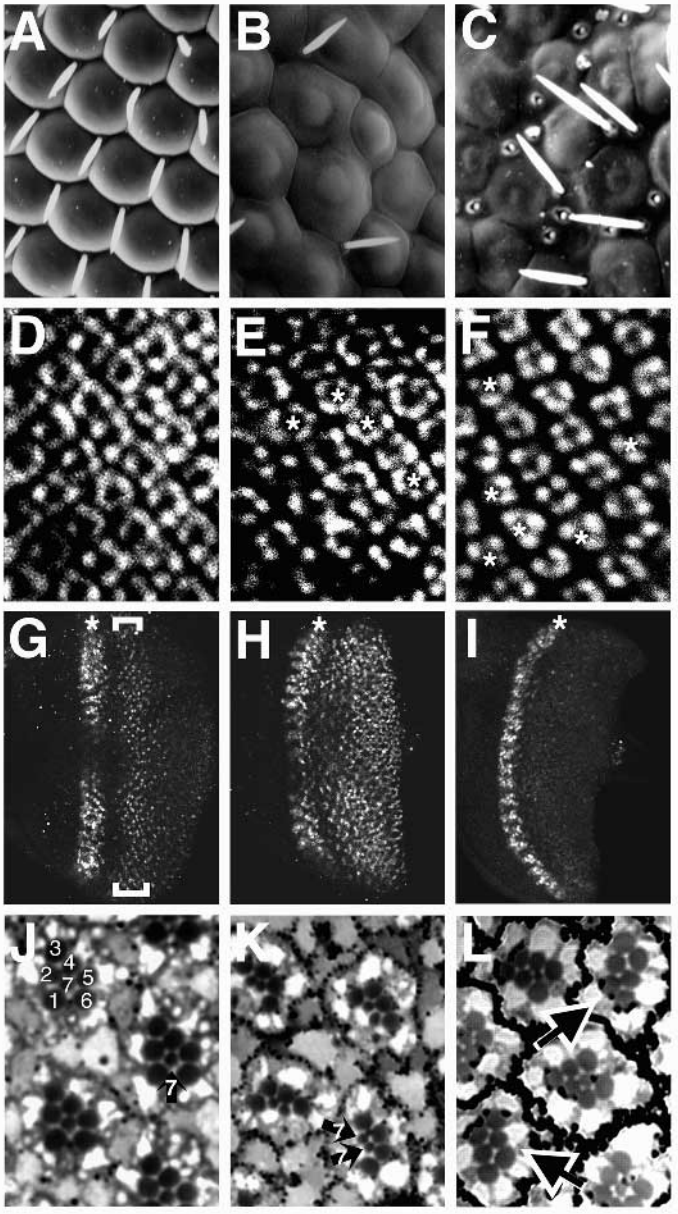


Fig. 2. Different phenotypes are associated with *sev-N^{act}* and *sev-ANK* expression. (A-C) Scanning electron microscopy of adult eyes. (A) Wild-type; (B) *sev-N^{act}*/+ ; (C) *sev-ANK/sev-ANK*. (D-F) Groucho staining of cone cells of the third-instar larval eye discs. (D) Wild type; (E) *sev-N^{act}*/+, * indicates the ommatidial cluster containing five cone cells; (F) *sev-ANK/sev-ANK*, * indicates the ommatidial cluster with one or two missing cone cells. (G-I) Optical tangential sections of third-instar larval whole eye discs of: (G) Mδ staining of a wild-type eye disc at the basal level, brackets approximately indicate cells in columns 5-12; (H) Mδ staining of *sev-N^{act}*/+; (I) mδ staining of *sev-ANK/sev-ANK* at the basal level. * points to the expression of *mδ* in the morphogenetic furrow. (J-L) Tangential sections through the apical retina of: (J) wild type, arrows indicate R7 cells; (K) *sev-N^{act}*/+, arrows indicate R7 cells; (L) *sev-ANK/sev-ANK*, arrows indicate ommatidia which contain no R7 cell.

Further trimming of the intracellular Notch sequences resulted in a construct, *sev-ANK*, which permitted the expression of only the ANK repeat region (aa1890-2108, Fig. 1). Expression of this construct in the eye gave a rough eye phenotype distinct from that associated with *sev-N^{act}* expression (Fig. 2C). *sev-ANK* eyes have ommatidial clusters lacking one or two cone cells (Fig. 2F) and an adult eye photoreceptor phenotype lacking R7 (Fig. 2L).

Table 1. Genetic interactions involving *sev-ANK* and *sev-N^{act}*

	Genetic interactions	
	sev-ANK	*sev-N^{act}*
sev-ANK	E	E
sev-N^{act}	E	E
sev-Deltex	+/–	+/–
Dl/+	E	E
dx/Y	+/–	E
Su(H)^{GOF}/+	+/–	E
Su(H)^{LOF}/+	+/–	S

Genetic interactions between *sev-ANK* and *sevN^{act}* were examined at 25°C under *trans*heterozygous conditions with the exception of the X-linked deltex. The effect the various mutations in the vertical column have on the rough eye phenotypes associated with *sev-ANK* and *sev-N^{act}* were scored according to: enhanced (E), suppressed (S) or no detectable effect (+/–). For these experiments the following alleles were used: *Dl^{5F102}*, *dx*, *Su(H)^{T4}* and *Su(H)^{IB115}* are respectively gain-of-function (GOF) and loss-of-function (LOF) Su(H) alleles (Fortini and Artavanis-Tsakonas, 1994). *sev-Dx* carries a construct expressing a full-length Deltex under the control of a *sev* promoter (Matsuno and Artavanis-Tsakonas, unpublished data).

The ankyrin repeats trigger Su(H)-independent events

The transcription factor Su(H) has been established as an effector of Notch since the expression of bHLH genes encoded by *E(spl)* depends on both Notch signaling and the presence of Su(H)-binding sites in the promoters (Jennings et al., 1994; Lecourtois and Schweisguth, 1995; Bailey and Posakony, 1995). Truncation of the extracellular domain of Notch results in an activated form of Notch as demonstrated by the fact that *N^{act}* and *N^{nucl}* expression under the *sev* promoter induces the activation of Mδ, a bHLH member of the E(spl) complex (Fig. 2H; Sun and Artavanis-Tsakonas, 1996). In contrast, the *sev-ANK* transgene fails to induce ectopic Mδ expression (Fig. 2I). This is consistent with the observation that the *sev-ANK* mutant phenotype is distinct from that induced by *N^{act}*.

In *sev-ANK* eye discs, the overall expression of Mδ is suppressed (Fig. 2, compare G and I). Mδ protein is detected only in clusters within the morphogenetic furrow where the *sev* promoter is not active (Fig. 2I; see below). Four lines of evidence argue against the possibility that this suppression reflects a dominant negative behavior associated with an overexpression of the ANK repeats. First, if overexpression of the ANK repeat peptide was interfering with wild-type Notch receptor function, one might expect that overexpressing the wild-type receptor would suppress the *sev-ANK* phenotype. Double mutants carrying *sev-ANK* and *sev-Notch* (full-length Notch, Fortini et al., 1993) do not show any modification of the rough-eye *sev-ANK* phenotype (data not shown). Second, the same is true for double mutant combinations between *sev-ANK* and *sev-Deltex* (K. Matsuno and S. Artavanis-Tsakonas, unpublished data), a positive regulator of Notch signaling (Table 1). Third, the constitutive activation of Notch via the expression of *sev-N^{act}* or *sev-N^{nucl}*, rather than suppressing the

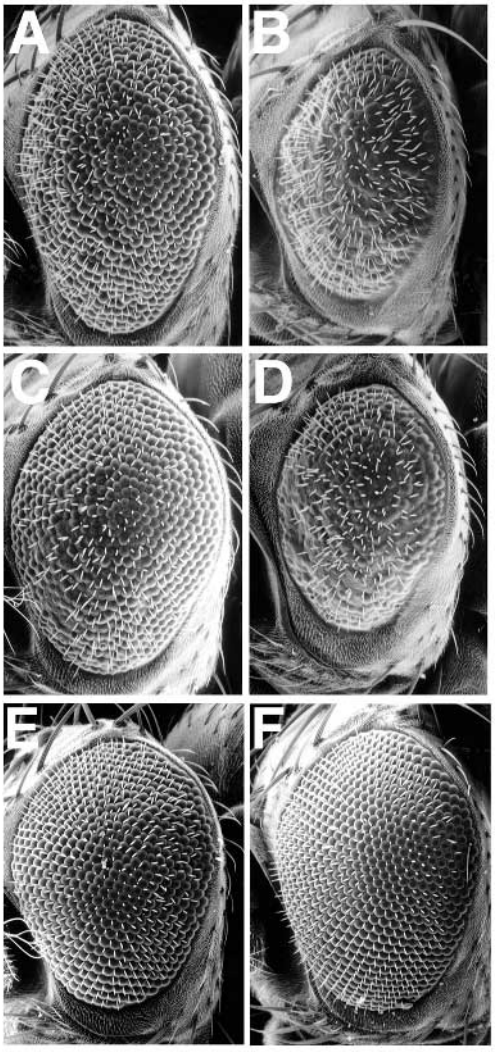


Fig. 3. The *sev-ANK* phenotype is enhanced by an activated Notch receptor. Scanning electron micrographs (SEM) of: (A) *sev-N^{nucl}*/+ eye; (B) *sev-N^{nucl}*/+; *sev-ANK*/+ eye; (C) *sev-N^{act}*/+ eye; (D) *sev-N^{act}*/+; *sev-ANK*/+ eye. (E) *sevANK*/+ eye (F) wild-type eye.

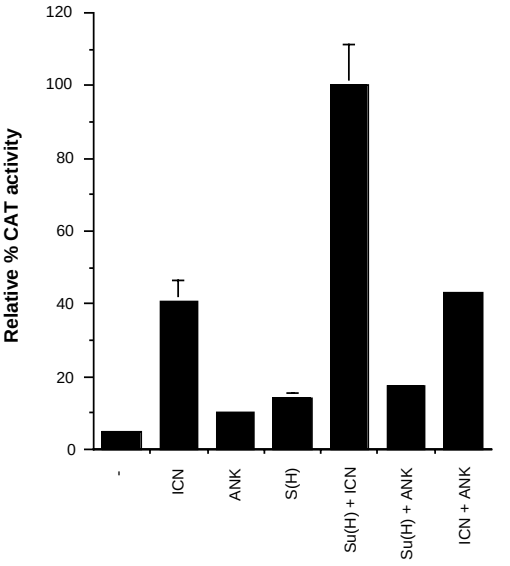


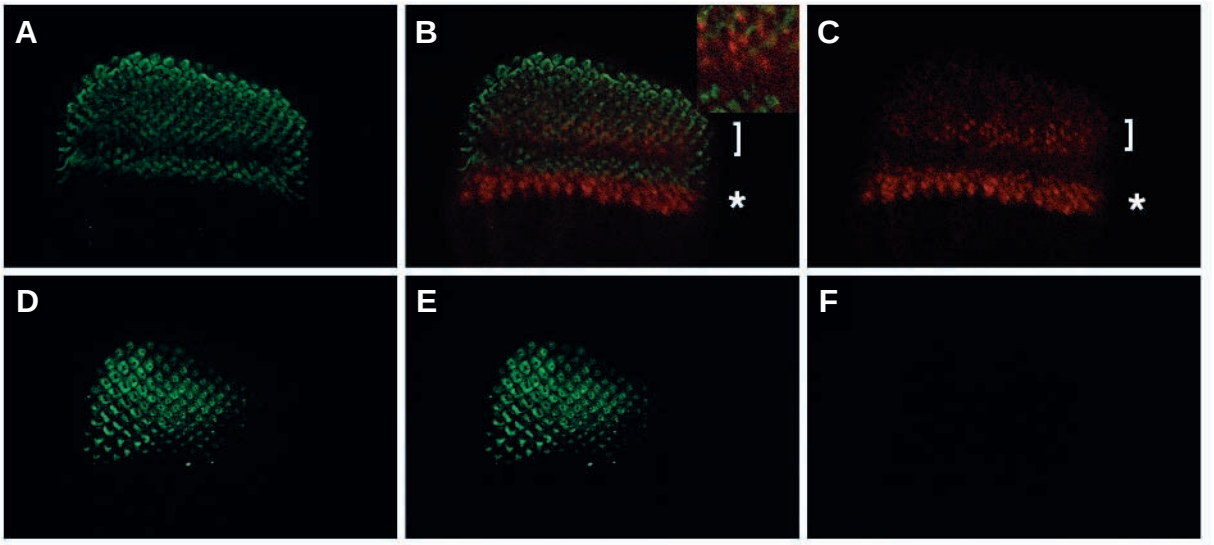
Fig. 4. The ankyrin repeats do not affect SuH-dependent transcription. In this transcription assay, a reporter construct containing the *chloramphenicol acetyl transferase* (*CAT*) gene cloned after 1.2 kb of the proximal promoter region of the *E(spl) mγ* gene was used (Eastman et al., 1997). The reporter was transfected into S2 cells alone and together with heat-shock-inducible expression plasmids containing Su(H), ankyrin repeats of Notch (ANK) or a Notch fragment containing the entire intracellular domain (ICN) as described in Eastman et al. (1997). The % CAT activity from a representative experiment done with triplicate samples is presented here. Bars represent the standard error.

sev-ANK phenotype, enhances it, suggesting that the ANK repeat peptide does not simply cancel the activity of the activated forms of Notch (Fig. 3). Finally, we find that Su(H)-dependent transcription of reporter genes in S2 cultured cells, while enhanced by N^{act}, is not affected by the expression of the ANK peptide. Fig. 4 summarizes the results of a reporter assay involving the Suppressor-of-Hairless-dependent transcription of a reporter gene driven by the E(spl) promoter m gamma. Previous work demonstrated that the promoter activity of the *E(spl)* gene *mγ* depends on the synergistic action of the intracellular domain of Notch and Su(H) (Eastman et al., 1997 and Fig. 4: Su(H)+ICN). Consistent with the idea that the ankyrin repeats exert their action independent of Su(H), we do not observe a modulation of *mγ* transcription by the ankyrin repeats peptides and Su(H) (Fig. 4, Su(H)+ANK).

The above observations suggest that the ANK repeat peptide triggers novel downstream events, apparently bypassing Su(H)-dependent Notch signaling. Consistent with this interpretation is also the finding that *sev-ANK* and either gain-of-function or loss-of-function *Su(H)* alleles do not interact genetically (Table 1). On the contrary, the *sev-N^{act}* phenotype is clearly enhanced by gain-of-function *Su(H)* alleles and suppressed by loss-of-function mutations (Table 1 and Verheyen et al., 1996). Analysis involving *Su(H)* mutant clones was uninformative since the Notch pathway is disrupted and they do not survive to adulthood (M. E. Fortini, personal communication). It is finally noted that while a polypeptide encompassing the entire Notch intracellular domain localizes to the nucleus (Struhl et al., 1993; Lieber et al., 1993; Fortini et al., 1993; Jarriault et al., 1995), the ANK repeat peptide was found to be cytoplasmic both in cultured cells and in vivo (data not shown), suggesting that, unlike N^{nucl}, the ANK repeats is not directly involved in transcriptional regulation with Su(H).

The ANK repeats act in a non-cell-autonomous fashion

In the wild-type eye disc, Mδ expression is detected in a subset of cells with basal nuclei located roughly in columns 5 to 12 (Fig. 5A,B). The Mδ staining that we observe is identical to that described by Baker et al. (1996). In the *sev-ANK* lines, Mδ expression is suppressed in the same cells (Fig. 2I). In order to accurately determine the cells in which the *sev* promoter is active relative to those producing Mδ, we used a transgenic line expressing β-galactosidase (β-Gal) under the *sev* promoter (*sev-β-Gal*). In *sev-β-Gal* eye discs, β-Gal protein was detected in the cytoplasm as well as in the nucleus, whereas Mδ was observed only in the nucleus. In order to corroborate the identity and relative position of the sev-expressing cells, we



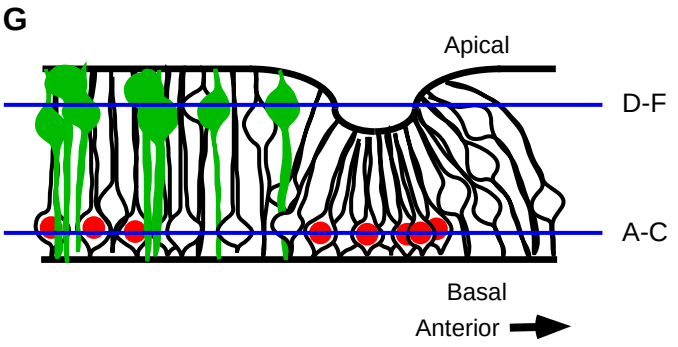


Fig. 5. The Notch ankyrin repeats suppress Mδ protein expression in a cell-non-autonomous manner. (A-F) Confocal images of third instar eye discs; (G) a diagram of a developing eye disc, which indicates approximately the apical and basal focal planes shown in A-F. (A-F) Eye discs of a *sev-β-Gal* transgenic line (a gift from Gerald M. Rubin, University of California, Berkeley) which express β-Gal under the control of the *sev* promoter were stained with rabbit IgG (green) and mouse monoclonal antibody, mAb174 (red) directed against β-Gal and Mδ, respectively. Optical tangential sections of basal (A-C, also see G, blue line indicated by A-C) and apical (D-F, also see G, blue line indicated by D-F) regions are shown: (A) basal β-Gal staining (green); (B) merged image of A and C showing basal staining of β-Gal (green) and Mδ (red) with a higher magnification inset shown in the top of right, note that green and red signals do not overlap; (C) staining of Mδ (red). The morphogenetic furrow is indicated by an asterisk and the region covered by approximately columns 5 to 12 is indicated by the bracket; (D) apical β-Gal staining (green); (E) merged image of D and F showing apical staining of β-Gal (green) and Mδ (red); (F) Mδ staining, note the absence of Mδ signal in the apical region. (G) Schematic diagram of the developing eye disc (adapted from T. Wolff and D. F. Ready, 1993) depicting the cells expressing β-Gal (indicated in green) under the control of the *sev* promoter and endogenous Mδ protein (indicated in red). Blue lines correspond to focal planes shown in A-C and D-F.

have also examined the nuclear staining of sev Delta intra expressing discs, a nuclear fragment deriving from the Delta protein (data not shown; Sun and Artavanis-Tsakonas, 1996). Fig. 5A-C shows that the β-Gal-expressing cells (green) are distinct from those cells producing Mδ (red), in the basal region of *sev-β-Gal* eye discs (see also Fig. 5G). An apical focal view (Fig. 5D-F; see also Fig. 5G), which shows cells in which the *sev* promoter is known to be active (green), lacks Mδ expression. Therefore in the *sev-ANK* discs, Mδ expression is affected by the action of the ANK repeat peptide expressed in neighboring cells under the *sev* promoter. These observations demonstrate that *sev-ANK* triggers downstream events that are independent of Su(H) and influence Notch signaling in a non-autonomous fashion.

Molecular interactions involving the ankyrin repeats

Given the distinct biological activity of the ANK repeats, especially the fact that the *sev-ANK* phenotype is not affected by loss-of-function Su*(H)* mutations, we were interested in examining the biochemical properties of the ANK repeats, which have been implicated in interactions with both Su(H) and Dx (Fortini and Artavanis-Tsakonas, 1994; Diederich et al., 1994; Matsuno et al., 1995). Earlier data from our laboratory have indirectly implicated the ANK repeats with the interactions between Su(H) and Notch, whereas more recent data have convincingly demonstrated a Notch Su(H)-binding site outside the ANK repeat region (Tamura et al., 1995). We have further examined the binding of Notch and Su(H) using the 'interaction trap' system (Zervos et al., 1993).

In agreement with the report of Tamura et al. (1995), Su(H) binds to a subtransmembrane region of Notch (aa 1769-1825) which excludes the ankyrin repeats (Fig. 6, construct 2). However, a Notch peptide (aa 1827-2259) that does not include this subtransmembrane region but instead carries a region C-terminal to it, including the ANK repeats, also binds to Su(H) (Fig. 6, construct 3; Fortini and Artavanis-Tsakonas, 1994). The ANK repeats are necessary for this second binding site, since their deletion eliminates Su(H) binding (compare Fig. 6, construct 3 and 14). On the contrary, a peptide encompassing just the ANK repeats (aa 1891-2113) does not bind to Su(H) (Fig. 6, construct 16, and see Tamura et al., 1995) but is capable of binding to Deltex (Matsuno et al., 1995). The in vivo

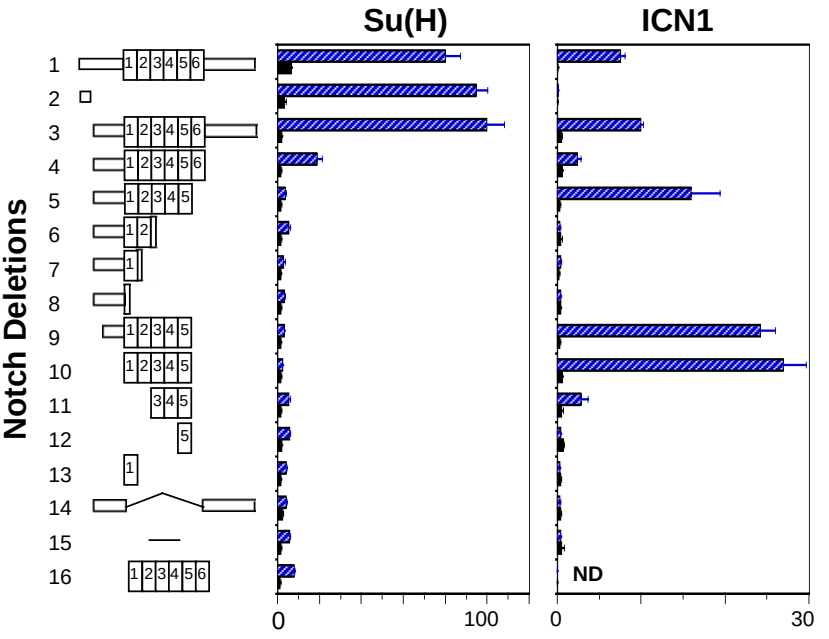


Fig. 6. The ankyrin repeats (ANK) support homotypic interactions and are necessary but not sufficient for one of the two Su(H)-binding sites. Deletion constructs of Notch intracellular domain in pJGSTOP were co-transfected with either pLEXSu(H) or pLEXICN1. The average value of β-galactosidase activity (with standard deviation) normalized to an arbitrary value of 100 for the interaction with pJGICN1 (construct 3) is shown. The activities recorded for induced (galactose) and non-induced (glucose) cultures are represented by stippled and solid bars, respectively. Numbers next to pJG constructs refer to amino acids (Wilkinson et al., 1994): 1; pJGICN1769-2259, 2; pJGSu(H)BS-2 (1769-1825), 3; pJGICN1, 4; pJGICN1827-2109, 5; pJGICN1827-2076, 6; pJGICN1827-2109, 7, pJGICN1827-2076, 8; pJGICN1827-1963, 9; pJGICN1846-2076, 10; pJGANK1-5 (1889-2076), 11; pJGANK3-5 (1969-2076), 12; pJGANK5 (2036-2076), 13; pJGANK1 (1846-1963), 14; pJGICN1ΔANK (aa 1827-2259 and 2111-2259), 15; pJGSTOP (no insert), 16; pJGANK1-6 (1891-2113).

expression of an ANK repeat polypeptide (aa 1890-2108) elicits the dominant phenotypes described above. These results also support the idea that the activity observed in the eye discs of *sev-ANK* is independent of Su(H).

We conclude that *Drosophila* Notch has at least two distinct binding sites for Su(H). The first is independent of the ANK repeats, while for the second the ANK repeat region appears to be necessary but not sufficient. We note that the yeast assay data are consistent with co-localization assays in *Drosophila* tissue culture cells (Fortini et al., 1994).

The ANK repeats mediate homotypic interactions

The interaction trap study reveals that the ANK repeats are involved in homotypic interactions, a property that may underlie the biological function of the ANK repeats (Table 2, construct 1). Deletion studies define the Notch region encompassing only ANK repeats 1-5 as necessary and sufficient to mediate these interactions (Table 2, constructs 7, 10 and 14). Also, the Notch ANK repeats from *Drosophila* interact with the analogous regions of two human Notch homologs, Notch-1 and Notch-2 (Ellisen et al., 1991; Stifani et al., 1992; Table 2, constructs 11 and 12). This interaction seems specific since the ANK repeats of *Drosophila* Notch do not associate with the ANK repeats of Cactus (Kidd, 1992; Table 2, construct 13), even though the Cactus ANK repeats do show homotypic interactions (Table 2, construct 14).

The yeast-based interaction trap analysis was corroborated using *Drosophila* cultured cells. Full-length Notch was co-expressed with Myc-tagged proteins with and without the Notch ANK repeats (shown schematically in Fig. 7A). The relative subcellular localization of the two proteins was then monitored. When full-length Notch was co-expressed with a Myc-tagged fragment containing the ANK repeats (NTMICN1Myc and DlTMICN1Myc in Fig. 7A), the two polypeptides co-localized in the majority of cells (Fig. 7D and E). Co-localization was independent of the Myc-tag and depended on the presence of the ANK repeats since Myc-

Table 2. Homotypic interactions between cytoplasmic domains of Notch protein

	Expression constructs	Media: galactose	Media: glucose
1.	pLEXICN1/pJGICN1	1500 (687)	1 (1)
2.	pLEXICN1/pJG	47 (4)	8 (6)
3.	pLEXICN1/pJGICN2	59 (11)	5 (2)
4.	pLEXICN1/pJGHairless	88 (9)	7 (1)
5.	pLEX/pJGICN1	79 (1)	7 (1)
6.	pLEXHairless/pJGICN1	81 (10)	6 (1)
7.	pLEXANK1-5/pJGANK1-5	1638 (190)	10 (3)
8.	pLEXANK1-5/pJGICN1ΔANK	42 (8)	11 (2)
9.	pLEXICN1ΔANK/pJGANK1-5	97 (24)	30 (7)
10.	pLEXICN1ΔANK/pJGICN1ΔANK	112 (49)	33 (1)
11.	pLEXICN1/pJGhN-1ANK	1381 (172)	10 (1)
12.	pLEXICN1/pJGhN-2ANK	861 (197)	5 (1)
13.	pLEXICN1/pJGCactusANK	68 (5)	7 (2)
14.	pLEXCactusANK/pJGCactusANK	768 (72)	10 (1)
15.	pLEXCactusANK/pJGHairless	109 (21)	14 (2)
16.	pLEXHairless/pJGCactusANK	55 (6)	5 (1)
17.	pLEXICN1/pJGICN1	1305 (411)	11 (1)
18.	pLEXICN1Su42c/pJGICN1	3831 (382)	23 (2)
19.	pLEXICN1/pJGDx	3419 (896)	22 (7)
20.	pLEXICN1Su42c/pJGDx	4469 (687)	53 (16)
21.	pLEXICN1/pJGSu(H)	1037 (154)	8 (5)
22.	pLEXICN1Su42c/pJGSu(H)	596 (48)	10 (6)

Amino acid numbers (Wharton et al., 1985). pLEXICN1 and pJGICN1 encode the Notch intracellular domain including the ankyrin repeats (aa 1827-2259). pJGICN-2 encodes the C-terminal region of the Notch intracellular domain (aa 2109-2704). pLEXHairless and pJGHairless encode the entire Hairless protein (Bang and Posakony, 1992). pLEXANK1-5 and pJGANK1-5 encode the ankyrin repeats 1-5 (aa1889-2076). pLEXICN1DANK and pJGICN1DANK encode an ICN1 derivative which has a deletion of the entire ANK repeats region (aa 1827-1884 and 2111-2259). pJGhN-1 and pJGhN-2 encode the ankyrin repeat regions of human Notch-1 (aa 1826-2147) and Notch-2 (aa 1772-2084), respectively (Ellison et al., 1991; Stifani et al., 1992). pLEXCactusANK and pJGCactusANK encode the ankyrin repeats region of the *Drosophila* Cactus protein (aa173-500) (Kidd, 1992). pLEXICN1Su42c contains an ICN1 derivative, ICN1Su42c which has a point mutation in the fifth ankyrin repeats (aa2060)[4]. pJGDx and pJGSu(H) encode full-length Deltex and Su(H), respectively. Standard deviations are shown in parenthesis.

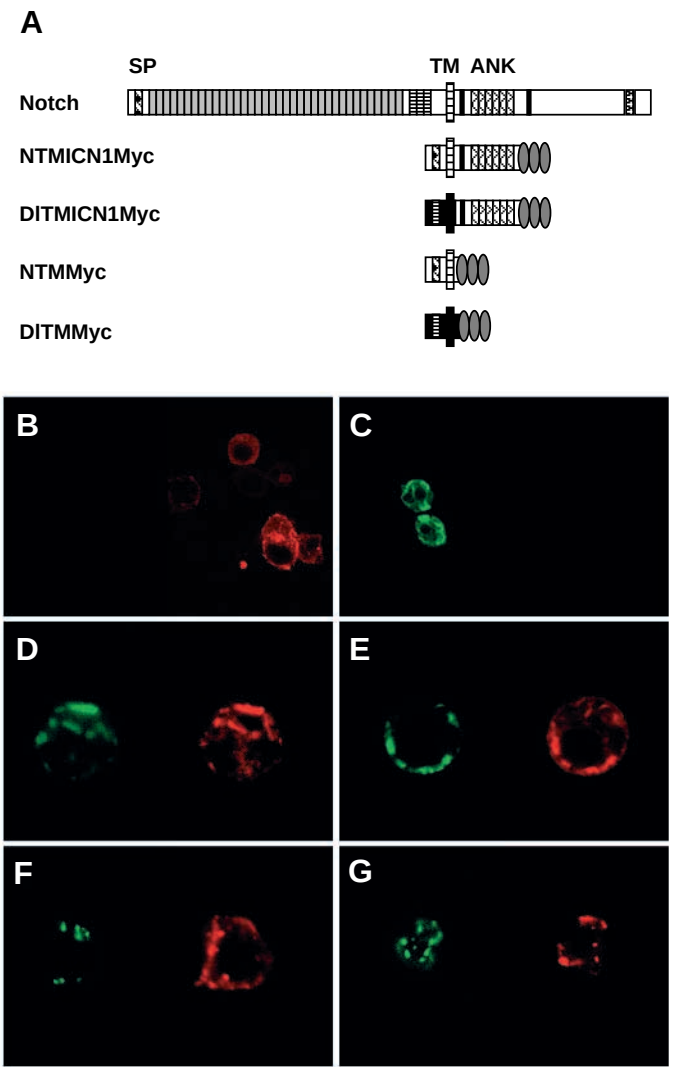


Fig. 7. Co-localization assays of Notch polypeptides in *Drosophila* tissue culture cells. (A) Schematic diagrams of Notch deletion and Notch/Delta chimeric proteins tagged with Myc-epitopes. NTMICN1Myc consists of the signal peptide (SP), transmembrane domain (TM) and ankyrin repeats (ANK) of Notch. DlTMICN1Myc is a Notch/Delta chimeric protein containing the signal peptide and transmembrane domain of Delta and the ankyrin repeats of Notch. NTMMyc contains a signal peptide and a transmembrane domain of Notch. DlTMMyc contains a signal peptide and a transmembrane domain of Delta. Hatched ellipses show tagged Myc epitopes (Fortini and Artavanis-Tsakonas, 1994). Open and filled rectangles represent portions of the proteins originating from Notch and Delta, respectively. (B-G) Co-localization of Notch proteins in *Drosophila* tissue culture cells. Confocal microscope images of *Drosophila* S2 cells are presented as split images showing the distributions of Myc epitope tagged Notch deletions or Notch/Delta chimeric proteins in green and full-length Notch in red. Each panel represents single transfection or co-transfection experiment involving Myc-tagged proteins or full-length Notch expression plasmids: (B) pMTNcDNA (full-length Notch), note that there is no bleedthrough from red channel to green channel; (C) NTMICN1Myc, note that there is no bleedthrough from green channel to red channel; (D) pMTNcDNA and NTMICN1Myc; (E) pMTNcDNA and DlTMICN1Myc; (F) pMTNcDNA and NTMMyc; (G) pMTNcDNA and DlTMMyc.

tagged polypeptides consisting of the Notch or Delta transmembrane domain (NTMMyc and DlTMMyc) did not co-localize with Notch (Fig. 7,F,G).

The mutation N^{Su42c} affects ankyrin homotypic interactions

Insight into the possible functional consequences of the homotypic interaction involving the Notch ANK repeats was provided by N^{Su42c}, the only point mutation known to map in the *Drosophila* Notch ANK repeats (Diederich et al., 1994). Table 2 summarizes the results of an interaction trap study showing that the N^{Su42c} mutation does not significantly affect the interaction of Notch with Deltex (compare constructs 19 and 20), while it strengthens Notch homotypic interactions (compare constructs 17 and 18). The N^{Su42c} mutation was isolated as a suppressor of the lethality associated with certain heteroallelic combinations of the *Abruptex* (*Ax*) mutations (Diederich et al., 1994; Xu et al., 1990). Since negatively complementing combinations of *Ax* alleles appear to reflect an overactive state of the Notch receptor, it is reasonable to assume that strengthening the ANK homotypic interactions leads to the reduction of Notch signaling.

DISCUSSION

The development of the compound eye in *Drosophila* rests upon sequential cell fate choices that gradually recruit precursor cells into ommatidial assemblies (Wolff and Ready, 1993). Genetic studies have demonstrated that Notch signaling controls cell fates throughout the development of the eye (Cagan and Ready, 1989). Given the precise cellular architecture of the eye disc, this preparation has proven useful in analyzing the activity of mutant Notch forms. Expression of activated forms of the Notch receptor in the eye have helped evaluate the action of Notch signaling at a single cell level (Fortini et al., 1993). Moreover, the phenotypes resulting from the expression of such mutant Notch forms contributed to the dissection of the Notch pathway (Verheyen et al., 1996). Significantly, known elements of the pathway such as *Dl*, *mastermind*, *deltex* and *Su(H)* were also identified as modifiers of activated *N* expressed under the *sev* promoter, validating the use of these gain-of-function phenotypes as a Notch pathway dissection tool (Verheyen et al., 1996). We were thus encouraged to use the same approach in combination with biochemical protein interaction assays to further dissect the function of the intracellular domain of Notch, in particular the activity of the ANK repeats region.

The protein interaction studies reported here clearly reveal the existence of two distinct Su(H)-binding sites on the Notch receptor, resolving what on the surface appeared to be contradictory results reported in independent studies (Fortini et al., 1993; Tamura et al., 1995). Our analysis indicates that one of the Su(H)-binding sites maps outside the ANK repeats, whereas for the second site the ANK repeats appear necessary but not sufficient. In vitro assays involving cultured cells have shown that Su(H) can be sequestered in the cytoplasm by virtue of its binding to Notch (Fortini and Artavanis-Tsakonas, 1994). Upon the interaction of Notch with its ligand Delta, Su(H) translocates into the nucleus (Fortini and Artavanis-Tsakonas, 1994). Nuclear translocation of Su(H) can also be triggered by

the overexpression of Dx in the same cell, for which the ANK repeats are both necessary and sufficient in order to associate with Dx (Matsuno et al., 1995). Therefore, despite the distinct binding sites of Su(H) and Dx on Notch, there is an interplay between these molecules (Matsuno et al., 1995).

The protein interaction assays that we describe suggest that the ANK repeats are involved in homotypic interactions. Extrapolating from what is commonly believed to be true in other transmembrane receptors, e.g., the EGF receptor, as well as the genetic behavior of certain *Notch* mutant alleles, e.g., the *Abruptex (Ax)* mutations, it is thought that Notch acts as a homomultimer (Foster, 1975; Portin, 1975). Specific *Ax* alleles, which involve point mutations in the extracellular EGF homologous region, can be viable in a homozygous form, yet they are lethal in *trans*heterozygous combinations. This negative complementation could be explained by homotypic interactions between the two mutant Notch molecules. However, direct evidence demonstrating the existence of homotypic interactions between Notch molecules had been elusive. The mutation N^{Su42c}, which maps within the ANK repeats, was in fact isolated as an intragenic suppressor of the lethality associated with two negatively complementing *Ax* alleles (Diederich et al., 1994). The mutant phenotype is paralleled by stronger homotypic interactions in the yeast assay, suggesting an involvement of the ANK repeats in the oligomerization of the Notch receptor. Given these observations, it is reasonable to postulate that homotypic interactions of the ANK repeats can modulate Notch signaling activity.

The transgenic analysis of the ANK repeats revealed an unexpected activity which acts in a non-cell-autonomous fashion. It should be noted that studies involving Glp-1, a *C. elegans* counterpart of Notch, indicated that a peptide consisting of the ANK repeats as well as short flanking sequences (52 amino acids N-terminal to the ANK repeats and 33 amino acids to C-terminal portion) mimicked gain-of-function mutations (Roehl and Kimble, 1993). More recent results demonstrated that this activity of GLP-1 requires the presence of the ANK flanking sequences (Roehl et al., 1996). Deletion analyses in *Drosophila* have shown that constructs lacking the ANK repeats behave as dominant negative mutations, mimicking loss-of-function phenotypes of *N* (Rebay et al., 1993; Lieber et al., 1993). These constructs, however, involve sequences immediately flanking the ANK repeats whose function is unknown. The ANK repeat peptide that we study here does not include flanking sequences, does not bind to Su(H) and fails to induce Mδ expression. Consistent with our findings, the expression of an analogous ANK peptide deriving from rat Notch-1 shows a CBF1 (a mammalian homologue of Su(H))-independent activity (Shawber et al., 1996).

Activation of Notch, as evidenced by the expression of the entire intracellular domain, results in the expression of Mδ which, in turn, depends on a downstream effector Su(H) (Jennings et al., 1994; Lecourtois and Schweisguth, 1995; Bailey and Posakony, 1995). The Ankyrin repeats fail to induce Mδ activity, suggesting that their biological action is independent of this effector, yet the ANK peptide activity affects the Notch pathway, since ANK expression in one cell was found to suppress Mδ expression in a neighbor. The simplest way to account for these observations is to postulate the existence of a yet to be identified effector whose expression is modulated by *sev-ANK*. As a consequence, the Notch pathway in a neighboring cell is inactivated ('X' in the model depicted in Fig. 8).

In this model, Notch is involved in at least two signaling events: one is dependent on Su(H), the other is not. The first requires the entire intracellular domain and induces the expression of *E(spl) bHLH* genes in a cell-autonomous, Su(H)-dependent fashion. The second depends on the expression of the ANK repeats only and does not involve Su(H). Cells expressing the ANK repeats are capable of antagonizing Notch signaling in the neighboring cells. The simplest way to explain this non-autonomous, antagonistic action of the ANK sequences is to suggest that the ANK-expressing cells down-regulate the endogenous Delta activity. Consequently, the Notch receptor in the neighboring cell is inactivated and Mδ expression is down-regulated. It is therefore not necessary to postulate novel antagonistic activities on the surface of the ANK-expressing cells. Irrespective of the molecular nature of the surface changes induced by the ANK sequences, the action of the ANK repeats must trigger the modulation of an effector, i.e., 'X'. Since the phenotype associated with ANK expression cannot be modified by either the overexpression of Dx, which binds to the ANK repeats, or by wild-type Notch, we postulate that the effector X is unknown. The possibility that this unknown component down-regulates endogenous Dl is particularly interesting since genetic evidence in *C. elegans* and *Drosophila* has suggested the possible existence of a regulatory loop in the Notch pathway (Wilkinson et al., 1994; Heitzler and Simpson, 1996).

It is worth pointing out that yeast two-hybrid-based screens for proteins capable of interacting with the ANK repeats of Lin-12 resulted in the identification of a protein that shows homology to EMB5, a chromatin-associated protein in mammals (Hubbard et al., 1996). At this point, we do not know if this protein mediates the activity of the ANK repeats. However, the rough eye phenotype associated with the ANK

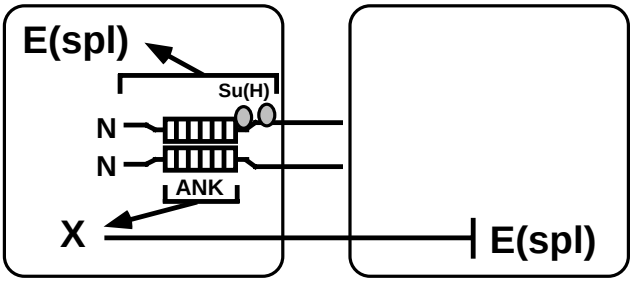


Fig. 8. A model for the activity of the Notch ankyrin repeats. The expression of E(spl) Mδ is regulated by Su(H), which has two distinct binding sites on Notch and acts as an effector of Notch signaling. The ankyrin repeats (ANK) are necessary and sufficient for Notch (N)/Deltex (Dx) and Notch/Notch interactions. However, they are only necessary for one of the two Su(H)-binding sites. Constitutively activated, truncated forms of the Notch receptor consisting of the entire intracellular domain induce the expression of Mδ. Cells expressing only the ankyrin repeats fail to induce Mδ expression. Mδ expression is suppressed in what appears to be a cell-nonautonomous fashion. We postulate the existence of Su(H)-independent events, which are mediated by the action of the ankyrin repeats. We suggest that the ankyrin repeats regulates the action of an unknown Notch pathway component 'X' and consequently down-regulates Notch receptor activity in a neighboring cell.

repeat expression make genetic modifier screening possible, providing the means to further dissect the novel Notch downstream events suggested by the action of the ANK repeats.

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