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Nuclear Access and Action of Notch In Vivo

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Summary

The *Drosophila Notch* (*N*) gene encodes a conserved single-pass transmembrane receptor that transduces extracellular signals controlling cell fate. Here, we present evidence that the intracellular domain of Notch gains access to the nucleus in response to ligand, possibly through a mechanism involving proteolytic cleavage and release from the remainder of the protein. In addition, our results suggest that signal transduction by Notch depends on the ability of the intracellular domain, particularly the portion containing the CDC10 repeats, to reach the nucleus and to participate in the transcriptional activation of downstream target genes.

Introduction

Notch belongs to a conserved family of transmembrane receptors that transduce intercellular signals controlling cell fate (reviewed in Weinmaster, 1997; Greenwald, 1998). All of the members of this family are single-pass transmembrane proteins that respond to ligands of the Delta-Serrate-Lag2 (DSL) family and contain several conserved sequence motifs. The large extracellular domain contains many tandem repeats of an epidermal growth factor (EGF) motif as well as three copies of a "Lin-12/N repeat" (LNR). In addition, they contain intracellular domains that include a block of six CDC10 (or ankyrin) repeats, one or two nuclear localization signals (NLS), a homopolymer repeat of glutamine (an "OPA" domain), and a proline-glutamate-serine-threonine–rich "PEST" domain. However, the intracellular domains of Notch proteins lack any recognizable catalytic motif, and it remains unclear how they transduce extracellular signals.

Truncated forms of Notch that consist of only the intracellular domain have constitutive transducing activity (Lieber et al., 1993; Struhl et al., 1993) and localize predominantly in the nucleus (Fortini et al., 1993; Lieber et al., 1993; Struhl et al., 1993; Kopan et al., 1994). Hence, it has been proposed that interactions between ligand and the extracellular domain of Notch induce the release of the intracellular domain from the membrane and that the intracellular domain translocates to the nucleus, where it transduces N signals by regulating the transcription of downstream target genes (Lieber et al., 1993; Struhl et al., 1993; Kopan et al., 1994).

The possibility of such a direct mechanism of signal transduction remains controversial. On the positive side, Notch cleavage products that include portions of the Notch intracellular domain have been identified in tissue culture cells and in whole animal extracts (e.g., Aster et al., 1994; Kopan et al., 1996; Blaumueller et al., 1997). Moreover, portions of the Notch intracellular domain have been shown to associate physically with the Suppressor of Hairless (Su[H]) protein, a sequence-specific DNA-binding protein, which appears responsible for activating the transcription of downstream target genes in response to Notch receptor activity (reviewed in Honjo, 1996; Weinmaster, 1997).

On the negative side, all attempts to obtain direct evidence for ligand-dependent nuclear access of the Notch intracellular domain have failed, despite the existence of antisera specific for epitopes within the intracellular domain and the ability to examine altered forms of Notch (e.g., lacking portions of the extracellular domain including the LNRs) that have constitutive transducing activity (Fehon et al., 1991; Lieber et al., 1993; Rebay et al., 1993). Also, there is no direct evidence that the productive interaction between Notch and Su(H) occurs within the nucleus. Indeed, analyses of the physical relationships between Notch and Su(H) protein in tissue culture and in vivo have led to proposals in which Notch tethers Su(H) at the cell surface, releasing and perhaps modifying Su(H) in response to ligand (Fortini and Artavanis-Tsakonas, 1994; Lecourtois and Schweisguth, 1995; Kopan et al., 1996; Roehl et al., 1996).

Here, we describe experiments designed to test whether the Notch intracellular domain normally gains access to the nucleus in response to ligand and whether it acts within the nucleus to regulate the transcription of downstream target genes. Our results provide evidence that such access occurs, possibly by a mechanism involving proteolytic cleavage that leads to the release of the intracellular domain from the rest of the protein. In addition, they support the hypothesis that the intracellular domain of Notch, and particularly the CDC10 repeats, transduce Notch signals by acting within the nucleus to activate gene transcription.

Results

Evidence for Nuclear Access of the Notch Intracellular Domain in Vivo

As noted in the Introduction, attempts to detect the physical presence of intracellular portions of Notch in the nucleus by conventional immunological or biochemical means have generally been unsuccessful, even in cells in which the receptor is active. We have therefore employed a potentially more sensitive approach in which the chimeric transcription factor Gal4-VP16 (GV) is inserted at various positions in otherwise wild-type Notch protein (Figure 1) and the resulting N^{+}-GV proteins expressed under heat shock control in embryos that also carry a *UAS-lacZ* transgene (Experimental Procedures). The Gal4-VP16 protein contains the DNA-binding domain of the yeast Gal4 transcription factor coupled to

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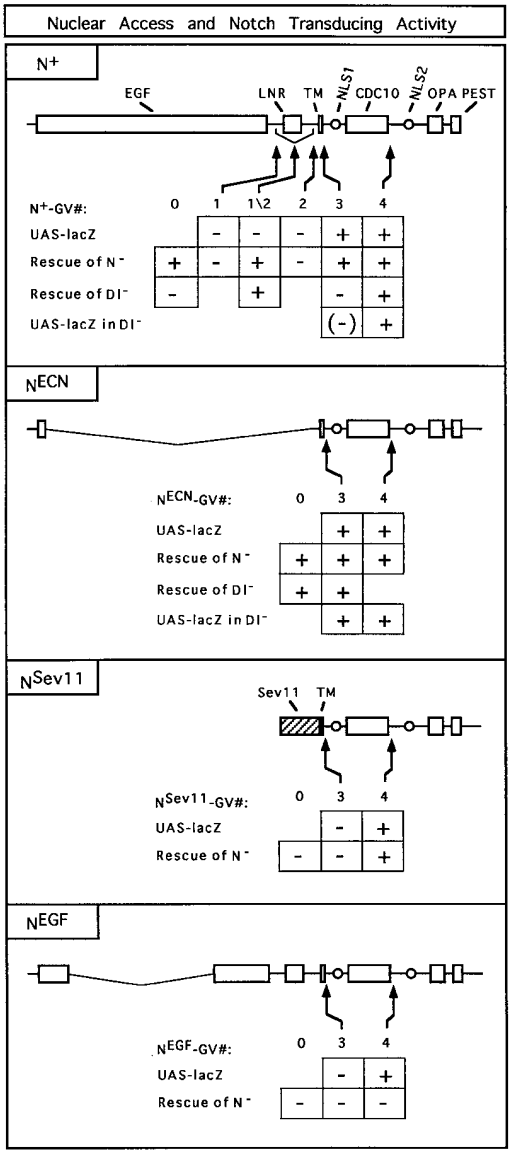

Figure 1. Summary of Experiments Assaying Nuclear Access and Transducing Activity of Notch

The general structural features of Notch are diagrammed in the top panel with the EGF repeats, Lin12/Notch repeats (LNR), transmembrane domain (TM), NLSs, CDC10 repeats, OPA repeats, and PEST sequences indicated. The Gal4-VP16 domain was inserted at four sites (arrows) and the resulting N^+-GV proteins designated by the insertion site (1–4; 0 = no Gal4-VP16 insertion; 1\2 = an insertion of Gal4-VP16 in place of the domain normally positioned between sites 1 and 2). The N^{ECN}, N^{Sev11}, and N^{EGF} derivatives of these proteins are diagrammed in the panels underneath (details of these constructs are given in Table 1; note that the N^{Sev11} derivative is composed of the extracellular and transmembrane domains of the Sev11 protein [hatched and black, respectively] joined to the intracellular domain of Notch). All of the derivatives shown were tested for nuclear access of the inserted Gal4-VP16 domain by assaying their ability to activate transcription of a *UAS-lacZ* target gene. In addition, all were tested for their ability to activate the Notch transduction pathway by assaying whether they could rescue epidermal development in N^{XK11} arm^{YD35} embryos. Finally, selected derivatives were tested for ligand-dependent nuclear access and Notch transducing activity by assaying their ability to activate *UAS-lacZ* expression in Dl^- versus *hsp70-Dl* Dl^- embryos and to rescue epidermal development in Dl^{X43} hh^{10E} embryos. The assays used (see Experimental Procedures) generally yielded qualitatively distinct results as illustrated in Figures 2, 5, and 6, except for *UAS-lacZ* expression activated by N^+-GV3 protein in Dl^- embryos. In this case, rare *UAS-lacZ*–expressing cells were observed (Figure 2) and the result indicated as "(−)" rather than "−".

the transcriptional activating domain of the viral VP16 protein (Sadowski et al., 1988). The *UAS-lacZ* gene contains four copies of the UAS-binding site for Gal4 and is transcribed in response to Gal4 as well as the Gal4-VP16 protein in *Drosophila melanogaster* (Fischer et al., 1988; data not shown). We reasoned that expression of the *UAS-lacZ* gene would provide a sensitive assay for nuclear access of the inserted Gal4-VP16 domain and hence for events that lead to nuclear import of the Notch intracellular domain.

The Gal4-VP16 coding sequence was inserted at either of two positions in the intracellular domain: just carboxy-terminal to the transmembrane domain to generate the chimeric protein N^+-GV3 and after the domain containing the CDC10 repeats to generate the chimeric protein N^+-GV4 (Figure 1). Heat shock–induced expression of each of these proteins during embryogenesis caused expression of the *UAS-lacZ* gene in the ventral ectoderm and the developing central nervous system, as well as in other tissues (Figures 2 and 6; data not shown). Cells in each tissue appeared to respond in a salt and pepper fashion similar to that caused by low level expression of the Gal4-VP16 protein alone (data not shown). Our initial assays were performed using a severe heat shock (37°C for 1 hr, followed by a 2 hr recovery), which generates levels of N^+-GV protein that are similar to that of endogenous Notch (data not shown). However, mild heat shock (e.g., 33°C for 60 min, followed by a 2 hr recovery) resulted in detectable expression of the *UAS-lacZ* gene, even though the level of N^+-GV protein is severalfold lower than the level of endogenous Notch (data not shown). We have examined the subcellular localization of the Gal4 DNA-binding domain inserted in both proteins, as well as in all of the remaining Gal4 derivatives shown in Figures 1 and 4, in the embryonic ectoderm. In all cases, the Gal4 DNA-binding domain appears to be localized predominantly at the cell periphery (data not shown), as is the case for both extracellular and intracellular epitopes of the endogenous Notch protein (Fehon et al., 1991).

We also assayed the ability of these chimeric proteins to provide Notch transducing activity. Specifically, we have asked whether their expression can suffice to rescue formation of the ventral epidermis in N^- embryos in which all cells of the ventral ectoderm would otherwise develop as neuroblasts. To facilitate the analysis, we used arm^- N^- embryos: when Notch function is restored in such embryos, they secrete a ventral cuticle that displays the Armadillo segmentation phenotype allowing them to be identified unambiguously (Lieber et al., 1993; Figure 5). For both the N^+-GV3 and N^+-GV4 proteins, we observed rescue (data not shown).

To test whether nuclear access of the inserted Gal4-VP16 domain depends on its being located within the intracellular domain, we assayed the activities of the

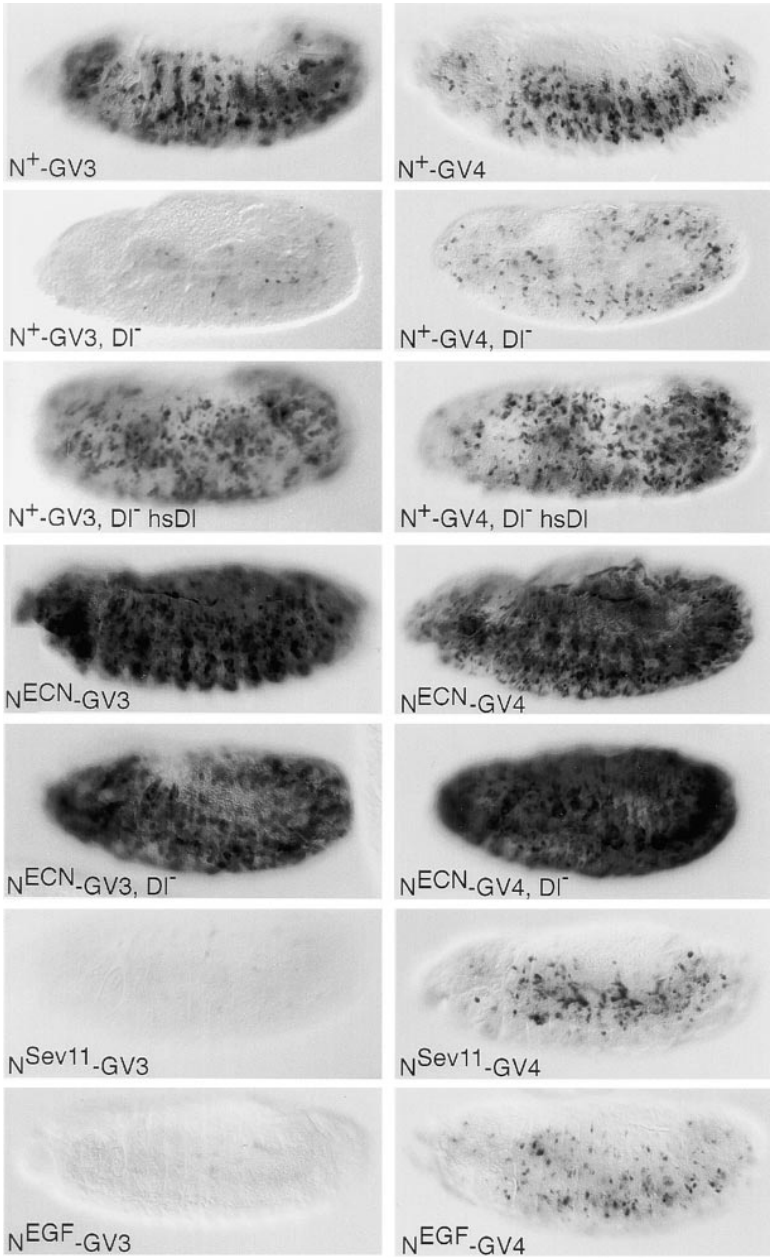

Figure 2. Ligand Dependence of Notch Nuclear Access

Embryos carrying the *UAS-lacZ* reporter gene and expressing the wild-type, ECN, Sev11, and EGF derivatives of N-GV3 and N-GV4 protein (Figure 1) are shown stained for β-gal protein expression. For the N-GV3 derivatives, only the wild-type and ECN forms induce β-gal expression. Moreover, the wild-type form does so in a ligand-dependent fashion, as indicated by the relative absence of β-gal expression in *Dl*$^-$ embryos compared to *Dl*$^+$ embryos and to *Dl*$^-$ embryos supplemented with ectopic Delta expressed under heat shock control (the few cells that express β-gal in *Dl*$^-$ embryos may reflect a response of the N$^+$-GV3 protein to another ligand, possibly Ser). In contrast, the ECN form induces similar amounts of β-gal expression in *Dl*$^-$ embryos compared to *Dl*$^+$ embryos, indicating that it does so in a ligand-independent fashion. For the N-GV4 derivatives, all induce β-gal expression even in the absence of ligand (N$^+$-GV4 in *Dl*$^-$ embryos) or when portions of the extracellular domain normally required for ligand-dependent activation of Notch are deleted (N^{Sev11}-GV4 and N^{EGF}-GV4). However, the ability of N$^+$-GV4 protein to induce β-gal expression retains some dependence on ligand (compare β-gal expression in *Dl*$^-$ with *Dl*$^-$ *hsDl* embryos). Here, as in Figure 6, all embryos are staged around the end of germband shortening, with anterior to the left; similar results were obtained in embryos staged as early as the completion of germband extension and as late as the completion of dorsal closure.

chimeric proteins N$^+$-GV1 and N$^+$-GV2, which contain Gal4-VP16 insertions in either of two sites in the extracellular domain, just before the LNR domain and just before the transmembrane domain (Figure 1). In contrast to the results obtained with the N$^+$-GV3 and N$^+$-GV4 proteins, no *UAS-lacZ* expression was observed in embryos expressing N$^+$-GV1 or N$^+$-GV2 protein (data not shown). However, neither the N$^+$-GV1 nor the N$^+$-GV2 protein was able to restore epidermal development in *arm*$^-$ *N*$^-$ embryos (Figure 1), indicating that the chimeric protein cannot function normally to transduce Notch signals. We therefore expressed another chimeric protein, N$^+$-GV1\2, in which the Gal4-VP16 coding sequence was inserted in place of the LNR domain. As previously shown, deletion of the LNR domain renders Notch constitutively active (Lieber et al., 1993), and we find that expression of the N-GV1\2 chimeric protein can rescue the formation of ventral epidermis not only in *N*$^-$ embryos but also in *Dl*$^-$ embryos (Figure 1). Nevertheless, the N$^+$-GV1\2 chimeric protein does not drive *UAS-lacZ* expression (Figure 1). We conclude that the Gal4-VP16 domain present in the N$^+$-GV1\2 protein does not have access to the nucleus, even though the chimeric receptor is constitutively active.

Thus, Gal4-VP16 insertions within the Notch intracellular domain, both amino-terminal and carboxy-terminal to the CDC10 domain, appear to have access to the nucleus in vivo, in contrast to Gal4-VP16 insertions in the extracellular domain, which do not. In principle, this access could be afforded by translocation of the entire receptor from the membrane to the nucleus. Alternatively, access may be afforded by one or more cleavage

events that occur amino-terminal to the inserted Gal4-VP16 domain and allow release and nuclear import of carboxy-terminal portions of the protein.

Evidence for Ligand-Dependent Nuclear Access of Notch

We have performed two sets of experiments to determine whether nuclear access of the Gal4-VP16 domain in N^+-GV3 protein depends on ligand.

In the first set of experiments, we have assayed *UAS-lacZ* expression in embryos expressing a series of N-GV3 proteins that have alterations of the extracellular domain, which block their ability to respond to ligand or which render them constitutively active irrespective of ligand (Figure 1). One derivative, referred to as N^{ECN}-GV3, lacks virtually all of the extracellular domain, including all 36 EGF repeats and the LNR domain. N^{ECN} protein has previously been shown to have ectopic transducing activity, suggesting that it is constitutively active irrespective of ligand (Fortini et al., 1993; Rebay et al., 1993). A second, referred to as N^{EGF}-GV3, consists of a deletion of EGF repeats 4-26: like other forms of Notch that have internal deletions of the EGF region (Lieber et al., 1993; Rebay et al., 1993), N^{EGF} protein cannot rescue the absence of endogenous Notch activity (Figure 1), indicating that it is unable to respond to ligand. The third, referred to as N^{Sev11}-GV3, lacks the entire extracellular domain as well as the transmembrane domain of Notch and has in its place the extracellular and transmembrane domains of Sev11, a truncated form of the receptor tyrosine kinase Sevenless (Basler et al., 1991). Like N^{EGF} protein, N^{Sev11} protein also appears unable to transduce ligand (Figure 1).

The main result we have obtained is that the N^+-GV3 and N^{ECN}-GV3 proteins can activate *UAS-lacZ* transcription and, in addition, can provide Notch transducing activity to otherwise N^- embryos. In contrast, the N^{EGF}-GV3 and N^{Sev11}-GV3 proteins can do neither (Figures 1 and 2). Thus, nuclear access of the Gal4-VP16 domain inserted in N-GV3 proteins correlates with Notch transducing activity: forms of N-GV3 protein that behave as if they cannot transduce Notch signals do not allow access, whereas forms that have ligand-dependent or constitutive transducing activity do allow access.

In the second set of experiments, we have assayed whether nuclear access and Notch transducing activity associated with the N^+-GV3 and N^{ECN}-GV3 proteins depend on Delta (Dl), the primary ligand for activating Notch in embryos (reviewed in Weinmaster, 1997; Greenwald, 1998). To test whether nuclear access depends on Delta, we compared the ability of the N^+-GV3 and N^{ECN}-GV3 proteins to activate *UAS-lacZ* expression in heat-shocked Dl^- embryos versus heat-shocked Dl^- embryos in which Delta expression is restored by a *hsp70-Dl* transgene (see Experimental Procedures). For the N^+-GV3 protein, we find that only very few cells show *UAS-lacZ* expression in the absence of Delta (Figure 2). However, in the presence of heat shock–induced Delta, *UAS-lacZ* is widely expressed (Figure 2), indicating that the response is ligand-dependent. By contrast, the N^{ECN}-GV3 protein activates *UAS-lacZ* expression irrespective of Delta (Figure 2).

To test whether the Notch transducing activities of the N^+-GV3 and N^{ECN}-GV3 proteins are Delta-dependent, we asked whether heat shock–induced expression of either protein can rescue epidermal differentiation in Dl^- embryos (for these experiments, the Dl^- mutation was linked with a hh^- mutation so that rescued embryos could be unambiguously identified by the Hedgehog segmentation phenotype; see Experimental Procedures). We find that expression of the N^+-GV3 protein fails to rescue epidermal differentiation in Dl^- hh^- embryos, in contrast to expression of the N^{ECN}-GV3 protein, which does (Figure 1; data not shown). Consequently the transducing activity of N^+-GV3 protein, but not N^{ECN}-GV3 protein, appears to be ligand-dependent.

Thus, both sets of experiments provide evidence that nuclear access of the Notch intracellular domain, as assayed by ability of the Gal4-VP16 domain of N-GV3 proteins to activate *UAS-lacZ* expression, depends on ligand and correlates with Notch transducing activity.

Evidence for Nuclear Access of Notch in the Absence of Ligand

We also analyzed nuclear access of the Gal4-VP16 domain in N-GV4 proteins, using the same tests for ligand dependence employed for the N-GV3 proteins. As shown in Figure 2, the main result we have obtained is that nuclear access of this domain, which is inserted carboxy-terminal to the CDC10 repeats, appears to depend on both ligand-dependent and ligand-independent mechanisms. For example, the N^{Sev11}-GV4 and N^{EGF}-GV4 proteins can activate *UAS-lacZ* expression, even though they should not be able to respond to ligand. Similarly, the N^+-GV4 protein can activate the *UAS-lacZ* gene in many cells in Dl^- embryos, despite the absence of Delta protein. Both sets of results indicate that carboxy-terminal portions of the Notch intracellular domain that include the GV4 insertion have access to the nucleus even in the absence of normal ligand stimuation. However, we also find evidence that ligand stimulates nuclear access of the Gal4-VP16 domain in N^+-GV4 protein. In particular, we consistently observe that more cells express the *UAS-lacZ* gene in heat-shocked *hsp70-*N^+*-GV4* Dl^- embryos when these embryos carry the *hsp70-Dl* transgene compared to when they do not.

Thus, at least some portions of the Notch intracellular domain appear to gain access to the nucleus in a ligand-independent fashion, possibly as a consequence of proteolytic cleavages that occur carboxy-terminal to the site of the GV3 insertion. We note that this ligand-independent access is not likely to be productive in terms of normal Notch signaling because wild-type Notch has no transducing activity in the absence of ligand, and both the N^{EGF} and N^{Sev11} proteins similarly lack transducing activity even in the presence of ligand (Figure 1; see Discussion).

Transducing Activity of the CDC10 Domain of Notch Depends on Access to the Nucleus

The ability of Notch to transduce extracellular signals depends critically on the integrity of the CDC10 repeats within the intracellular domain (Greenwald and Seydoux, 1990; Kodoyianni et al., 1992; Lieber et al., 1993; Rebay

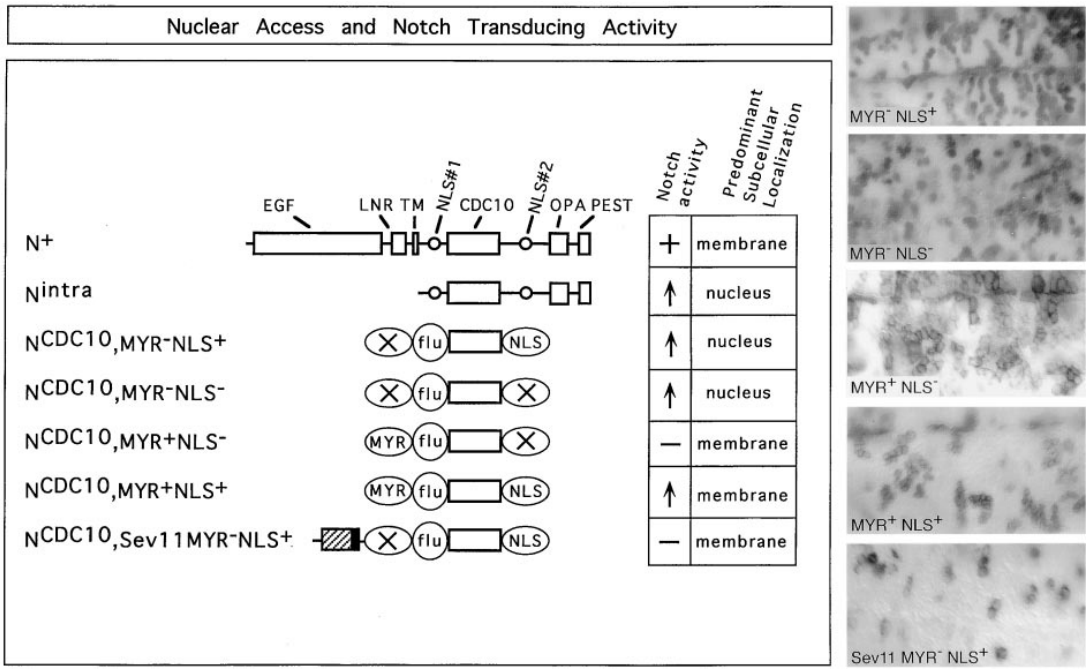

Figure 3. Predominant Subcellular Localization and Notch Transducing Activity of N^{CDC10} Proteins

The structures of Notch, N^{intra}, and the various derivatives of Flu-tagged N^{CDC10} proteins are diagrammed as in Figure 1 (the results for Notch and N^{intra} have been published previously; Struhl et al., 1993). The top four N^{CDC10} derivatives are identical except for a single G→A substitution, which inactivates the MYR signal in the MYR^- derivatives, and adjacent K→T substitutions, which inactivate the NLS signal in the NLS^- derivatives. The last derivative, N^{CDC10},Sev11MYR^-NLS^+ (bottom) is composed of the extacellular and transmembrane domains of the Sev11 protein fused immediately upstream of the N^{CDC10},MYR^-NLS^+ protein. All of the proteins shown were tested for Notch transducing activity by examining whether they could rescue epidermal development in *arm*$^-$ *N*$^-$ embryos (assay 1), whether their expression could rescue epidermal development in *Dl*$^-$ *hh*$^-$ embryos (assay 2), and whether their expression could suppress neuroblast segregations in early *N*$^+$ embryos (assay 3). Wild-type Notch protein rescues the absence of the endogenous gene (1), but does not rescue the absence of Delta (2) or cause an antineurogenic phenotype (3): it is scored as "+". In contrast, the remaining proteins either have all three activities (indicating that they have intrinsic activity, scored as ("↑"), or none of these activities (scored as "−"). In addition, the predominant subcellular localization of each of the N^{CDC10} derivatives was assayed using antisera against the CDC10 (in *N*$^-$ embryos) and Flu epitopes. The ventral ectoderms of embryos having clones of cells expressing each of the five forms of N^{CDC10} proteins are shown on the right stained for expression of the Flu epitope (embryos are staged at the end of the early phase of germband extension). Note that the two derivatives that are found predominantly in the nucleus have intrinsic Notch transducing activity, in contrast to two of the three derivatives that appear to be largely excluded from the nucleus and lack detectable activity. The remaining derivative, N^{CDC10},MYR^+NLS^+, is exceptional in that it retains intrinsic transducing activity even though it appears to be predominantly membrane associated. However, this protein differs from the N^{CDC10},MYR^+NLS^- protein in that it carries a wild-type rather than a mutant NLS sequence. We infer that the presence of the active NLS signal in this protein allows a small amount to reach the nucleus despite the presence of the MYR^+ signal, accounting for its intrinsic transducing activity.

et al., 1993), and in at least one case, a relatively small fragment of the intracellular domain that contains these repeats has intrinsic transducing activity (Roehl and Kimble, 1993). We have therefore asked whether the intrinsic transducing activity of such a fragment of Notch, termed N^{CDC10}, depends on whether it can gain access to the nucleus.

As diagrammed in Figure 3, we have coupled a polypeptide containing the N^{CDC10} fragment and two copies of the Flu-epitope tag (Wilson et al., 1984) with wild-type or mutated versions of the myristylation signal (MYR) of *Drosophila* Src (Cross et al., 1984; Simon et al., 1985) at the amino terminus and of a nuclear localization signal (NLS) from SV40 T antigen (Kalderon et al., 1984) at the carboxy terminus to create four tagged forms of the CDC10 domain. In principle, these should be targeted to membranes (MYR^+NLS^-) or the nucleus (MYR^-NLS^+), or to neither (MYR^-NLS^-) or both (MYR^+NLS^+). These four proteins are identical except for changes in a single amino acid (Gly or Ala) within the MYR signal and in two adjacent amino acids (Lys-Lys or Thr-Thr) within the NLS.

Each of the four proteins was expressed using a combination of the Gal4/UAS (Fischer et al., 1988; Brand and Perrimon, 1993) and Flp-out (Basler and Struhl, 1994) techniques to create embryos in which all, or only some, cells express the tagged protein (see Experimental Methods) and the results of these experiments shown in Figure 3. The MYR^-NLS^+ protein is localized predominantly in the nucleus and has intrinsic Notch transducing activity, as indicated by its ability to block neuroblast segregations in wild-type embryos and to rescue epidermal differentiation in *arm*$^-$ *N*$^-$ and in *Dl*$^-$ *hh*$^-$ embryos. Similarly, MYR^-NLS^- protein accumulates predominantly in the nucleus, despite the absence of an active NLS, and has intrinsic signal-transducing activity. In contrast, the MYR^+NLS^- protein appears to be excluded from the nucleus and lacks detectable Notch transducing activity as it fails to rescue *arm*$^-$ *N*$^-$ embryos or to block neuroblast segregations. In the case of the

MYR^+NLS^+ protein, most of the protein accumulates outside of the nucleus, indicating that the MYR^+ signal predominates over the NLS^+ signal. Nevertheless, this protein retains Notch transducing activity, perhaps because the presence of an active NLS allows a small amount to gain access to the nucleus despite the presence of an active MYR signal. To test this possibility, we created a fifth chimeric protein in which the Sev11 extracellular and transmembrane domains were fused immediately upstream of the MYR^-NLS^+ protein. The resulting protein, $Sev11MYR^-NLS^+$, has a conventional signal sequence at its amino terminus and should be inserted into the membrane as it is synthesized. As a consequence, it should remain stably membrane associated, despite the presence of an active NLS at its carboxy terminus. As expected, the $Sev11MYR^-NLS^+$ protein appears to be excluded from the nucleus. Moreover, it lacks detectable Notch transducing activity, in contrast to MYR^+NLS^+ protein.

Thus, a discrete CDC10-containing portion of the Notch intracellular domain has intrinsic transducing activity. However, this transducing activity appears to require that the protein has access to the nucleus, as it is enhanced by nuclear targeting and abolished by membrane targeting. Because the CDC10 repeats are essential for signal transduction in the context of the wild-type protein, we interpret these results as evidence that transduction by Notch normally requires access of the CDC10 repeats to the nucleus.

Evidence for Transcriptional Regulation by the Notch Intracellular Domain

One reason why Notch signal transduction may depend on nuclear access of the CDC10 domain is that this domain is directly involved in regulating transcription in response to ligand. To test this possibility, we have assayed the consequences of inserting protein domains that have well-characterized roles in mediating transcriptional activation or repression into the intracellular domain of otherwise intact Notch. We reasoned that if the Notch intracellular domain normally participates in transcriptional regulation, adding such activating or repressing domains might have opposite effects on the regulation of downstream target genes, yielding phenotypes corresponding to gain or loss of activity of the Notch pathway.

To assay the effects of inserting an activator domain, we compared the consequences of expressing N^+-GV4 protein, which contains the transcriptional activating domain of VP16, with those of expressing a control chimeric protein, N^+-G4, which contains the Gal4 DNA-binding domain at the same site but lacks the VP16 activation domain (Figure 4). We find that both proteins have Notch transducing activity, as indicated by their ability to rescue the neurogenic phenotype of *arm*$^-$ *N*$^-$ embryos (Figures 4 and 5; data not shown). Moreover, both also are capable of directing *UAS-lacZ* expression in similar numbers of cells, although the level of expression is significantly lower in the case of N^+-G4 (Figures 4 and 6). However, two lines of evidence indicate that the added presence of the VP16 domain in N^+-GV4 protein renders it constitutively active in terms of Notch signal transduction, in contrast to N^+-G4 protein, which lacks this domain and is ligand-dependent.

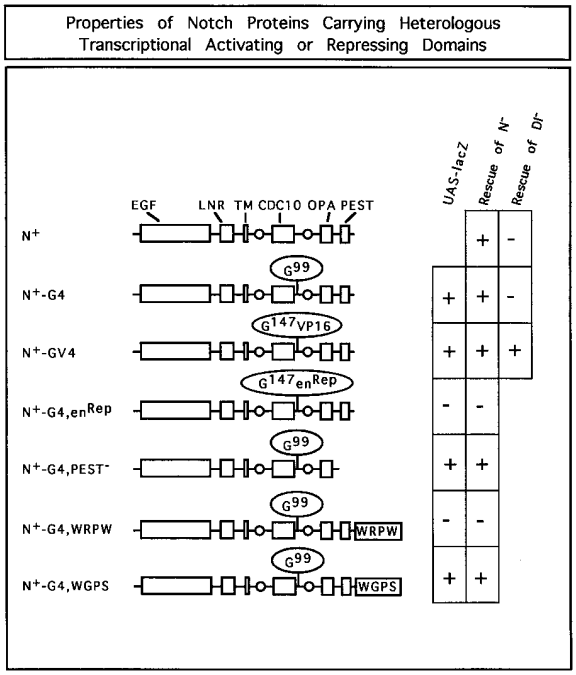

Figure 4. Summary of Experiments Providing Evidence for a Direct Involvement of the Notch Intracellular Domain in Transcriptional Regulation

A series of N^+-G4 derivatives that carry activator (VP16) or repressor (en^{Rep},WRPW) motifs, as well as appropriate control derivatives, are diagrammed on the left, and the results of assays for nuclear access and Notch transducing activity (see Figure 1; Experimental Procedures) are indicated on the right (see also Figures 5 and 6). G99 refers to the presence of the first 99 amino acids of Gal4 protein, which contain principally the DNA-binding domain. G147 refers to the presence of the first 147 amino acids of Gal4 protein; these additional amino acids are present in the Gal4-VP16 domain inserted in the N-GV proteins diagrammed in Figure 1.

First, we tested whether heat shock–induced expression of either N^+-G4 or N^+-GV4 protein can suffice to rescue the formation of ventral epidermis in *Dl*$^-$ *hh*$^-$ embryos. We observe that expression of the N^+-GV4 protein, but not N^+-G4, has rescuing activity (Figures 4 and 5; data not shown). Second, we tested whether the expression of either protein can block neuroblast segregations in embryos, an assay for constitutive Notch transducing activity (Struhl et al., 1993). We find that heat shock–induced expression of N^+-GV4 protein represses neuroblast segregations, both in wild-type and *Dl*$^-$ embryos (data not shown). In contrast, we could not detect an effect of heat shock–induced expression of N^+-G4 protein on these segregations.

Further evidence that insertion of the VP16 activator domain can cause constitutive activity of Notch comes from comparing the Notch transducing activities of the N^{Sev11}, N^{Sev11}-GV3, and N^{Sev11}-GV4 proteins (Figure 1). None of these proteins should be able to respond to ligand, owing to the substitution of the extracellular and transmembrane domains of the Sev11 protein for those of Notch. Nevertheless, the presence of the Gal4-VP16 domain inserted in N^{Sev11}-GV4 protein allows this protein to rescue epidermal development in *arm*$^-$ *N*$^-$ embryos, correlating with the ability of the Gal4-VP16 domain to

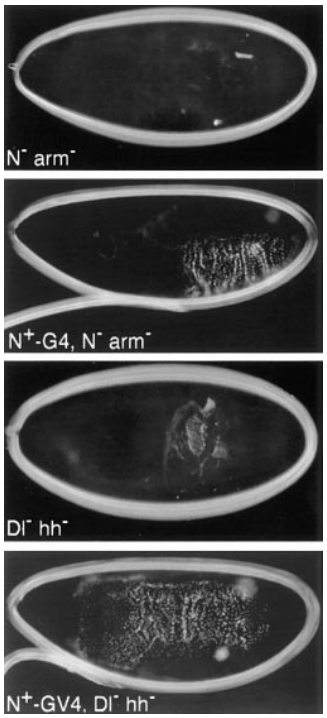

Figure 5. Rescue of Epidermal Differentiation in *arm*$^{-}$ *N*$^{-}$ and *Dl*$^{-}$ *hh*$^{-}$ Embryos by N^{+}-G4 and N^{+}-GV4 Proteins

In *arm*$^{-}$ *N*$^{-}$ and *Dl*$^{-}$ *hh*$^{-}$ embryos, virtually all ectodermal cells differentiate as neural tissue at the expense of epidermis, leading to absence of cuticular structures. However, epidermal differentiation is restored in *arm*$^{-}$ *N*$^{-}$ embryos that express N^{+}-G4 protein and in *Dl*$^{-}$ *hh*$^{-}$ embryos that express N^{+}-GV4 protein. The *arm*$^{-}$ and *hh*$^{-}$ mutations are linked, respectively, to the *N*$^{-}$ and *Dl*$^{-}$ mutations and cause distinctive "lawn" phenotypes of disorganized ventral hairs, confirming the genotypes of the "rescued" embryos.

gain access to the nucleus even in the absence of normal ligand interactions (Figure 1). One interpretation of this finding is that all three proteins are cleaved carboxy-terminal to the Sev11 transmembrane domain, but only intracellular cleavage products derived from the N^{Sev11}-GV4 protein include the VP16 activation and hence acquire Notch transducing activity. Extending this intepretation to the constitutive activity of N^{+}-GV4 protein, we suggest that addition of the VP16 activator domain confers transducing activity to intracellular cleavage products of Notch that arise in the absence of ligand stimulation and would otherwise lack activity. We note that the added presence of the VP16 activation domain is not sufficient to restore Notch transducing activity in the case of N^{EGF}-GV4 protein. Nevertheless, this protein can activate *UAS-lacZ* expression, indicating that the Gal4-VP16 domain does have access to the nucleus (Figures 1 and 2). It is possible that N^{EGF} proteins may be processed differently than N^{Sev11} or N^{+} proteins, leading to intracellular cleavage products that lack Notch transducing activity even when coupled to a VP16 activating domain.

To assay the effects of inserting a repressor domain, we used two different motifs: an alanine-rich portion of the homeodomain protein Engrailed (en^{Rep}) and the WRPW tetrapeptide that is present at the C terminus of basic helix-loop-helix repressor proteins such as Hairy

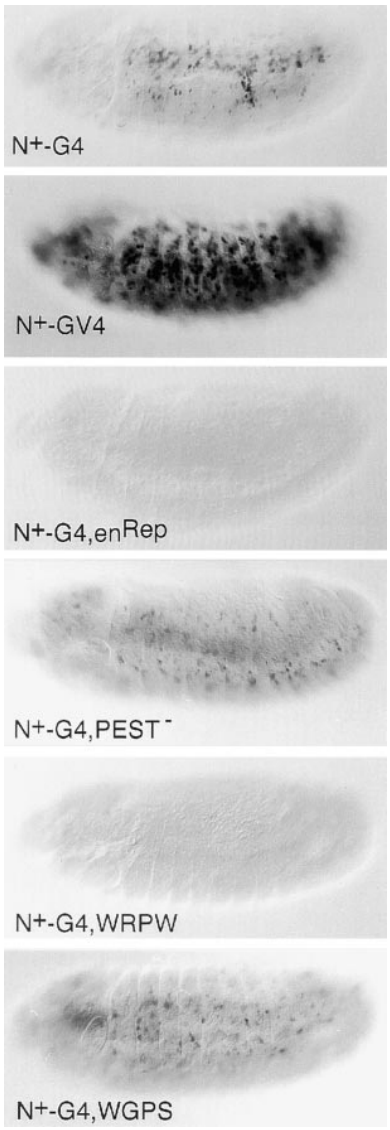

Figure 6. Regulation of *UAS-lacZ* Expression by N^{+}-G4 Proteins Carrying Heterologous Transcriptional Activating or Repressing Domains

Embryos carrying the *UAS-lacZ* reporter gene and expressing the various derivatives of N^{+}-G4 protein diagrammed in Figure 4 are shown stained for β-gal protein expression. The presence of just the DNA-binding domain of Gal4 in N^{+}-G4 protein leads to moderate levels of β-gal expression. The further addition of the VP16 activation domain enhances this level of expression, while the addition of the en^{Rep} or Hairy (WRPW) repression domains completely blocks this expression. By contrast, the WGPS and PEST derivatives of N^{+}-G4 protein behave similarly to the canonical N^{+}-G4 protein.

and mediates transcriptional repression at least in part by recruiting the protein Groucho (Gro) (reviewed in Fisher and Caudy, 1998). In the first case, the en^{Rep} domain was inserted in place of the VP16 domain in N^{+}-GV4 protein to generate the protein N^{+}-G4,en^{Rep}; in the second case, the WRPW peptide was added to the end of N^{+}-G4 protein to create the protein N^{+}-G4,WRPW (Figure 4). As controls for the N^{+}-G4,WRPW experiment we also examined the consequences of adding a mutated form of this peptide, WGPS (to generate the protein

N^+-G4,WGPS), or deleting the C-terminal 80 amino acids of Notch (to generate the protein N^+-G4,$PEST^-$). We find that both the N^+-G4,en^{Rep} and N^+-G4,WRPW proteins lack detectable Notch signal–transducing activity, as indicated by their failure to rescue epidermal development in *arm*$^-$ *N*$^-$ embryos (Figure 4; data not shown). In addition, they are unable to activate expression of the *UAS-lacZ* gene (Figures 4 and 6). In contrast, the N^+-G4,WGPS and N^+-G4,$PEST^-$ proteins behave similarly to N^+-G4: both have Notch signal–transducing activity and can direct *UAS-lacZ* expression (Figures 4 and 6; data not shown).

Thus, adding well-defined transcriptional activating or repressing domains to intact Notch has opposing effects on Notch signal transduction, causing gain or loss of activity, respectively. In a corresponding fashion, they also cause the enhancement or loss of *UAS-lacZ* expression mediated by a Gal4 DNA-binding domain inserted within Notch. We interpret these results as evidence that Notch signal transduction normally involves the direct participation of the intracellular domain in activating the transcription of downstream target genes.

Discussion

Eukaryotic cells employ many strategies to link the reception of extracellular signals to changes in gene expression. In general, these strategies depend on transmembrane receptors, which receive signals at the cell surface and modify one or more intracellular effector proteins that transduce these signals to the nucleus. The same mechanism may also apply for Notch signal transduction. In this case, interactions between ligand and Notch at the cell surface would modify an effector such as the DNA-binding protein Su(H), which then translocates to the nucleus and regulates gene expression. However, the Notch intracellular domain contains conserved nuclear localization signals and, when expressed on its own, accumulates predominantly in the nucleus and has constitutive transducing activity. These observations have suggested an alternative and unusual mechanism for Notch signal transduction. In this case, ligand binding to the extracellular domain of the receptor would induce translocation of the intracellular domain to the nucleus, where it acts, in association with Su(H) or other DNA-binding proteins, to regulate transcription.

As outlined in the Introduction, all previous attempts to obtain direct evidence for ligand-dependent nuclear access of the Notch intracellular domain have failed. Hence, if Notch transduces extracellular signals through a mechanism that depends on nuclear import of the intracellular domain, one would have to argue that the amount of protein that accumulates in the nucleus is so small that it cannot be readily detected by conventional biochemical or immunological means. Here, we have used potentially more sensitive in vivo assays for nuclear access and action to ask whether the Notch intracellular domain does indeed gain access to the nucleus in response to ligand and whether it transduces Notch signals within the nucleus by regulating the transcription of downstream target genes.

Evidence for Ligand-Dependent Nuclear Access of the Notch Intracellular Domain

In the first series of experiments, we have used insertions of the Gal4-VP16 transcriptional activator at different sites within Notch protein to assay whether any portions of the receptor normally have access to the nucleus. The premise of this approach is that the Gal4-VP16 domain must gain access to the nucleus to activate transcription of a *UAS-lacZ* target gene and hence serves as an indicator for nuclear access of adjoining Notch sequences. We have found that Gal4-VP16 domains inserted in the intracellular domain, but not the extracellular domain, of Notch do indeed have access to the nucleus, as judged by their ability to activate *UAS-lacZ* transcription. Moreover, we show that access of a specific Gal4-VP16 insertion, GV3, positioned just carboxy-terminal to the Notch transmembrane domain is ligand-dependent and correlates with Notch signal–transducing activity. Thus, our results provide in vivo evidence that the intracellular domain of Notch gains access to the nucleus in response to ligand stimulation.

Evidence that Nuclear Access of the Intracellular Domain Is Necessary for Signal Transduction

We performed a second series of experiments to investigate whether nuclear access is required for Notch signal transduction. We first defined a minimal fragment of the Notch intracellular domain containing the CDC10 repeats that has intrinsic transducing activity (see also Roehl and Kimble, 1993) and then asked whether the intrinsic transducing activity associated with this fragment depends on its having access to the nucleus. We find that addition of sequences that permit or target this polypeptide to accumulate in the nucleus retain transducing activity, whereas sequences that target this polypeptide to membranes block the activity. Because the CDC10 repeats are essential for signal transduction by Notch (Greenwald and Seydoux, 1990; Kodoyianni et al., 1992; Lieber et al., 1993; Rebay et al., 1993), we interpret these results as evidence that the Notch intracellular domain must normally reach the nucleus to transduce Notch signals.

Evidence for a Direct Role of the Notch Intracellular Domain in Transcriptional Activation

We performed a third set of experiments to examine the role of the Notch intracellular domain in the nucleus and, specifically, whether it acts directly to regulate transcription. We inserted structural motifs that have well-defined roles in transcriptional activation and repression into the intracellular domain of intact Notch. We find that adding the VP16 activator domain renders the protein constitutively active, provided that it is inserted at a position which allows it to gain access to the nucleus irrespective of ligand. Moreover, the presence of the VP16 domain enhances the ability of Notch derivatives that carry the Gal4 DNA-binding domain to activate transcription of the *UAS-lacZ* target gene. In contrast, adding repressor motifs from either Engrailed or Hairy blocks the signal-transducing activity of the resulting Notch proteins as well as their ability to activate *UAS-lacZ* expression via the Gal4 DNA-binding domain. These results support the

hypothesis that the Notch intracellular domain directly transduces Notch signals within the nucleus by activating the transcription of downstream target genes.

Possible Mechanisms for Ligand-Dependent Nuclear Access of Notch

In principle, interactions between ligand and the extracellular domain of Notch might allow the intracellular domain to gain access to the nucleus by either of two mechanisms. In the first, these interactions would induce translocation of the entire receptor from the membrane to the nucleus. To be consistent with our findings, the translocated receptor would have to retain a membrane topology that would allow Gal4-VP16 domains inserted in the intracellular domain to bind and transcriptionally activate *UAS-lacZ* target genes while precluding the same activity by Gal4-VP16 domains inserted in the extracellular domain. In the second mechanism, which we favor, nuclear access would be afforded by one or more ligand-dependent proteolytic cleavages, which occur amino-terminal to the Notch intracellular domain and lead to its ability to move from the membrane to the nucleus.

A precedent for such a proteolytic processing mechanism is provided by the sterol-dependent nuclear import of DNA-binding domains of the Sterol Regulatory Element Binding Proteins 1 and 2 (SREBP-1 and SREBP-2) (reviewed in Brown and Goldstein, 1997). Each of the SREBPs is composed of three domains, an amino-terminal cytosolic domain that includes a transcription factor of the basic-helix-loop-helix class, a central domain consisting of two transmembrane domains flanking a short extracytosolic domain, and a carboxy-terminal cytosolic domain. A reduction in cholesterol abundance is sensed by the SREBP cleavage-activating protein (SCAP), which mediates a sequence-specific cleavage event in the extracytosolic domain. This first cleavage then precipitates a second cleavage, which occurs within the amino-terminal transmembrane domain. As a consequence of the second cleavage, the amino-terminal cytosolic domain is released from the membrane and translocates to the nucleus, where it binds cholesterol response genes and activates their transcription.

We suggest that the Notch intracellular domain may gain access to the nucleus by a similar mechanism to that of the SREBPs with ligand binding precipitating one or more cleavages, leading to the release of the intracellular domain from the membrane. Because the Gal4-VP16 domain inserted just downstream of the transmembrane domain of N^{+}-GV3 protein gains access to the nucleus in response to ligand, these cleavages would presumably occur in the extracellular or transmembrane domains, as in the case of SREBPs, or just carboxy-terminal to the transmembrane, within or amino-terminal to three basic amino acids that comprise a putative stop-transfer sequence.

The relationship of the proposed ligand-dependent cleavage(s) to other biochemically defined cleavages in Notch proteins is uncertain. When expressed in mammalian tissue culture cells, a truncated derivative of mouse Notch1 protein equivalent to *Drosophila* N^{ECN} protein, mNΔE, has constitutive activity. Moreover, this protein appears to be cleaved in a region close to the junction between the transmembrane and intracellular domains resulting in nuclear localization of at least some of the cleaved protein in at least some cells (Kopan et al., 1996). Although this cleavage has been assumed to occur within the intracellular domain (Kopan et al., 1996), the biochemical evidence is also compatible with a cleavage event within the transmembrane domain. Thus, it is possible that both the mNΔE and *Drosophila* N^{ECN} proteins are constitutively active because they mimic an intermediate normally generated during ligand-dependent processing of the extracellular domain of Notch and are further processed, like endogenous Notch protein, by cleavages that occur within or just carboxy-terminal to the transmembrane domain.

Notch proteins also appear to be cleaved at a second site in the extracellular domain as they mature and are exported to the cell surface to yield a disulfide bond–linked heterodimer composed of a large amino-terminal portion of the extracellular domain and the remainder of the protein (Blaumueller et al., 1997). This cleavage appears to depend on the activity of a membrane-associated metalloprotease, Kuzbanian, which is required for Notch signal transduction (Pan and Rubin, 1997). However, there is no evidence at present that Kuzbanian actually cleaves Notch or is involved in ligand-dependent processing of Notch.

We note that processing of β-amyloid precursor protein (β-APP) involves an intramembrane cleavage that depends on the function of Presenilin proteins (De-Strooper et al., 1998), which also appear to be required for normal Notch activity (reviewed in Greenwald, 1998). Presenilins may therefore have a common role in facilitating cleavages within or adjacent to the transmembrane domains of both Notch and β-APP proteins.

Previous genetic studies of Lin-12 and Notch have suggested that ligand activates Notch proteins by facilitating oligomerization (Greenwald and Seydoux, 1990; Heitzler and Simpson, 1993). If signal transduction by Notch depends on ligand-dependent cleavages within or amino-terminal to the transmembrane domain, it follows that oligomerization may serve to regulate the proteolytic activity responsible for this cleavage. For example, oligomerization may allow the recruitment of an extracellular or transmembrane protease. Alternatively, such a protease may always be associated with Notch, but it requires oligomerization to act.

Nuclear Access of Carboxy-Terminal Portions of the Notch Intracellular Domain in the Absence of Ligand

Our findings using N-GV4 proteins suggest that some carboxy-terminal fragments of the Notch intracellular domain gain access to the nucleus as a consequence of cleavages within the intracellular domain. However, in contrast to the ligand-dependent nuclear access we detect using N-GV3 proteins, none of the carboxy-terminal processing events suggested by our experiments with N-GV4 proteins appear to depend on ligand. Nor do they appear to correlate with Notch signal–transducing activity, except in proteins in which the resulting carboxy-terminal portions of the intracellular domain would

Table 1. Amino Acid Sequences at Joins in Deleted or Chimeric Proteins

Protein[a]	Joins	Amino Acid Sequence (/Linker/)[b]
N^{ECN}	**Notch**/**Notch**	**SVGCQN**/id/**TAAKHQ**
N^{Sev11}	Sev11/**Notch**	LVLVRK/**RAHGVT**
N^{EGF}	**Notch**/**Notch**	**CEIAVP/EDCTES**
N^{+}-GV1	**Notch**/Gal4VP16	**NGGSGS**/ppp/KLLSSI
	Gal4VP16/**Notch**	IDEYGG/**SGNDRY**
N^{+}-GV2	**Notch**/Gal4VP16	**EAAEFL**/gsppp/KLLSSI
	Gal4VP16/**Notch**	IDEYGG/s/**TAAKHQ**
N^{+}-GV1/2	**Notch**/Gal4VP16	**NGGSGS**/ppp/KLLSSI
	Gal4VP16/**Notch**	IDEYGG/s/**TAAKHQ**
N^{+}-GV3	**Notch**/Gal4VP16	**STORKR**/sgpp/KLLSSI
	Gal4VP16/**Notch**	IDEYGG/isgv/**RKRAHG**
N^{+}-GV4	**Notch**/Gal4VP16	**QAMIGS**/ppp/KLLSSI
	Gal4VP16/**Notch**	IDEYGG/**SPPPGQ**
N^{+}-G4	**Notch**/Gal4	**QAMIGS**/ppp/KLLSSI
	Gal4/**Notch**	TGLFVQ/**GSPPPG**
N^{+}-G4,en^{Rep}	**Notch**/Gal4	**QAMIGS**/ppp/KLLSSI
	Gal4/engrailed	QLTVSI/slaag/ALEDRC
	engrailed/**Notch**	PEKSAL/**GSPPPG**
N^{+}-G4,$PEST^{-}$	**Notch**	**QHNQQA**/s
N^{+}-G4,WRPW	**Notch**/WRPW	**SEAIYI**/qp/WRPW
N^{+}-G4,WGPS	**Notch**/WGPS	**SEAIYI**/qp/WGPS
N^{Sev11}-GV3	Sev11/Gal4VP16	LVLVRK/rrsgpp/KLLSSI
	Gal4VP16/**Notch**	IDEYGG/isgv/**RKRAHG**
N^{CDC10},$MYR^{+}NLS^{+}$	MYR^{+}/Flu	MGNKCCSKRQ/gtmagni/$[YPYDVPDYAG]_2$
	Flu/**Notch**	$[YPYDVPDYAG]_2$/sma/**PPAHQD**
	Notch/NLS^{+}	**QAMIGS**/PPKKKRKVED
MYR^{-} signal		MANKCCSKRQ
NLS^{-} signal		PPKTTRKVED
Sev11MYR^{-} signal	Sev11/MYR^{-}	LVLVRK/rrsagrt/MANKCCSKRQ

[a] As indicated in Figures 1 and 4 and in text. For the N^{+}-G4,$PEST^{-}$, N^{+}-G4,WRPW, and N^{+}-G4,WGPS proteins, the G4 insertion is the same as that shown for N^{+}-G4 protein and is composed of the first 99 amino acids of the Gal4 protein. For the N^{+}-G4,en^{Rep} protein, the G4 insertion is composed of the first 147 amino acids of the Gal4 protein (all of the GV insertions contain the same 147 amino acid domain fused at the carboxyl terminus of the VP16 activation [Sadowski et al., 1988]). For the N^{CDC10} series, all five proteins are identical to N^{CDC10},$MYR^{+}NLS^{+}$ except for the substitution of the mutated MYR or NLS signals, or the Sev11MYR^{-} signal. The Sev11 and N^{ECN} proteins are described, respectively, in Basler et al. (1991) and Rebay et al. (1993).

[b] Six amino acids of each protein adjacent to the join are shown (N sequences are in boldface), and the joins are listed in the amino-terminal to carboxyl-terminal order. The Flu, MYR, and NLS signals are shown in full. Linkers between the joins, when present, are indicated in lower case.

also contain insertions of the VP16 activating domain. At present, a minimalist view of these putative intracellular cleavages is that they reflect nonspecific degradative events that do not normally play a role in signal transduction, except perhaps in the potentially important process of down-regulating receptor activity. However, it remains possible that one or more of these processing events is essential for N signal–transducing activity once a ligand-dependent cleavage occurs upstream.

Signal Transduction by the Notch Intracellular Domain

In principle, the only limiting step in the mechanism of signal transduction by Notch may be the proposed ligand-dependent cleavage event that releases the intracellular domain from the membrane and allows it to enter the nucleus. In favor of this view, the Notch intracellular domain has intrinsic signal-transducing activity and is found predominantly in the nucleus when expressed on its own (Lieber et al., 1993; Struhl et al., 1993). Moreover, the intrinsic activity of a smaller domain composed primarily of the CDC10 repeats depends on its being allowed to enter the nucleus (Figure 3). However, Notch signal transduction also appears to depend on several proteins that associate physically with the Notch intracellular domain, such as Su(H), Dishevelled, Deltex, and Numb, as well as other proteins, such as Mastermind and Hairless (reviewed in Weinmaster, 1997; Greenwald, 1998). Hence, interactions between ligand and the Notch extracellular domain may be required not only to release the intracellular domain but also to initiate a series of other events within the cell that are required to generate a productive complex of this domain with other proteins.

What is the nature of this complex, and what is its role in the nucleus? At least in the embryonic ventral ectoderm, our findings support the view that the Notch intracellular domain functions in the nucleus to activate transcription of downstream target genes. Key targets for Notch-dependent transcriptional activation are genes of the *Enhancer of split* complex (*E[spl]*). These genes are required in the ventral ectoderm to specify epidermal versus neural differentiation (reviewed in Weinmaster, 1997; Greenwald, 1998). Morever, they have been shown to be direct targets for binding by the Su(H) protein (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995) and to be transcriptionally activated in response to Notch signaling (Jennings et al., 1994; Jarriault et al., 1995; Kopan et al., 1996; Eastman et al., 1997).

By analogy with EBVNA2, a viral coactivator protein that interacts with a mammalian Su(H) homolog CBF1 to convert it from a transcriptional repressor to a transcriptional activator (Hsieh and Hayward, 1995), the Notch intracellular domain, perhaps in association with other proteins, may combine with Su(H) to contribute an activation domain that allows Su(H) to activate the transcription of *E(spl)* genes as well as other target genes. We note that Su(H) is not required for all Notch-dependent developmental decisions (Lecourtois and Schweisguth, 1995; Wang et al., 1997), raising the possibility that Notch mediates some cell fate choices by associating with other DNA-binding transcription factors or by employing other mechanisms to transduce extracellular signals.

Experimental Procedures

Construction of Notch Transgenes

Wild-type and deleted derivatives of the *N* and *N-GV* coding sequences were inserted into a *Casper-hsp70* vector, which contains the *hsp70* promoter upstream of the insertion site and a 3′ UTR sequence from the *SV40* T-antigen gene downstream of the insertion site. The *N* coding sequence was derived from a *N* minigene (as in Struhl et al., 1993). The Gal4-VP16 coding sequence encodes the first 147 amino acids of Gal4, including the DNA-binding domain, and the carboxy-terminal 78 amino acids of VP16, including the activation domain (Sadowski et al., 1988). The Sev11 (Basler et al., 1991), Myristylation (Cross et al., 1984; Simon et al., 1985), NLS (Kalderon et al., 1984), and Flu epitope (Wilson et al., 1984) sequences have been described previously (Table 1). For some experiments, we also used a *pUAST* vector (Brand and Perrimon, 1993) to place *N* sequences under the control of a promoter that responds to Gal4. In these contructs, a $>y+>$ flp-out cassette (Basler and Struhl, 1994) was introduced immediately upstream of the Notch sequences and excised after the constructs were inserted as stable transgenes in the *Drosophila* genome. The deduced amino acid sequences at the joins between Notch, Gal4-VP16, En, Hairy, and Sev11 proteins as well as between these sequences and the Myristylation, SV40 NLS, and Flu sequences in all of these constructs are listed in Table 1.

In Vivo Assays of Notch Transducing Activity, *UAS-lacZ* Expression, and Notch Expression

UAS-lacZ expression: embryos carrying one copy of the given *hsp70-N-GV* or *hsp70-N-G* transgene and one copy of the *UAS-lacZ* reporter (Fischer et al., 1988) were heat shocked for 1 hr at 37°C (or lower temperatures, as indicated), allowed to recover for 2 hr at 25°C, and then fixed and stained for β-gal expression by standard immunohistochemical methods (as in Struhl et al., 1993).

UAS-lacZ expression in Dl^{X43} versus Dl^{X43} *hsp70-Dl* embryos. Embryos derived from a cross between *hsp70-N-GV* Dl^{X43}/TM3, *ftz-lacZ* males and either *UAS-lacZ*; Dl^{X43}*/TM3, ftz-lacZ* or *UAS-lacZ;* Dl^{X43} *hsp70-Dl/TM3, ftz-lacZ* females were heat shocked and stained for β-gal expression as described above. Dl^{X43} mutant embryos were identified by the absence of striped *ftz-lacZ* expression. The *hsp70-Dl* transgene contains the coding sequence for Delta (Kopczynski et al., 1988) inserted in a *Carnegie20*-derived *hsp70* vector (Struhl et al., 1993).

Neuroblast segregation assay: embryos carrying a single copy of the given *hsp70-N* or *hsp70-N-GV* transgene were heat shocked for 30 min at 37°C, allowed to recover for 90 min at 25°C, and stained for Hunchback (Hb) expression, which marks newly segregated neuroblasts, by immunhistochemistry (as in Struhl et al., 1993). In experiments involving Notch derivatives that lack Gal4 or Gal4-VP16 domains, the Gal4/UAS system was also used to drive ubiquitous expression. For these experiments, embryos carrying a single copy of the given *UAS-N* transgene and either of two Gal4 driver genes, *arm-Gal4* (Sanson et al., 1996) or *Tubα1>Gal4-VP16*^{F442A} were stained for Hb expression. The *Tubα1>Gal4-VP16*^{F442A} transgene is composed of the *Tubα1* promoter, which is active in most or all cells (Basler and Struhl, 1994), positioned upstream of a *Gal4-VP16* coding sequence that contains the F442A mutation, which reduces the activity of the VP16 activation domain (Regier et al., 1993).

Epidermal rescue assay for N^- embryos: N^{XK11} arm^{YD35}*/FM7* females were crossed to males carrying a given *hsp70-N* or *hsp70-N-GV* transgene and embryos subjected to three 1 hr 37°C heat shocks interspersed with 3 hr recovery times at 25°C. Larval cuticles formed by the resulting embryos were assayed for the Armadillo segmentation phenotype (Lieber et al., 1993). For the various Notch derivatives that do not contain Gal4, the Gal4/UAS method was also used: for these experiments, N^{XK11} arm^{YD35} embryos carrying a given *UAS-N* gene and the *Tubα1>Gal4-VP16*^{F442A} transgene were derived from N^{XK11} arm^{YD35}*/FM7* females. In all cases, identical results were obtained using the *hsp70* promoter or the Gal4/UAS technique to express the same Notch protein.

Epidermal rescue assay for Dl^- embryos: Dl^{X43} hh^{10E}*/TM3* females were crossed to *hsp70-N-GV/+;* Dl^{X43} hh^{10E}/+ males, treated as above for N^{XK11} arm^{YD35} embryos, and the resulting cuticles scored for the Hedgehog segmentation phenotype. For all of the Notch derivatives that do not contain Gal4, equivalent experiments were also performed using the Gal4/UAS method (as described above for epidermal rescue of N^- embryos) and identical results obtained.

Assays for the subcellular distribution of Notch-derived proteins: a monoclonal antisera Mab179C6 (Fehon et al., 1991) was used to detect the intracellular domain of Notch. The epitope is present in the CDC10 bearing domain used in the experiments shown in Figure 3. Commerically available polyclonal anti-Gal4 (Santa Cruz) and monoclonal anti-Flu (BabCo) antisera were used to detect the Gal4 and Flu epitopes. For the N^{CDC10}*,MYR NLS* series of transgenes, embryos carrying a *hsp70-flp* transgene, the *69B-Gal4* transgene (which drives the expression of *UAS*-target genes in most ectodermal cells; Brand and Perrimon, 1993), and a given *UAS*$>y^+>$ N^{CDC10}*,MYR NLS* transgene were heat shocked to remove the $>y^+>$ Flp-out cassette, generating clones of cells that express the N^{CDC10}*,MYR NLS* coding sequence.

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