Analysis of Notch Lacking the Carboxyl Terminus Identified in *Drosophila* Embryos

Cedric S. Wesley and Lino Saez

Department of Genetics, The Rockefeller University, New York, New York 10021

Abstract. The cell surface receptor Notch is required during development of *Drosophila melanogaster* for differentiation of numerous tissues. Notch is often required for specification of precursor cells by lateral inhibition and subsequently for differentiation of tissues from these precursor cells. We report here that certain embryonic cells and tissues that develop after lateral inhibition, like the connectives and commissures of the central nervous system, are enriched for a form of Notch not recognized by antibodies made against the intracellular region carboxy-terminal of the CDC10/Ankyrin repeats. Western blotting and immunoprecipitation analyses show that Notch molecules lacking this region are produced during embryogenesis and form protein complexes with the ligand Delta. Experiments with cultured cells indicate that Delta promotes accumulation of a Notch intracellular fragment lacking the carboxyl terminus. Furthermore, Notch lacking the carboxyl terminus functions as a receptor for Delta. These results suggest that Notch activities during development include generation and activity of a truncated receptor we designate NΔCterm.

Key words: Notch • Delta • neurogenesis • *daughterless* • differentiation

Introduction

Notch (N)[1] is required throughout development of *Drosophila melanogaster* for differentiation of tissues as diverse as the nervous systems, cuticle, internal organs, and muscles (for a review of Notch signaling, see Artavanis-Tsakonas et al., 1999; see also Zecchini et al., 1999; Wesley, 1999; Brennan et al., 1999a,b). N is a cell surface receptor which generates intracellular signals when a ligand binds its extracellular domain (Artavanis-Tsakonas et al., 1999). During embryogenesis, N is required to produce neuronal and epidermal precursor cells in a process termed lateral inhibition (Cabrera, 1990; Skeath and Carroll, 1992).

During lateral inhibition, the ligand Delta (Dl) binds the extracellular domain of N, leading to transmission of signals to the nucleus by the intracellular protein, Suppressor of Hairless (Su(H)). Cells that respond to these signals by turning on the expression of *Enhancer of split Complex* genes (*E(spl)C*), and turning off the expression of the proneural *Achaete scute Complex* genes, become the epidermal precursor cells; cells that do not turn on the expression of *E(spl)C* but continue to express *Achaete scute Complex* genes, become the neuronal precursor cells (see Artavanis-Tsakonas et al., 1999). N function continues to be required during differentiation of neurons from the neuronal precursor cells (Giniger et al., 1993; Giniger, 1998) and epidermis from the epidermal precursor cells (Hoppe and Greenspan, 1990; Couso and Martinez-Arias, 1994; Wesley, 1999). Requirement of N function at successive stages is also observed during differentiation of tissues like the adult compound eyes and sensory bristles (Cagan and Ready, 1989; Guo et al., 1996; Wang et al., 1997). This implies that N is required continuously during differentiation of a cell lineage to maintain the cell fates specified during lateral inhibition and/or generate additional differentiation signals at post-lateral inhibition stages.

Su(H) activity is affected by some proteins that also bind the N intracellular domain. Deltex contributes to the Su(H)-mediated N signaling pathway (Matsuno et al., 1995), while Numb, Dishevelled, and Hairless antagonize this pathway (Axelrod et al., 1996; Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996; Wang et al., 1997). On the other hand, Disabled, which functions with N during differentiation of neurons from neuronal precursor cells (i.e., after lateral inhibition), is not known to affect Su(H) activity (Giniger et al., 1993; Giniger, 1998). Su(H) interacts with the RAM 23 region and the CDC10/Ankyrin re-

Address correspondence to Cedric S. Wesley, Department of Genetics, The Rockefeller University, 1230 York Avenue, New York, NY 10021. Tel.: (212) 327-8233. Fax: (212) 327-7420. E-mail: wesleyc@rockvax.rockefeller.edu

[1]*Abbreviations used in this paper:* CNS, central nervous system; Dl, Delta; *da, daughterless* gene; *E(spl)C, Enhancer of split Complex* genes; N, Notch; Su(H), Suppressor of Hairless protein.

peats region in the N intracellular domain (Fortini and Artavanis-Tsakonas, 1994; Tamura et al., 1995; see Fig. 1). Deltex interacts with the CDC10/Ankyrin repeats region (Diederich et al., 1994; Matsuno et al., 1995), Numb with the RAM 23 and PEST regions (Guo et al., 1996), Dishevelled with the unique region carboxy-terminal of the CDC10/Ankyrin repeats (Axelrod et al., 1996), and Disabled with the RAM 23 region (Giniger, 1998). The binding site of Hairless has not been mapped (Wang et al., 1997; see Fig. 1 a). These different activities and affinities suggest that regulation of activities of different proteins that bind the intracellular domain might be an important component of N functions at successive stages of differentiation.

In this study, we describe results showing that a truncated form of N lacking the sequence carboxy-terminal of the CDC10/Ankyrin repeats is produced during embryogenesis. This truncated receptor, which would lack the Dishevelled and one of the two Numb-binding sites, can function as a receptor for Dl. Its differential accumulation in interacting cells may play a role in choice of cell fates during lateral inhibition and regulation of activities of different proteins that bind the N intracellular domain.

Materials and Methods

Immunostaining of N Protein in Embryos

αNPCR antibody was generated against the intracellular segment of N, amino acids 2,115–2,536, between the CDC10/Ankyrin repeats and the OPA repeats (Lieber et al., 1993; Kidd et al., 1998; numbering of the 2,703–amino acid-long N protein is according to Kidd et al., 1986). The αN203 antibody was generated in rats against a glutathione-*S*-transferase fusion peptide including N EGF-like repeats 1–3 (amino acids 59–177) following standard procedures (Harlow and Lane, 1988). αN203 immunoprecipitates and detects only N forms from embryos and S2-Notch cells. It gives N immunostaining patterns in embryos, imaginal discs, and larval brains that is indistinguishable from other published N staining patterns. All the N antibodies used in this study are N-specific antibodies: they do not give signals in N^- embryos or N molecules recognized by each are recognized by at least two other independently generated N antibodies (Kidd et al., 1989, 1998; Lieber et al., 1993). These polyclonal antibodies also recognize N only when their respective epitope regions are included and even a small terminal segment of the epitope region is sufficient for recognition by the respective antibody.

Immunostaining procedure described in Lieber et al. (1993) was followed and signals detected with HRP. An anti–β-galactosidase antibody made in rabbit and alkaline phosphatase reactions were used to sort out *FM7 lac-Z* or *TM6 lac-Z* chromosome carrying embryos laid by $N^{264\text{-}47}$/*FM7 lac-Z* or Dl^{X}/*TM6 lac-Z* flies. Embryos shown in Fig. 2, a–i and j–q were collected in separate batches and samples within each batch were processed identically.

Immunoprecipitations

For immunoprecipitation of N molecules from embryos, ~50–100-μl vol of dechorionated embryos, of appropriate ages (laid by circadian cycle entrained flies to minimize age variance in embryos), were crushed using a loose fitting pestle in a 1-ml Wheaton Dounce Grinder, in the presence of ice-cold pbBSS + protease inhibitors + 0.75% Triton X-100 (pbBSS: 55 mM NaCl, 40 mM KCl, 15 mM Mg_2SO_4, 10 mM $CaCl_2$, 20 mM glucose, 50 mM sucrose, 0.74 mM KH_2PO_4, 0.35 mM Na_2HPO_4; protease inhibitors: 20 ng/ml each of leupeptin, pepstatin, trypsin inhibitor, and E-64, 5 ng/ml of aprotinin, 2 mM phenylmethylsulfonyl fluoride). After 20 min of incubation on ice, deoxycholate was added to a final concentration of 0.5% and incubated on ice for 25 min. The extract was precleared for ~2 h at 4°C with GammaBind Plus beads (Amersham Pharmacia Biotech), and incubated overnight at 4°C with the immunoprecipitation antibody. Immunocomplexes were captured with GammaBind Plus beads, the beads rinsed four times with 1 ml of cold pbBSS + protease inhibitors + 0.1% Triton X-100. Bound complexes were eluted with 40 μl of 1× Laemmli buffer + protease inhibitors, boiled for 6 min, separated by SDS-PAGE in 4% gels, Western blotted according to standard procedures (Harlow and Lane, 1988; Sambrook et al., 1989), and signals were detected with the ECL kit (Amersham Pharmacia Biotech).

For immunoprecipitation of N-Dl cross-linked complexes, ~800-μl vol of dechorionated embryos of appropriate ages (laid by circadian cycle entrained flies) were partially crushed with a loose fitting pestle in a 1-ml Wheaton Dounce Grinder, in the presence of 400 μl of ice-cold pbBSS + protease inhibitors, with or without ~2 mM BS^3 (Bis[sulfosuccinimidyl] suberate; Pierce Chemical Co.). After 45 min of incubation on ice, 12 μl of cold 2-M Tris-HCl, pH 7.5, was added to quench the cross-linking reaction. Membrane proteins were extracted in 0.75% Triton X-100 and 0.5% deoxycholate. The rest of the procedure was identical to that described for immunoprecipitation of Notch proteins from embryos except that the wash buffer included 10 mM Tris, pH 7.5. 100 μl of the monoclonal αDl was used per immunoprecipitation. The amounts of proteins in different extracts were standardized using absorbance values at 280 nM and the BioRad D_C protein assay kit. See also Wesley (1999) for description of these immunoprecipitation procedures.

Western Blot Analyses

Embryos. Populations of flies were transferred to the appropriate temperature, eggs collected for 2 or 3 h (or 6 h at 18°C), and reared for the indicated period of time at the indicated temperatures (with appropriate corrections for differences in developmental rate).

Cultured Cells. Cells were heat-shocked for 30 min at 37°C, allowed to synthesize proteins for 1 or 2 h at room temperature, and washed 2× in Shields and Sang's M3 media plus antibiotics.

N and Dl Cell Aggregates. 1×10^6 S2-N, S2-N^{60g11}, S2-$N^{1–2155}$, or S2-N^{2262} cells were mixed with 1.5×10^6 S2-Dl or S2 cells, transferred to 14-ml round-bottom Falcon tubes or siliconized Falcon multiwell plates, and shaken gently for 1 or 2 h. Total proteins from embryos, cells, or cell aggregates were extracted in pbBSS + protease inhibitors + 0.75% Triton X-100 + 0.5% deoxycholate as described above for immunoprecipitation of N molecules. Proteins were separated in either 4 or 8% SDS-PAGE, Western blotting was performed as described (Harlow and Lane, 1988; Sambrook et al., 1989), and signals were detected with the ECL kit (Amersham Pharmacia Biotech). The amount of proteins in different extracts was standardized using absorbance values at 280 nM and the BioRad D_C protein assay kit. The Western blotting procedures followed are also described in Wesley (1999).

Cloning of N^{60g11}. Nco1-Nar1 (amino acids ~1,996 and ~2,323, respectively) fragment was Pfu-PCR amplified from N^{60g11}/*FM7 lac-Z* DNA and cloned into pGEM7z vector (Promega). Clones carrying the ~175-bp N^{60g11} fragment including the site of mutation (see Lyman and Young, 1993) were distinguished from the 154-bp wild-type fragment carrying clones (derived from the *FM7 lac-Z* chromosome) by PCR analysis. The Nco1-Nar1 fragment of the N^{60g11} gene was checked by sequencing and used to replace the wild-type Nco1-Nar1 fragment in a hsCasper-N clone. Cloning of $N^{1–2155}$ and $N^{1–2262}$: *N* fragments were Pfu-PCR amplified with a primer 5′ of the Nco1 site (amino acid ~1,996) and with a primer containing a stop site either at the third BamH1 site (amino acid ~2,155) or at the first EcoR1 site (amino acid ~2,262). These fragments were sequenced to confirm that there were no PCR-related mutations and used to replace the Nco1-Nar1 fragment in a pGem7z-N clone. The whole N fragment was excised and cloned into the hsCasper vector. Cloning of the $N^{2155–2703}$: the carboxy-terminal BamHI-XbaI fragment of the *N* gene was cloned into hsCasper vector. It was expressed transiently in S2 cells. UAS-$N^{intra1790}$ and UAS-$N^{1893–2155}$ are described in Kidd et al. (1998) and Lieber et al. (1993), respectively. These were cotransfected into S2 cells with hsGal4 for transient expression. hsGal4 alone was transfected to obtain S2-hsGal4 only.

Northern Blot Analysis

Embryos. 0–24-h embryos laid by +/FM7 lac-Z × *FM7lac-Z/Y* crossed flies and N^{60g11}/*FM7 lac-Z* flies, reared at 18°C (~0–12-h staged embryos reared at 25°C) were used for Fig. 6 d. 0–6-h embryos laid by UAS-$N^{intra1790}$; hsGal4, UAS-$N^{1893–2155}$; hsGal4, or *yw* Canton S embryos (collected at 25°C, heat-shocked at 37°C for 30 min, and incubated at room temperature for 45 min) were used for Fig. 6 e.

Cultured Cells. Cells were heat-shocked for 30 min at 37°C and allowed to synthesize proteins for 1 or 2 h. The cells were washed 2× in Shields and Sang's M3 media plus antibiotics (M3 medium), and resuspended in M3 medium at a concentration of 10^7 cells/ml. 0.7 ml of S2, S2-Dl, S2-N, S2-N^{60g11}, S2-N^{1-2155}, or S2-N^{2262} cells were mixed with 0.7 ml of S2 cells or S2-Dl cells. The mixtures were transferred to siliconized Falcon multiwell tissue culture plates and gently rotated for 2 h. UAS-$N^{intra1790}$, hsGal4, UAS-$N^{1893-2155}$, hsGal4, and hs$N^{2155-2703}$ cells were heat-shocked for 30 min and allowed to synthesize proteins for 45 min. Total RNAs from embryos and cells were extracted using RNAzol B (Tel-test, Inc.) according to manufacturer's protocol. 20 μg (UAS and *yw* embryos) or 40 μg of total RNA was loaded in each lane. Standard Northern blot procedures were followed (Sambrook et al., 1989).

Results

An Antibody Made against the Carboxyl Terminus of N Does Not Stain Certain Embryonic Tissues Expressing N

Immunostaining experiments were done with αN203, which recognizes the amino terminus of N, and with αNPCR, which recognizes the carboxyl terminus of N (see Fig. 1 and Materials and Methods for information about these antibodies). Stage 8-9 Canton S embryos immunostained with αN203 showed relatively intense punctate staining in the region involved in lateral inhibition, whereas the embryos stained with αNPCR showed a homogenous staining of the same region (Fig. 2, a–e). The intense punctate signals in embryos treated with αN203 are derived from segregating neuroblasts: cell morphology identify them as neuroblasts and the pattern of αN203 staining rapidly changed during this stage of embryogenesis (Fig. 2, compare b with c). Furthermore, αN203 staining corresponded with the expression pattern of the proneural *achaete* gene accompanying neuroblast segregation (Campos-Ortega and Hartenstein, 1985; Cabrera, 1990; Skeath and Carroll, 1992; data not shown). Differences in the staining patterns of αNPCR and αN203 were more striking at later stages of embryogenesis: αNPCR antibody did not stain the commissures and connectives of the central nervous system (CNS; Fig. 2 f), while αN203 showed strong staining of the same tissues as previous studies of N distribution have shown (Fig. 2 g; Kidd et al., 1989; Johansen et al., 1989; Fehon et al., 1991; Giniger et al., 1993; Kooh et al., 1993; Giniger, 1998). The previous studies cited used antibodies made against the first two EGF-like repeats, the last six EGF-like repeats, extensive regions of the EGF-like repeats, or intracellular regions including both the CDC10/Ankyrin repeats and the unique region in the carboxyl terminus. None of these studies used an antibody that is specific to the unique region carboxy-terminal of the CDC10/Ankyrin repeats, like αNPCR. At some other stages, the two antibodies gave similar patterns (Fig. 2, h and i).

Lack of staining of commissures and connectives of the CNS by αNPCR was not because this antibody fails to recognize N in the embryos: (a) omission of αNPCR from the immunostaining procedure resulted in complete loss of signals in the embryos (Fig. 2, compare k with j); (b) αNPCR failed to generate any signals in the neurogenic N^{264-47}/Y embryos which have lost expression of N (see Kidd et al., 1989), but generated strong signals in the neurogenic Dl^x/Dl^x embryos which continue to express N (Fig. 2, l and m); (c) αNPCR generates a patchy staining pattern in N^{ts1} embryos raised at 30°C (Fig. 2, n and o; patchy loss of N in N^{ts1} embryos is expected since only ~70% of these embryos fail to complete embryogenesis at the restrictive temperature of 30°C, see Shellenbarger and Mohler, 1978); and (d) non-recognition of N in connectives and commissures of the CNS was a property of αNPCR as these tissues were stained with αN203 (Fig. 2 g) and the nervous system–specific anti-HRP antibody (Fig. 2, compare q with p stained with αNPCR). If both αN203 and αNPCR antibodies recognized the same N molecules at all

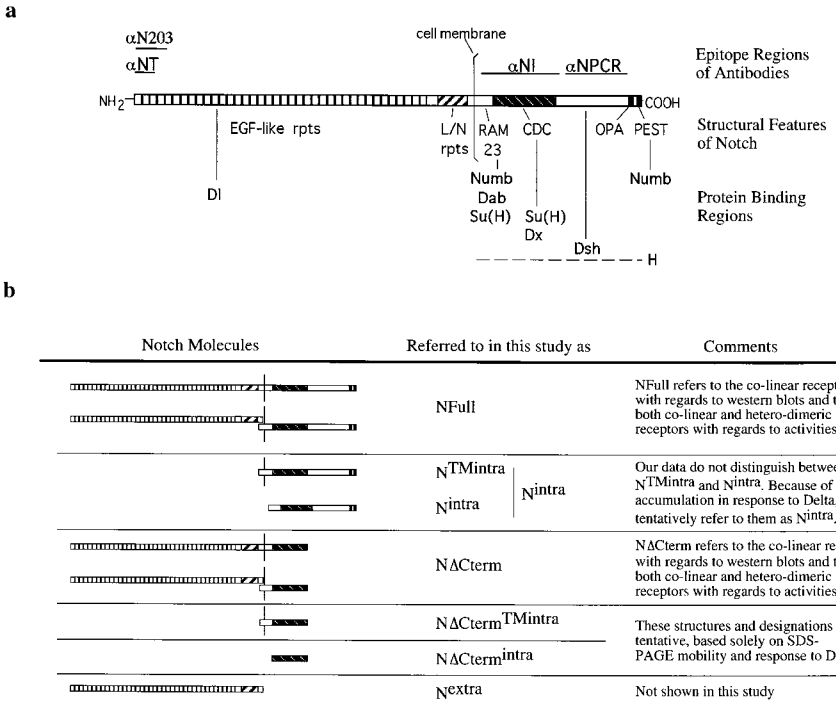

Figure 1. Features and structures of Notch molecules referred to in this study. (a) Features of the full-length N molecule (NFull) and N antibodies. EGF-like rpts, epidermal growth factor-like repeats; L/N rpts, Lin12/Notch repeats; CDC, CDC10/Ankyrin repeats; OPA, Glutamine-rich sequence; Dab, Disabled; Dx, Deltex; Dsh, Dishevelled; H, Hairless (exact binding site is not known). (b) Nomenclature used for different forms of Notch.

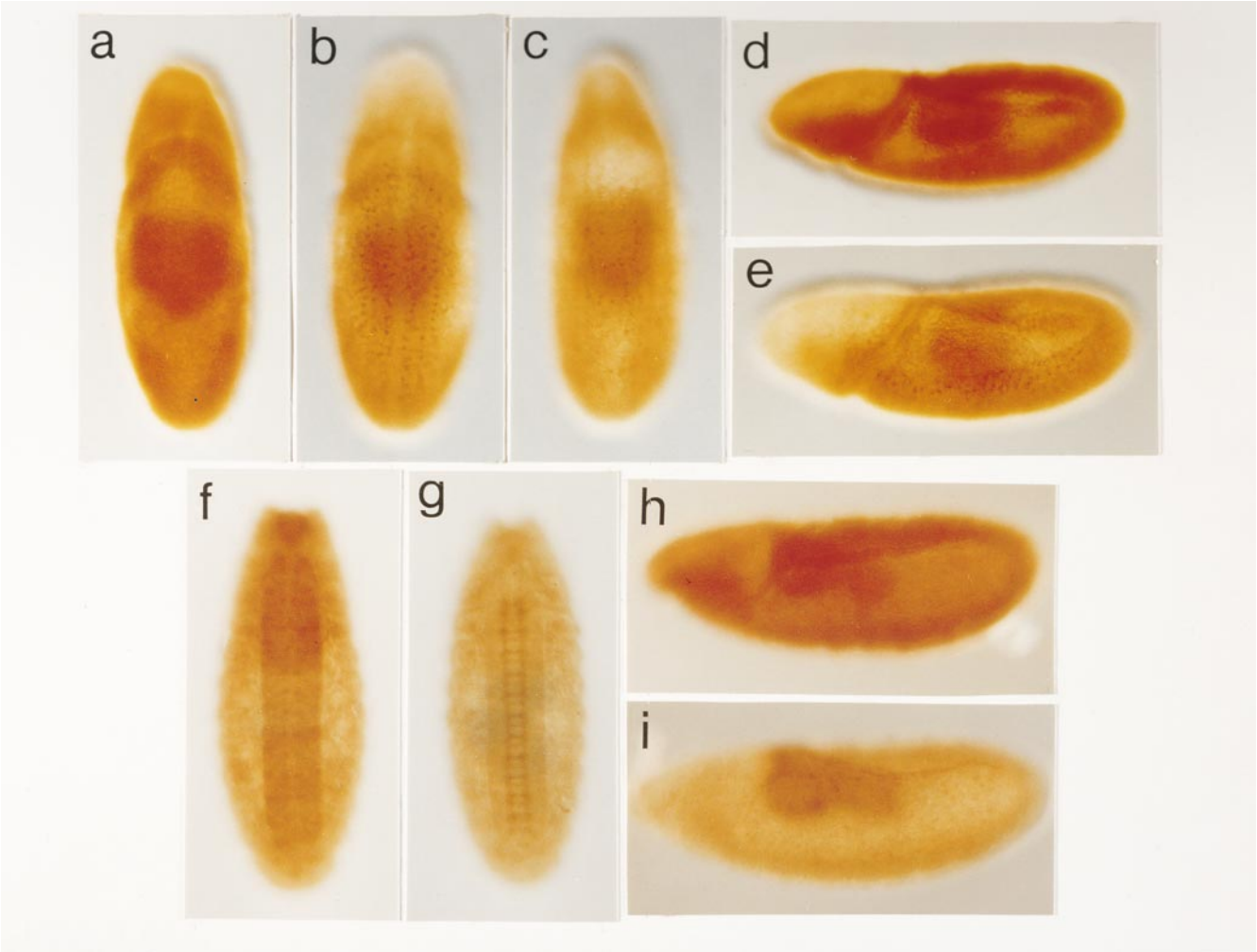

Figure 2 (continues on facing page).

stages of development, similar staining patterns would be expected at all stages. Instead, only αN203 showed higher levels of N in the connectives and commissures of the developing CNS (Fig. 2, b, c, e, and g, compare with αNPCR staining in a, d, and f). The pattern of N expression in *E(spl)C*$^{-}$ embryos deficient in lateral inhibition signaling was the same as in *Dl*$^{-}$ embryos (detected by αN203 and αNPCR antibodies): expression of N is higher than in Canton S embryos and limited to the neurogenic region (data not shown). These results indicated that a subset of differentiating tissues that express N, produced after lateral inhibition signaling, are enriched for a form of N that either does not contain the region known to be present carboxy-terminal of the CDC10/Ankyrin repeats, or has masked the antibody epitopes in that region.

Embryos Produce Notch Molecules Lacking Sequence Carboxy-terminal of the CDC10/Ankyrin Repeats

SDS-PAGE analysis of N immunoprecipitated from Canton S embryonic extracts showed that αN203 and αNI recover a triplet of N proteins in the ~350-kD range (Fig. 3 a, lanes 1 and 2; αNI is made against the intracellular region between the transmembrane domain and the end of CDC10/Ankyrin repeats, Lieber et al., 1993; see Fig. 1). The three forms are referred to as NFull, N350.2, and NΔCterm in increasing order of electrophoretic mobilities (see later for the basis for these names). Similar forms of N have been reported previously, detected using an antibody made against the last six EGF-like repeats (Johansen et al., 1989). However, αNPCR, made against the intracellular region carboxy-terminal of the CDC10/Ankyrin repeats, immunoprecipitated only NFull and N350.2 (Fig. 3 a, lane 3) indicating that NΔCterm is not recognized by this antibody.

As immunoprecipitations were done with a buffer approximating physiological conditions, it is possible that physiological NΔCterm masked αNPCR epitopes and this prevented immunoprecipitation by αNPCR. To evaluate this possibility, N was immunoprecipitated from Canton S embryos with αNI (which recovers all three forms), two equal aliquots of the immunoprecipitates were separated by SDS-PAGE, and the resultant Western blots probed with αNI and αNPCR. NΔCterm was detected by αNI (as

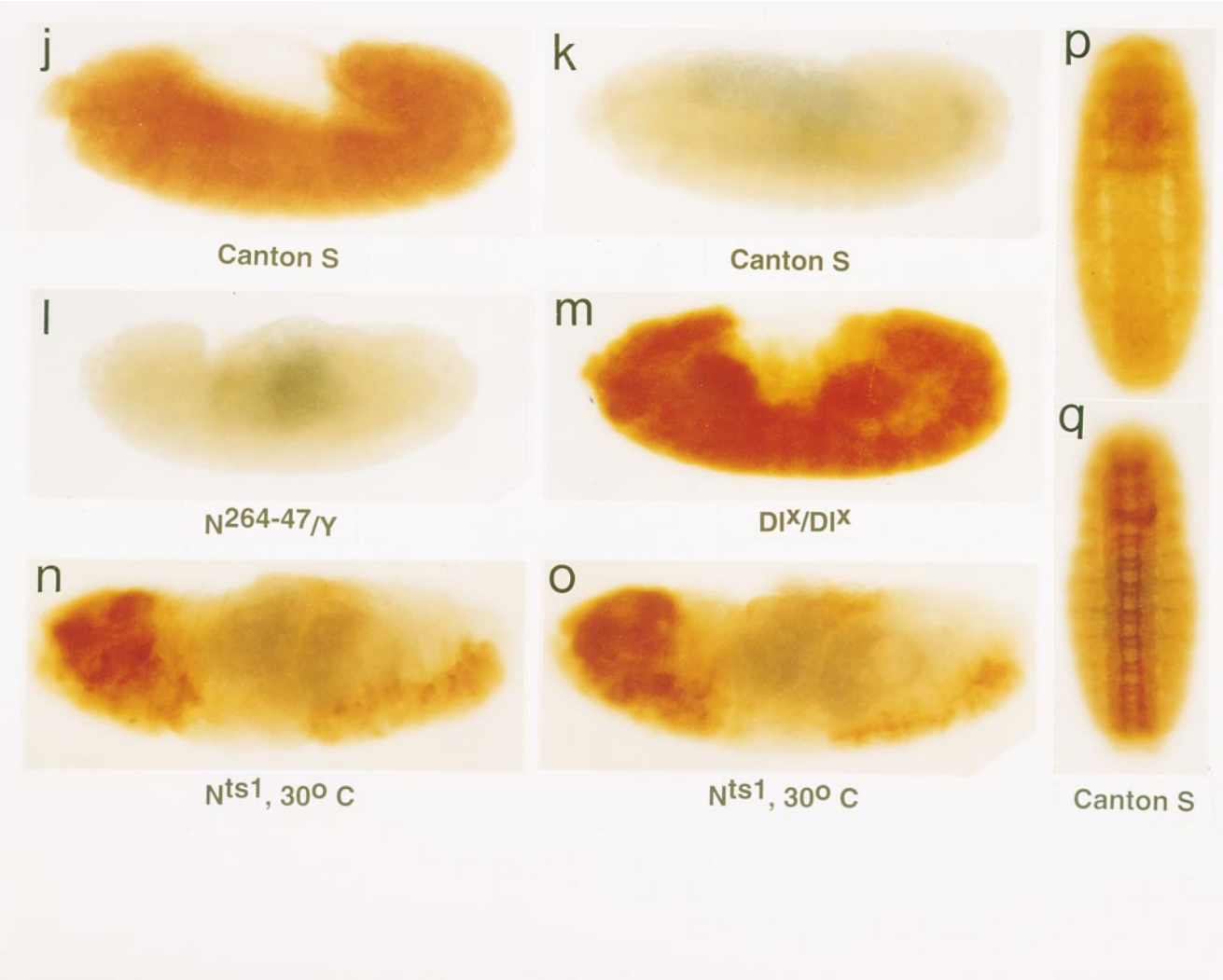

Figure 2. αNPCR, an antibody made against the intracellular sequence carboxy-terminal of the CDC10/Ankyrin repeats, does not recognize N enriched in certain cells and tissues of the developing Canton S embryos. (a–i) Immunostained embryos showing that αN203 detects a form of N enriched in the segregating neuroblasts and the commissures and connectives of the CNS (b, c, e, and g), but αNPCR does not (a, d, and f). αN203 and αNPCR show similar staining patterns at other stages of embryogenesis (compare h [treated with αNPCR] with i [treated with αN203]). (j–q) Immunostained embryos showing that αNPCR recognizes N in the developing embryos: embryos stained using mouse serum (instead of αNPCR made in mouse) failed to give any signals (k), while αNPCR gave signals as expected (j); the neurogenic $N^{264\text{-}47}/Y$ embryos, which have lost N expression, failed to give signals with αNPCR (l) but the neurogenic Dl^x/Dl^x embryos, which express N, gave strong signals with αNPCR (m); and N^{ts1} embryos, which produce low levels of N when reared at the restrictive temperature of 30°C, gave patchy signals with αNPCR (n and o). (p and q) Not only αN203, but also anti-HRP labels the commissures and connectives of the CNS (q) but not αNPCR (p). Embryos a–i and p and q are Canton S embryos; genotypes of others are indicated below the embryos. (a–e) Stage 8-9 embryos; (f, g, p, and q) about stage 15 embryos; (h and i) about stage 10 embryos; (j–o) about stage 12 embryos.

expected) but not by αNPCR (Fig. 3 b) indicating that non-recovery of NΔCterm with αNPCR is due to absence, rather than masking, of αNPCR epitopes.

The absence of αNPCR epitopes and the faster SDS-PAGE migration (compared with NFull containing the αNPCR epitopes) suggested that NΔCterm lacked the carboxyl terminus sequence. To determine whether N molecules truncated to remove the carboxyl terminus αNPCR epitope region migrate alongside NΔCterm in SDS-PAGE, and to get a rough estimate of how much of the carboxyl terminus region is lost in NΔCterm, the following cell lines were generated: S2-$N^{1–2155}$ cells producing N molecules truncated after amino acid 2,155, immediately after the CDC10/Ankyrin repeats, and S2-$N^{1–2262}$ cells producing N molecules truncated after amino acid 2262. Extracts from these cells were separated in SDS-PAGE alongside extracts from embryos, from S2 cells expressing N, and from S2 cells expressing N^{60g11}. N^{60g11} is N protein produced from the mutant N^{60g11} allele. N^{60g11} contains a frame shift mutation that results in deletion of the intracellular region carboxy-terminal of amino acid 2,123 (580 amino acids are deleted and 19 random amino acids added before termination; Lyman and Young, 1993). Western blotting analysis showed that NΔCterm migrates alongside $N^{1–2155}$ and

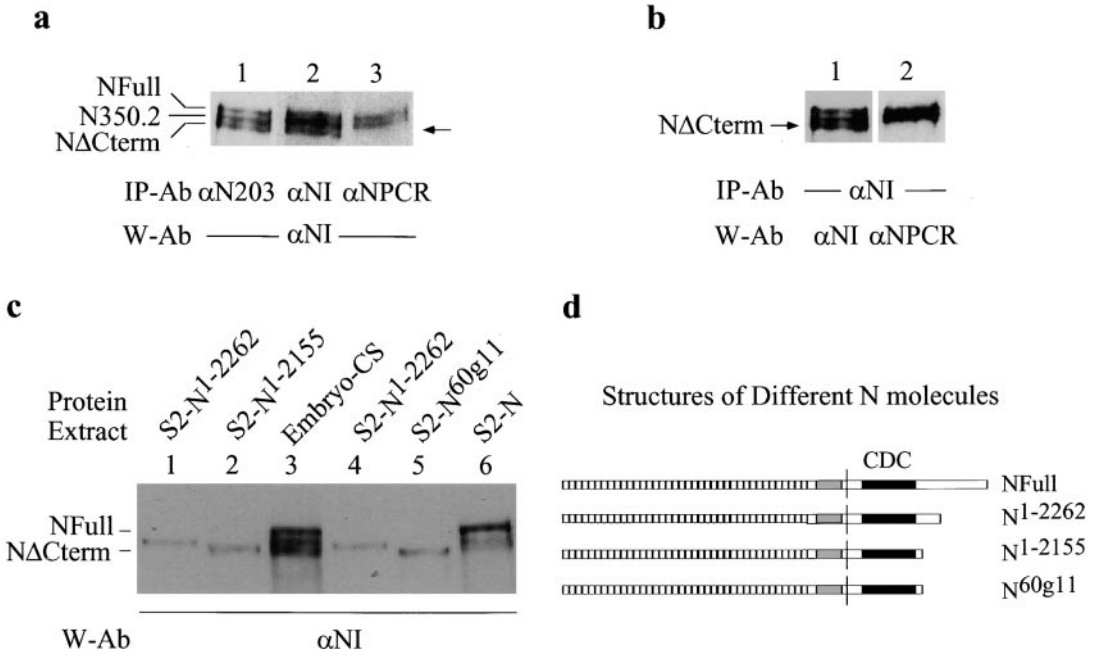

Figure 3. A form of N lacking sequence carboxy-terminal of the CDC10/Ankyrin repeats is present in Canton S embryos. (a) Autoradiographs of Western blots showing that αNPCR does not immunoprecipitate NΔCterm expressed in Canton S embryos. Protein extract was prepared from 0–2-h embryos (which produce relatively equal levels of different forms of N), split into three aliquots for immunoprecipitation with αN203, αNI, and αNPCR antibodies. Arrow points to NΔCterm missing in the αNPCR immunoprecipitate. The three lanes are adjacent lanes in the same blot, but lane 1 is exposed to film for a longer period of time than lanes 2 and 3. (b) Autoradiographs of Western blots showing that NΔCterm is not detected by αNPCR even when it is present in Western blots. N in 0–2-h Canton S embryonic extract was immunoprecipitated by αNI (which immunoprecipitates all three forms of N), electrophoresed in separate lanes, and the blots probed with the indicated antibodies. Note absence of signal from NΔCterm in αNPCR lane. (c) Autoradiograph of a Western blot showing that N truncated for intracellular sequence carboxy-terminal of the CDC10/Ankyrin repeats migrate alongside NΔCterm in SDS-PAGE. Note that $N^{1–2155}$ (lacking 548 amino acids) and N^{60g11} (lacking 560 amino acids) migrate alongside NΔCterm, whereas NFull (the full-length 2,703–amino acid-long protein) and $N^{1–2662}$ (lacking 441 amino acids) migrate slower than $N^{1–2155}$, N^{60g11}, and NΔCterm. NFull expressed in S2 cells and embryos show similar SDS-PAGE mobilities (compare NFull in lane 3 with lane 6). (d) Structures of different N molecules used in c. All N forms shown migrate in the 350-kD range. IP-Ab, antibody used for immunoprecipitations; W-Ab, antibody used in Western blot analysis.

N^{60g11}, but faster than $N^{1–2262}$ (Fig. 3 c). The migration of all N molecules in SDS-PAGE reflected the size of truncation in the carboxyl terminus (see diagram in Fig. 3 d). A difference in mobility due to a difference of ~107 amino acids (in ~2,300 amino acids) is clearly apparent in SDS-PAGE (Fig. 3 c, lanes 1, 2, 4, and 5). Thus, NΔCterm is not recognized by αNPCR because it is truncated for ~500 amino acids in the carboxyl terminus and therefore lacks the αNPCR epitope region. The nature of differences between N350.2 and NFull and between N350.2 and NΔCterm are presently unknown. The slowest migrating ~350-kD form is named NFull because it appears to contain the complete sequence; the fastest migrating ~350-kD form is called NΔCterm because it lacks the carboxyl terminus (half of the intracellular domain); and the form migrating between NFull and NΔCterm is named N350.2 because it is the second of three forms in the ~350-kD range.

NFull, N350.2, and NΔCterm are colinear N molecules as they are recognized by an amino terminus antibody (αN203) and at least one of the intracellular antibodies (αNI and αNPCR) in SDS-PAGE–based Western blot analysis. Therefore, these colinear forms may be substrates of Kuzbanian or Furin-like Convertase enzymes for production of heterodimeric cell surface molecules as proposed by Pan and Rubin (1997), Blaumueller et al. (1997), and Logeat et al. (1998). Our data related to activities of NFull and NΔCterm do not distinguish between the colinear and the proposed heterodimeric forms of the receptors. Therefore, NFull and NΔCterm would refer to the colinear receptors on Western blots but to both the colinear and the proposed heterodimeric receptors with regard to activities. N, without any numbers, acronyms, or abbreviated names, will be used to refer to the N protein in general (inclusive of all forms). The proposed or inferred structures of the various forms of N referred to in this study and the caveats, if any, associated with inference of their structures or usage of names are shown in Fig. 1 b.

NΔCterm Is Associated with Delta during Embryogenesis

Anti-Dl immunoprecipitations were performed from different stages of embryos to determine whether NΔCterm is associated with Dl during embryogenesis. Embryos laid by circadian cycle entrained adult flies were used to minimize age variance and maximize chances for detection of any developmental stage-specific recovery of different forms of N. Proteins interacting at the cell surfaces were

cross-linked, and the complexes immunoprecipitated by anti-Dl antibody were analyzed with antibodies made against different regions of N. The cross-linking/immunoprecipitation procedure employed recovers only complexes of proteins known to interact at cell surfaces during *Drosophila* embryogenesis (Wesley, 1999).

The monoclonal anti-Dl antibody used here (mAb 202, Fehon et al., 1990) does not recover detectable levels of NFull in the absence of cross-linkers (Fig. 4 a, lanes 1 and 2; see also Wesley, 1999). It does not recover even the intracellular and extracellular fragments of the proposed heterodimeric NFull (data not shown). This may be due to disruption of N-Dl complexes when cells are lysed for immunoprecipitation (Fehon et al., 1990; Wesley, 1999) or due to inefficient recovery of NFull by this anti-Dl antibody. Also, none of the anti-N antibodies produced in our laboratory (six have been tested), nor anti-Dl antibodies tested, detect or recover significant levels of the intracellular domain of the proposed heterodimeric NFull receptor, either from cultured cells expressing N or wild-type embryos (Kidd et al., 1998; Wesley, 1999). Significant levels of a 250–300-kD N extracellular fragment is detected in Western blots or immunoprecipitations with antibodies made against the extracellular domain (Wesley, C.S., personal observation). The ~180-kD extracellular domain fragment described in Blaumueller et al. (1997) is not detected by these extracellular N antibodies. We do not know the reason for this. Failure to recover the proposed intracellular domain of the heterodimeric NFull receptor may be due to the fact that most NFull molecules in these cells or embryos are nonfunctional (see Struhl and Adachi, 1998; Schroeter et al., 1998). In the cross-linking/immunoprecipitation procedure employed here, the intracellular and extracellular fragments composing the heterodimeric cell surface receptor are expected to be cross-linked along with the ligand.

N immunoprecipitated by anti-Dl antibody from 0- to 3-h embryonic extracts was recognized by αNPCR, αNI, and αNT (the last antibody was made against the first two EGF-like repeats, Kidd et al., 1989; see Fig. 1 for their epitope regions), indicating that N in these complexes contains all domains of N (Fig. 4 a, lanes 5, 7, 9). N immunoprecipitated from 3–6-h embryonic extracts was recognized by αNI and αNT, but not by αNPCR (Fig. 4 a, lanes 6, 10, 8) suggesting that this form of N is not recognized by αNPCR. Immunoprecipitation in the absence of cross-linkers, or without the anti-Dl antibody, failed to recover any N containing complexes (Fig. 4 a, lanes 1–4), indicating that the complexes recovered in these experiments contained both N and Dl. Recognition of N by αNPCR in one extract and not in the other (when both were extracted at the same time, with the same procedure) ruled out any experimental variation influencing antibody recognition and indicated that N molecules in the two complexes are indeed different.

Western blot analysis of a 3-h interval sampling of proteins showed that while NFull was the predominant form in 0–3-h embryos, it was expressed at very low levels in the 3–6-h-old embryos (Fig. 4 b, lanes 1 and 2; N350.2 and NΔCterm are present at similar levels in 3–6-h extracts and migrate close to each other in 4.25% SDS-PAGE gels). This suggested that the form of N associated with Dl

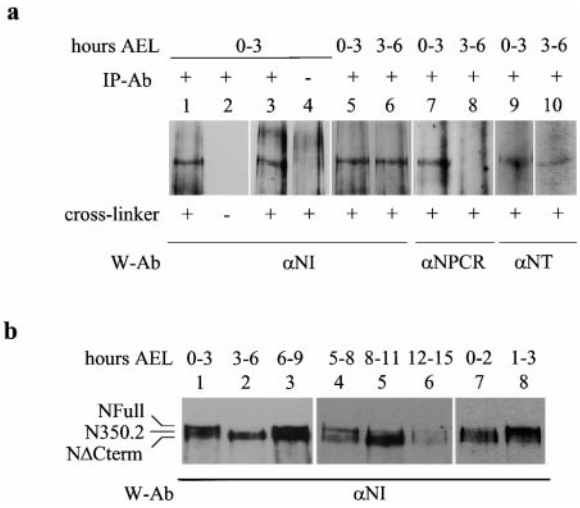

Figure 4. NΔCterm is associated with Dl during embryogenesis. (a) Autoradiographs of Western blots showing the form of N complexed with Dl in 3–6-h Canton S embryonic extracts is NΔCterm. Dl containing complexes were immunoprecipitated with mAb 202 anti-Dl antibody (Fehon et al., 1990) and the Western blots probed with the indicated N antibodies. N in the 0–3-h complexes is recognized by αNI (lane 5), αNPCR (lane 7), and αNT (lane 9), but N in the 3–6-h complexes is recognized by αNI (lane 6), αNT (lane 10), and not by αNPCR (lane 8). N was not detected on the blots when the cross-linker was not used (lanes 1 and 2) or when the anti-Dl antibody was not used (lanes 3 and 4; an anti-βGalactosidase monoclonal antibody was used instead). Thus, N complexed with Dl in 3- and 6-h embryonic extracts is NΔCterm. All N/Dl complexes shown migrate in the ~350-kD range. Presence or absence of the carboxyl terminus did not measurably affect the electrophoretic mobility of N/Dl complexes as cross-linking renders the complexes partially resistant to denaturation during SDS-PAGE (see Wesley, 1999). (b) Autoradiographs of Western blots showing that the level of NFull is low in 3- and 6-h and 8–11-h Canton S embryonic extracts (compare lanes 2, 5 with 1, 3, 4). The same amount of total proteins (quantitated by absorbance values at 280 nM and the BioRad D_C protein assay kit) was loaded in all the lanes. Note similar levels of N350.2 and NΔCterm in lanes 1 and 2. The two blots are exposed to film for different periods. 4.25% SDS-PAGE gels were used for all Western blots. N containing complexes and N forms shown migrate in the ~350-kD range. IP-Ab, antibody used for immunoprecipitations; W-Ab, antibody used in Western blot analysis; AEL, after egg laying; Cross-linker, Bis(sulfosuccinimidyl) suberate (Pierce).

in 0–3-h embryos is NFull and the form of N associated with Dl in 3–6-h embryos is NΔCterm. The form of N associated with Dl in 0–3-h embryos is unlikely to be N350.2 (which is also recognized by αNPCR) because it is present at equivalent levels in both 0–3- and 3–6-h embryos (see Fig. 4 b) and would have been recovered from both embryos if it associated with Dl. The low level of NFull in 3–6-h embryos and the association of Dl with NΔCterm in embryos of the same age are consistent with the observations that a form of N not recognized by αNPCR is enriched in 3–6-h embryos (Fig. 2, a–e) and that both N and Dl are required for neurogenesis after lateral inhibition (Giniger et al., 1993; Giniger, 1998). The majority of embryos in the 3–6-h sample will be between 4 to 5 h of devel-

opment at 25°C and past the lateral inhibition stage (see Campos-Ortega and Hartenstein, 1985). The low levels of NFull in 3–6-h embryos also indicate that most of αNPCR signals in embryos at these stages (Fig. 2, a and d) derive from N350.2 and N200. N200 is a form of N lacking >18 amino-terminal EGF-like repeats (thereby the Dl-binding region) and associates with Wingless during embryogenesis (Wesley, 1999).

Fig. 4 b reveals an interesting feature of N: the level of NFull appears to fluctuate significantly in relation to levels of N350.2 and NΔCterm. The depletion of NFull in 3–6-h embryos is not due to depletion of maternal contribution. Zygotic contribution appears to start at ~1.5 h of embryogenesis as the level of NFull increases in 1–3-h embryos (Fig. 4 b, lanes 7 and 8). Furthermore, NFull is required for lateral inhibition (Lieber et al., 1993; Struhl et al., 1993; Axelrod et al., 1996; Struhl and Adachi, 1998; Kidd et al., 1998), embryos lacking the zygotically contributed N (but having maternally contributed N) are deficient in lateral inhibition (Cabrera, 1990; Skeath and Carroll, 1992), and N^{60g11} embryos which produce NΔCterm-like protein (at 18°C) but have maternally contributed NFull are still deficient in lateral inhibition (Lyman and Young, 1993; Brennan et al., 1997; Wesley, C.S., unpublished observation). The depletion in levels of NFull is observed again in 8–11-h embryos (Fig. 4 b, lanes 5). Equal amounts of total proteins were loaded in all the lanes of Fig. 4 b. Since 8–11-h embryos express the highest level of N (see Johansen et al., 1989; Kidd et al., 1989), the blot containing lanes 4–6 was exposed to film for a shorter period than the blot containing lanes 1–3. Thus, it appears that NFull is produced intermittently during embryogenesis. If NFull was replenished continuously in embryos, or if the embryos were not synchronized in their development by circadian entrainment, the variation in levels of NFull would not have been apparent.

An Intracellular Fragment of Notch Lacking the Carboxyl Terminus Accumulates when S2 Cells Expressing NFull Are Treated with S2-Dl Cells

To determine whether NΔCterm or the intracellular domain of this cell surface receptor is produced when Dl binds NFull, in vitro experiments were performed with S2-Dl and S2-N cells. N and Dl produced in S2 cells bind each other (Fehon et al., 1990; Rebay et al., 1991). Most of N produced in S2-N cells is NFull (see later and Wesley, 1999). S2 cells (untransfected) and S2-Dl cells do not express N and the *Notch* gene in S2 cells is rearranged (Wesley, C.S., unpublished data; Fehon et al., 1990; Ye et al., 1999).

S2-N cells were treated with S2-Dl cells or S2 cells, and protein extracts analyzed by Western blotting with αNI and αNPCR antibodies. S2-N cells treated with S2-Dl cells for 2 h accumulated higher levels of a ~120-kD fragment (designated N^{intra}) and a ~55-kD fragment (designated $N\Delta Cterm^{TMintra}$ that are recognized by αNI (Fig. 5 a, lanes 1 and 2; see later for the basis for these names). The same blot probed with αNPCR recognized N^{intra} but not $N\Delta Cterm^{TMintra}$ (Fig. 5 a, lanes 3 and 4). N^{intra}, recognized by both αNI and αNPCR (see Fig. 5 a, lanes 2 and 4), is the full-length N intracellular domain. It migrates alongside the non-membrane–tethered $N^{intra1790}$ (Fig. 5 b). Both N^{intra} and $N\Delta Cterm^{TMintra}$ were not recognized by any of the extracellular domain antibodies (data not shown). Since $N^{intra1790}$ is rapidly depleted in cells, S2-N cells in this experiment were treated with Dl for only 45 min so that comparable levels of N^{intra} and $N^{intra1790}$ were obtained. $N\Delta Cterm^{TMintra}$ was not observed in this experiment as its accumulation requires ~2 h. These experiments did not show accumulation of NΔCterm (data not shown).

The ~120-kD N^{intra} produced in response to Dl is most likely the ~120-kD N intracellular domain that accumulates in embryos in a Dl-dependent manner (Kidd et al., 1998; Struhl and Greenwald, 1999; Ye et al., 1999). We have therefore tentatively designated it N^{intra} (see Fig. 1 b). $N\Delta Cterm^{TMintra}$ is not recognized by αNPCR, just like NΔCterm. Mobility in SDS-PAGE indicates that $N\Delta Cterm^{TMintra}$ lacks ~500 amino acids in the carboxyl terminus of the intracellular domain, also like NΔCterm. Expression of $N^{intra1790}$ fails to produce $N\Delta Cterm^{TMintra}$ (Fig. 5 b). Longer expression periods, longer exposure to film, or expression of membrane-tethered N^{intra} failed to show even a trace of $N\Delta Cterm^{TMintra}$ or smaller molecules (not shown). These observations strongly suggest that $N\Delta Cterm^{TMintra}$ is not derived from N^{intra} but derived from the full-length N molecules also present in the cells. The N segment from the amino terminus of the transmembrane domain to the carboxyl terminus of the CDC10/Ankyrin repeats (amino acids 1,745–2,145) would be ~45 kD. The size of ~55 kD for $N\Delta Cterm^{TMintra}$ suggests that it contains the transmembrane domain. Therefore, we have tenta-

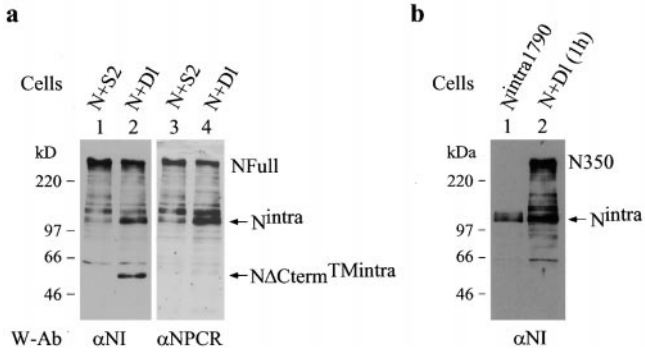

Figure 5. A N intracellular fragment lacking the carboxyl terminus accumulates in S2-N cells treated with Dl. (a) Autoradiographs of a Western blot showing that S2-N cells treated with S2-Dl cells accumulate a 55-kD fragment, $N\Delta Cterm^{TMintra}$, recognized by αNI (lanes 1 and 2) but not by αNPCR (lanes 3 and 4). A 120-kD fragment, N^{intra}, also accumulates in response to Dl (lanes 1 and 2) but is recognized by both αNI and αNPCR (lanes 2 and 4). S2-N cells were treated with S2-Dl cells for 2 h. $N\Delta Cterm^{TMintra}$ can be detected after 2 h of treatment and variably detected after 3 h of treatment. On the other hand, N^{intra} can be detected within half an hour of treatment and up to ~5 h of treatment. (b) Autoradiograph of a Western blot showing that N^{intra} migrates alongside the non-membrane–tethered $N^{intra1790}$. As expression of $N^{intra1790}$ rapidly declines compared with expression of N, S2-N cells here were treated with S2-Dl cells for only 1 h (and therefore $N\Delta Cterm^{TMintra}$ is not observed). 8% SDS-PAGE gels were used for the blots. W-Ab, antibody used in Western blot analysis.

tively designated it NΔCterm^{TMintra} (see Fig. 1 b). As the cell surface N molecules are proposed to be a heterodimers of the extracellular domain and the intracellular domain (Blaumueller et al., 1997; Pan and Rubin, 1997; Logeat et al., 1998), NΔCterm^{TMintra} could very well be the intracellular domain of heterodimeric NΔCterm receptor. In all experiments with S2 cells, the N extracellular domain (N^{extra}) detected by our antibodies (i.e., the ~250–300-kD fragment) did not enrich in response to Dl although its level relative to NFull increased (data not shown).

NΔCterm Promotes Expression of daughterless in Response to Dl

The staining pattern shown in Fig. 2 indicates that NΔCterm is involved in development of commissures and connectives of the CNS. This raised the possibility that NΔCterm might function as a receptor for Dl. We examined this possibility in cultured cells. S2-N cells express NFull, whereas S2-N^{60g11}, S2-N$^{1–2155}$, and S2-N$^{1–2262}$ express NΔCterm-like receptors (see Fig. 3 c). All N molecules have the complete extracellular domain and form aggregates with S2-Dl cells indicating that they bind Dl (data not shown; see Rebay et al., 1991). We treated S2-N, S2-N^{60g11}, S2-N$^{1–2155}$, and S2-N$^{1–2262}$ with S2-Dl cells and assayed RNA extracted from these cells for expression of numerous genes known to interact genetically with *Notch* and *Delta*. The RNAs of *Achaete Scute Complex*, *Enhancer of Split Complex*, and *wingless* were not detected in our experiments. RNA of many other genes were not responsive to Dl treatment. However, the expression of the *daughterless* (*da*) gene was responsive to NΔCterm-like receptors. Expression of both NFull and NΔCterm-like receptors in S2 cells suppressed *da* expression (Fig. 6 a, lanes 1–3, 8, and 10). This indicated that the presence or absence of sequence carboxy-terminal of the CDC10/Ankyrin repeats per se does not affect *da* expression. Treatment of NΔCterm-like receptors with Dl promoted accumulation of *da* RNA, while treatment of NFull did not (Fig. 6 a, lanes 2–5 and 8–11). Another comparison of NFull and NΔCterm-like receptor, N^{60g11}, treated with Dl is shown in Fig. 6 a,

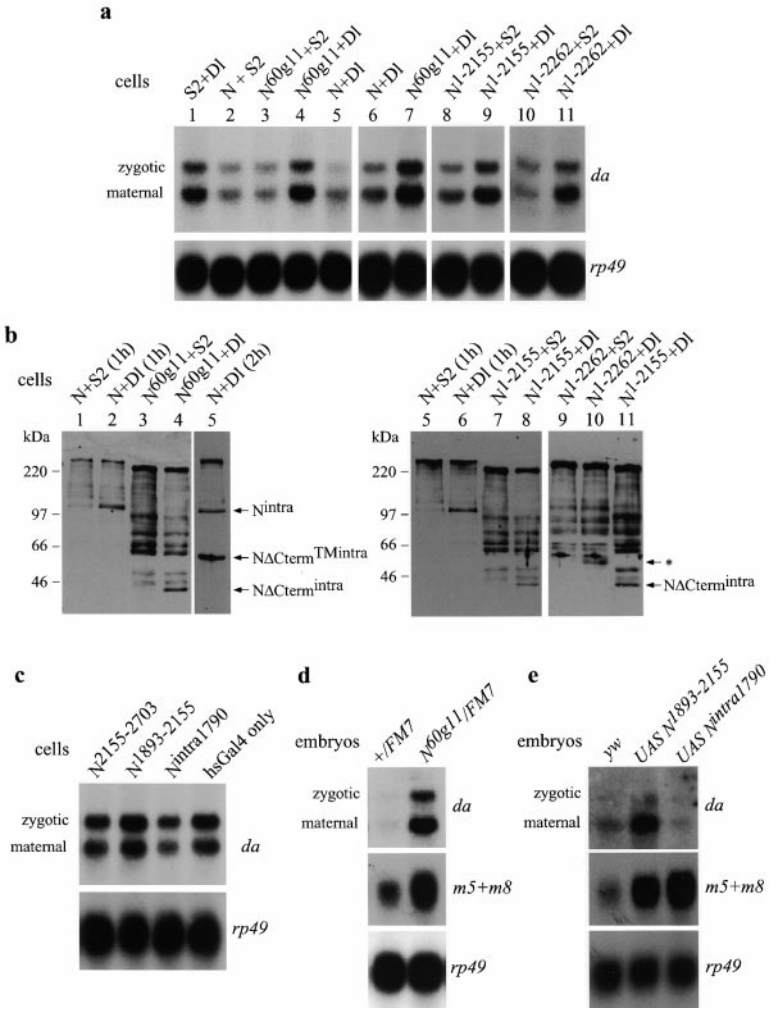

Figure 6. NΔCterm-like receptors promote expression of *da*. (a) Autoradiographs of Northern blots showing that aggregates of S2 cells expressing Dl and NΔCterm-like receptors promote expression of *da* (lanes 3, 4, 7–11) but not aggregates of S2 cells expressing Dl and the full-length N (lanes 2, 5, and 6). *da* expression in S2 cells is equivalent to that in S2-Dl cells (not shown). See Caudy et al., 1988b, for description of the two (maternal and zygotic) transcripts of *da*. (b) Autoradiograph of a Western blot showing that S2 cells expressing NΔCterm-like receptors, N^{60g11} and N$^{1–2155}$, treated with S2-Dl cells accumulate a 40-kD fragment, NΔCterm^{intra} (lanes 3, 4, 7, and 8). Expression of NΔCterm-like receptors rapidly declines. Therefore, the treatments were performed for only 1 h. S2-N cells treated with S2-Dl cells (for 1 h) are shown for the sake of comparison (lanes 1, 2, 5, and 6). S2-N cells treated with S2-Dl cell for 2 h is shown in lane 5. The proteolytic cleavage responsible for production of NΔCterm^{intra} occurs amino-terminal of the CDC10/Ankyrin repeats because S2-N$^{1–2262}$ cells treated with S2-Dl cells accumulate a 52–55-kD fragment (marked with an asterisk) instead of the 40-kD NΔCterm^{intra} accumulated by S2-N$^{1–2155}$ cells (lanes 9–11). αNI was used to detect N molecules on the blot. 8% SDS-PAGE gels were used to prepare the blots. (c) Autoradiograph of a Northern blot showing that N$^{1893–2155}$ promotes expression of *da* in S2 cells but not N^{intra1790} (lanes 2 and 3); the carboxyl terminus fragment, N$^{2155–2703}$, weakly suppresses expression of *da* (lane 1). (d) Autoradiograph of a Northern blot showing that N^{60g11}-expressing embryos (*N^{60g11}/FM7 lac-Z*) overproduce *da* and *E(spl)C* (*m5 and m8*) RNA when compared with embryos expressing only wild-type N (+/FM7 lac-Z). RNAs were extracted from embryos reared at 18°C. (e) Autoradiograph of a Northern blot showing that UAS N$^{1893–2155}$ expressed in embryos promotes expression of both *da* and *E(spl)C* (*m5 and m8*) RNA, whereas UAS N^{intra1790} promotes expression of only *E(spl)C* (*m5 and m8*) RNA. *yw*, the genetic background of UAS N$^{1893–2155}$ and UAS N^{intra1790} carrying flies, is used as control. All flies were crossed to hsGAl4 lines and RNA extracted from the resultant embryos. 20 μm RNA was loaded in each lane. In all Northern blots, *rp49* gene was used to show relative levels of total RNA.

lanes 6 and 7. Non-response of *da* to NFull receptor is consistent with the observation that mammalian full-length Notch suppresses the activity of a *da* related gene in mammalian cell lines (Ordentlich et al., 1998). The dependence on Dl for promotion of *da* expression indicated involvement of a ligand-induced, activated N intracellular molecule for signal transduction from the NΔCterm-like receptors.

To identify the ligand activated signaling molecule of NΔCterm-like receptors, Western blot analysis was performed after treatment of S2-N^{60g11} and S2-$N^{1–2155}$ cells with S2-Dl cells. These two N molecules are indistinguishable in Western blots (differing in length by only 12 amino acids). The cells were treated for only 1 h as the expression of N^{60g11} and $N^{1–2155}$ declines rapidly. The results show that a ~40-kD intracellular molecule, designated NΔCterm^{intra}, accumulates in S2-N^{60g11} and S2-$N^{1–2155}$ cells in response to Dl, and the expected N^{intra} accumulates in S2-N cells (Fig. 6 b, lanes 1–8; see later for the basis for the name NΔCterm^{intra}). S2-$N^{1–2262}$ cells treated with S2-Dl cells do not accumulate the ~40-kD molecule but instead accumulate a 52–55-kD molecule (Fig. 6 b, lanes 9–11, see band marked with an asterisk). The 12–15-kD size difference between this molecule and NΔCterm^{intra} is approximately the difference between the carboxyl termini of $N^{1–2155}$ and $N^{1–2262}$. This indicates that NΔCterm^{intra} is produced by a proteolytic cleavage amino-terminal of the CDC10/Ankyrin repeats. The ~40-kD size suggests that NΔCterm^{intra} does not contain the transmembrane domain. Since this molecule is produced in response to Dl, just like N^{intra} from NFull, we have tentatively designated it NΔCterm^{intra} (see Fig. 1 b).

NΔCterm^{intra} is closest in size to the ~35-kD intracellular fragment containing just the CDC10/Ankyrin repeats, $N^{1893–2155}$ (data not shown) suggesting that the CDC10/Ankyrin repeats, with little flanking sequence, transduces the signals from NΔCterm. If just the CDC10/Ankyrin repeats fragment is the activated signaling molecule associated with NΔCterm receptor, then $N^{1893–2155}$ was expected to promote expression of *da* in the absence of Dl. We tested this expectation. Results show that $N^{1893–2155}$ indeed promotes *da* expression in S2 cells in the absence of Dl, while $N^{intra1790}$, just like N, does not (Fig. 6 c). In several repetitions of the experiment, expression of *da* in S2-$N^{1893–2155}$ cells was consistently higher than in the control cells (S2 cells transfected with hsGal4 only) and always lower in S2-$N^{intra1790}$ cells. Expression of the N intracellular sequence carboxy-terminal of the CDC10/Ankyrin repeats, $N^{2155–2703}$, does not suppress *da* expression as strongly as $N^{intra1790}$ (Fig. 6 c).

Next, we examined whether NΔCterm-like receptor, N^{60g11}, and $N^{1893–2155}$ increase *da* expression in vivo. Northern blot analysis of RNA extracted from *N^{60g11}* embryos showed that overexpression of the NΔCterm-like receptor results in overproduction of *da* RNA (Fig. 6 d). As observed in S2 cells, expression of $N^{1893–2155}$ in embryos promotes expression of *da*, while expression of $N^{intra1790}$ does not (Fig. 6 e). As embryos in an early stage of embryogenesis were used here, only the expression of the maternal transcript is prominent.

Struhl and Adachi (1998) have shown that molecules like $N^{1893–2155}$ ($N^{CDC10MYR-NLS-}$) localize in the nucleus, rescue epidermal development in Notch^{-} embryos, rescue epidermal development in the absence of Dl, and suppress neuroblast segregations in early Notch^{+} embryos (i.e., antineurogenic effect). These activities are similar to those of N^{intra} and related to expression of *E(spl)C* (see also Roehl and Kimble, 1993; Kidd et al., 1998). This suggested that despite the different effects on *da* expression, both N^{intra} and $N^{1893–2155}$ should overproduce *E(spl)C* RNA. Probing of the same blots with *m5* and *m8* genes of *E(spl)C* shows that these genes are overexpressed in both (Fig. 6 e). As expected, *m5* and *m8* genes of *E(spl)C* are also overexpressed in N^{60g11} embryos (Fig. 6 d).

The differential response of *da* and the *E(spl)C* might be due to expression of N^{60g11} and $N^{1893–2155}$ in both neuronal and epidermal precursor cells, and expression of $N^{intra1790}$ only in epidermal precursor cells (see Fig. 2). Only the neuronal precursor cells increase *da* expression during embryogenesis (Vaessin et al., 1994). However, the differential expression could be also due to NΔCterm promoting *da* expression and not the full-length N (as in S2 cells). Activation of *E(spl)C* by NΔCterm may have come about through the proneural genes rather than through lateral inhibition signaling (see Discussion). Thus, it is possible that genes like *da* are responsive to signals from NΔCterm, not from NFull, and genes like *m5* and *m8* of *E(spl)C* are responsive to signals from both receptors.

nd^3 Embryos Overproduce NΔCterm and Related Molecules

nd^3 is a temperature-sensitive, homozygous viable allele of *N* (Shellenbarger and Mohler, 1975) with an amino acid replacing point mutation in the EGF-like repeat 2 (Lyman and Young, 1993). In a screen of *Notch* mutants, we discovered that *nd^3* embryos reared at 25°C accumulate higher levels of a form of N that is recognized by αNT and αNI but not by αNPCR, which migrates close to the full-length form (Fig. 7 a). 4% SDS-PAGE gels were used here as N that are recognized by αNPCR (NFull and N350.2), migrate together in these gels and the levels of NΔCterm can be unambiguously determined. Embryos heterozygous or hemizygous for the null allele, *N^{264-47}*, the homozygous viable allele, *split*, and several *Abruptex* alleles of *Notch* showed no alteration in levels of NΔCterm (data not shown). The overexpressed form in *nd^3* embryos (25°C) is NΔCterm because: (a) there is no other N molecule in *D. melanogaster* that migrates close to the full-length form and is recognized by αNT and αNI, but not by αNPCR (Wesley, C.S., unpublished data); (b) it is recognized by αNT made against the first two EGF-like repeats indicating that the amino terminus is intact in this form (Fig. 7 a); and (c) αNPCR failed to immunoprecipitate a form of N migrating alongside NΔCterm from *nd^3* embryos (25°C) (expected if the faster mobility is due to a truncation in the amino terminus rather than in the carboxyl terminus, data not shown).

A higher percentage SDS-PAGE analysis of extracts prepared from 25°C reared embryos revealed that ~55- and ~40-kD N intracellular fragments, having the same SDS-PAGE migration properties as NΔCterm^{TMintra} and NΔCterm^{intra} from cultured cells, are also overexpressed in *nd^3* (25°C) embryos (Fig. 7 b). Overexpression of these

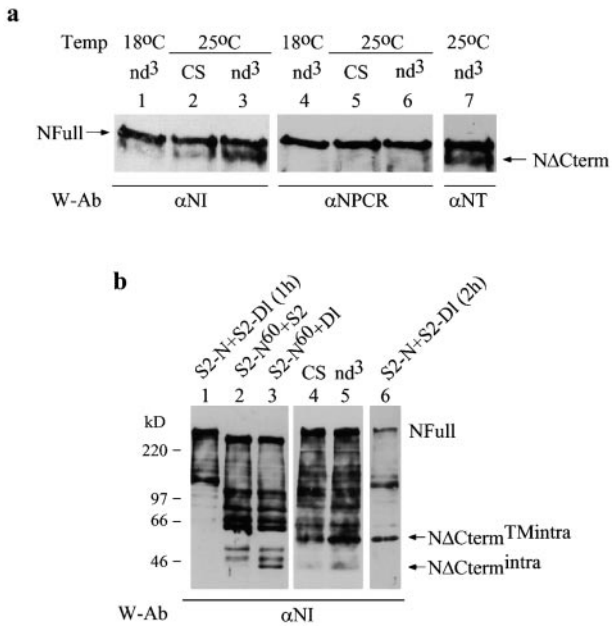

Figure 7. nd^3 embryos overexpress N molecules resembling NΔCterm, NΔCterm^{TMintra}, and NΔCterm^{intra}. (a) Autoradiographs of Western blots showing that nd^3 embryos reared at 25°C overproduce a form of N resembling NΔCterm (lanes 1–3). This form of N, just like NΔCterm, is recognized by αNI and αNT (lanes 3 and 7) but not αNPCR (lane 6). The same amount of total proteins (quantitated by absorbance and BioRad D_C protein assay kit) was loaded in all of the lanes (note similar levels of NFull in all lanes). 0–3-h (25°C) or 0–6-h (18°C) embryos were used. The blot probed with αNI (lanes 1–3) was reprobed with αNPCR (lanes 4–6). Lane 7 is an independent blot. The levels of NFull and NΔCterm in Canton S embryos at 18°C were similar to the levels observed at 25°C (data not shown). 4% gel was used because in these gels NFull and N350.2 migrate together resulting in unambiguous determination of the level of NΔCterm. All N forms shown migrate in the ~350-kD range. (b) Autoradiographs of Western blots showing that nd^3 embryos reared at 25°C also overproduce forms of N resembling NΔCterm^{TMintra} and NΔCterm^{intra}. Lanes 4–6 are from the same blot but lanes 2 and 3 are exposed to film for a shorter period than lane 1. Lanes 1–3 are an independent blot. S2-N in lane 1 was treated for 1 h, whereas S2-N cells in lane 6 were treated for 2 h. The different ~350-kD forms of N do not resolve in 8% gels used for this blot. W-Ab, Western blotting antibody; Temp, temperature at which embryos were reared after heat-shock.

and NΔCterm molecules in nd^3 (25°C) embryos suggest that the processes producing N molecules lacking the carboxyl terminus are interrelated and NΔCterm is the source of NΔCterm^{TMintra} and NΔCterm^{intra}. N^{intra} is not clearly detected in embryonic extracts (see Fig. 7 b, lanes 4–6). This may be because very low amounts of N^{intra} molecules are sufficient to transduce the Dl-mediated lateral inhibition signal in vivo (Schroeter et al., 1998; Struhl and Adachi, 1998; Kidd et al., 1998). The nd^3 experiments indicate that (1) production of N molecules lacking the carboxyl terminus is a normal feature of the *N* gene that can be altered by a point mutation, just like any other functional aspect of a protein, and (2) EGF-like repeat 2 (the site of mutation in the nd^3 allele; Lyman and Young, 1993) regulates production of these truncated molecules. Of no less significance, nd^3 allele provided us with a means to identify the putative in vivo NΔCterm^{TMintra} and NΔCterm^{intra} molecules from among the many minor N molecules generally detected in a N Western blot.

Discussion

Our analysis of N molecules in embryos and S2 cells show the following: (a) whereas the cells undergoing lateral inhibition in the developing embryo are enriched for N molecules recognized by both the amino and carboxyl terminus antibodies, the cells and tissues produced subsequent to lateral inhibition are enriched for N molecules not recognized by the carboxyl terminus antibody (Fig. 2). (b) Correspondingly, Dl forms complexes with the full-length N during lateral inhibition period, and with the N molecule lacking the carboxyl terminus in the period after lateral inhibition (Fig. 4). (c) N molecules lacking the carboxyl terminus (NΔCterm, NΔCterm^{TMintra}, and NΔCterm^{intra}) are produced during embryogenesis (Figs. 3 and 5–7). (d) S2 cells expressing N receptors containing the carboxyl terminus (NFull) treated with S2-Dl cells accumulate an intracellular N molecule lacking the carboxyl terminus, NΔCterm^{TMintra} (Fig. 5). (e) NΔCterm is the most likely substrate for production of NΔCterm^{TMintra} (Figs. 5 and 7). (f) NΔCterm functions as a receptor for Dl, with the NΔCterm^{intra} (comprised mostly of the CDC10/Ankyrin repeats) as its activated signaling molecule, and the *da* gene is responsive to its signals (Fig. 6).

Based on the results summarized above, we propose the following hypothetical model for N functions during embryogenesis. Lateral inhibition starts with NFull receptor containing the full signaling potential. The back and forth lateral inhibition signaling between interacting cells leads to carboxyl terminus processing of the full-length N molecules present inside the cells (i.e., those not involved in Dl binding) and production of the NΔCterm receptors. Cells expressing higher levels of NΔCterm become the neuronal precursor cells and cells expressing higher levels of NFull become the epidermal precursor cells. NFull disappears in neuronal precursor cells and NΔCterm, a secondary receptor with restricted signaling potential, functions during differentiation of the nervous system. Epidermal precursor cells expressing only NFull, or appreciable levels of both NFull and NΔCterm, continue the same process during differentiation of the epidermis. Advance from signaling by NFull to signaling by NΔCterm would mean that those cells have attained a degree of irreversibility in their differentiation process. For example, once NΔCterm becomes the sole N receptor in the neuronal precursor cells, these cells can only proceed along the neuronal differentiation path. N would continuously function in this manner to both specify and restrict cell fates during differentiation of a cell lineage.

NΔCterm would lack the Dishevelled-binding region, one of the Numb-binding regions, the OPA sequence, and the PEST sequence (see Fig. 1 a). Therefore, it is likely that loss of one or more of these features is involved in restricting the differentiation possibilities for a cell. Dishevelled and Numb are known to antagonize Su(H) activities (Axelrod et al., 1996; Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996; Wang et al., 1997). Proteolytic re-

moval of their binding sites is likely to eliminate antagonisms to Su(H) activities and promote activities of facilitators like Deltex. This might contribute to the lateral inhibition process and selection of precursor cells for neuronal fates. On the other hand, production of NΔCterm^{intra} lacking the Su(H)-binding sites from NΔCterm receptor might promote neuronal fates by promoting activities of Hairless or Numb or Achaete (through Daughterless; Schweisguth and Posakony, 1994; Schweisguth, 1995; Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996). It is also possible that Disabled, which functions with N during differentiation of the CNS after lateral inhibition, can signal from NΔCterm and not NFull. Thus, production and functions of NFull and NΔCterm might provide directionality to N functions at successive stages of differentiation. All these properties of NFull, NΔCterm, and the proteins interacting or not interacting with these two receptors, may be involved during differentiation of the adult sensory organ (bristle) wherein Su(H) activity is required for determination of some fates and not others (Schweisguth and Posakony, 1994; Schweisguth, 1995; Wang et al., 1997).

We have no evidence, one way or the other, about involvement of Su(H) in transducing signals from NΔCterm. Regulation of expression of *E(spl)C* genes by NΔCterm seems to indicate that the canonical Su(H)-mediated lateral inhibition pathway is involved. However, *E(spl)C* genes expression could be regulated by an alternate pathway. NΔCterm regulates *da*, not NFull. Daughterless protein, is an activator of proneural proteins (Dambly-Chaudiere et al., 1988; Murre et al., 1989; Cabrera and Alonso, 1991; van Doren et al., 1991) and proneural genes also activate expression of *E(spl)C* (Kramatschek and Campos-Ortega, 1994; Singson et al., 1994). Some differences in the activities of the intracellular domains of NFull and NΔCterm seem very likely. One, the RAM23 region in the intracellular domain of N (see Fig. 1 a) is important for Su(H) activities related to NFull, N^{intra} and lateral inhibition (Fortini and Artavanis-Tsakonas, 1994; Tamura et al., 1995; Matsuno et al., 1997). It appears that NΔCterm^{intra} lacks most of this region, if not all. Two, the sequence carboxy-terminal of the CDC10/Ankyrin repeats is required for transcriptional activation upon binding DNA (Kidd et al., 1998). Since NΔCterm lacks this sequence, it might activate genes indirectly through inactivation of a constitutive repressor or stabilization of RNA. NFull containing the carboxyl terminus would activate genes directly from DNA. Thus, it is possible that NFull and NΔCterm might signal through different pathways with some shared outcomes at certain stages of development, like expression of *E(spl)C* genes. Su(H) might be functioning with both pathways, albeit in different ways.

Production of N receptors with restricted signaling potential may be important for another reason. NFull binds different ligands and regulates different genes in response to them (see Artavanis-Tsakonas et al., 1999; Wesley, 1999). Removal of the carboxyl terminus after initiation of NFull signaling by one ligand might set the cell on a differentiation path specific to that ligand. For example, removal of the carboxyl terminus in neuronal precursor cells after Delta-specific lateral inhibition signaling might make NΔCterm in these cells either unresponsive to Wingless functioning in the epidermis differentiation pathway, or responsive to Wingless in the manner specific to neuronal differentiation pathway. Treatment of full-length N with Wingless results in accumulation of a N molecule lacking the Dl-binding region (Wesley, 1999). This secondary N receptor may be produced during epidermogenesis to eliminate the antagonism to Wingless functions presented by the Dl-binding site. Non-response or pathway-specific response to a second ligand may be necessary for development given the broad overlap in distributions of different N ligands. Thus, expression of a particular secondary N receptor might indicate both the differentiation path taken by a cell and the degree to which this cell has differentiated from cells in the parent population.

The molecular phenotypes of *nd^3* allele suggest that EGF-like repeat 2 might be an important component in the regulation of NΔCterm production during embryogenesis. It seems possible that the EGF-like repeat array of N might include two classes of repeats, one containing repeats that bind ligands outside the cells and the other containing repeats that target Notch for different kinds of processing inside the cell. Such a function for EGF-like repeats might explain why N^{intra} do not produce NΔCterm^{TMintra}. These molecules might lack the appropriate EGF-like repeats to target them to the right place for carboxyl terminus processing. An interesting extension of this possibility is that there are different targeting EGF-like repeats responsive to different ligands.

The regulation of *da* expression by NΔCterm may be significant for embryogenesis. *da* genetically interacts with *Notch* (Brand and Campos-Ortega, 1988, 1990), it is required for development of the nervous system from neuroblasts but not for lateral inhibition (Caudy et al., 1988a,b; Vaessin et al., 1994), and the Daughterless protein promotes DNA-binding activities of the proneural Achaete-Scute Complex proteins (Dambly-Chaudiere et al., 1988; Murre et al., 1989; Cabrera and Alonso, 1991; van Doren et al., 1991). Both NΔCterm and Daughterless protein (Vaessin et al., 1994) accumulate in segregating neuroblasts raising the possibility that NΔCterm is involved in this upregulation of *da* expression. Accordingly, *nd^3* embryos which overproduce NΔCterm also overproduce *da* RNA in the neuroblasts (data not shown).

In the embryo, *da* is expressed at low levels in almost all cells (Murre et al., 1989; CSW, personal observation) but is upregulated in certain cells including the segregating neuroblasts (Vaessin et al., 1994). In our experiments, S2 cells expressing NFull and NΔCterm receptors had lower levels of *da* RNA than S2 cells without N. In response to Dl, only S2-NΔCterm cells increased expression of *da* RNA, but only to the level observed in cells without N (Fig. 6 a). Therefore, it appears possible that with the expression of different forms of N, developing cells acquire an ability to differentially regulate the otherwise constitutive *da* expression. Such differential regulation might be important for suppressing the activities of Achaete-Scute Complex proteins in the developing epidermis where NFull is expected to function, but not in the developing nervous system where NΔCterm is expected to function. Since both N receptors have the ability to activate *E(spl)C*, the timing and sequence of expression of NFull and NΔCterm may also be important for development.

We thank Michael Young for his support of the study; Toby Lieber and Simon Kidd for generous sharing of research materials; and Justin Blau, Umadevi Wesley, and Monica Roth for comments on the manuscript. We are also thankful for the excellent comments and suggestions of two anonymous reviewers.

This work was supported by National Institutes of Health GM 25103 to Michael Young.

Submitted: 15 September 1999
Revised: 25 February 2000
Accepted: 17 March 2000

References

Artavanis-Tsakonas, S., M.D. Rand, and R.J. Lake. 1999. Notch signaling: cell fate control and signal integration in development. *Science.* 284:770–776.

Axelrod, J.D., K. Matsuno, S. Artavanis-Tsakonas, and N. Perrimon. 1996. Interaction between Wingless and Notch signaling pathways mediated by Dishevelled. *Science.* 271:1826–1832.

Blaumueller, C.M., H. Qi, P. Zagouras, and S. Artavanis-Tsakonas. 1997. Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell.* 90:281–291.

Brand, M., and J.A. Campos-Ortega. 1988. Two groups of interrelated genes regulate early neurogenesis in *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* 197:457–470.

Brand, M., and J.A. Campos-Ortega. 1990. Second-site modifiers of the *split* mutation of *Notch* define genes involved in neurogenesis in *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* 198:275–285.

Brennan, K., R. Tateson, K. Lewis, and A. Martinez-Arias. 1997. A functional analysis of *Notch* mutation in *Drosophila*. *Genetics.* 147:177–188.

Brennan, K., M. Baylies, and A. Martinez-Arias. 1999a. Repression by Notch is required before Wingless signalling during progenitor cell development in *Drosophila*. *Curr. Biol.* 9:707–710.

Brennan, K., R. Tateson, V. Zecchini, T. Lieber, J.P. Couso, V. Zecchini, and A. Martinez-Arias. 1999b. The Abruptex mutations of Notch disrupt the establishment of proneural clusters in *Drosophila*. *Dev. Biol.* 216:230–242.

Cabrera, C.V. 1990. Lateral inhibition and cell fate during neurogenesis in *Drosophila*: the interactions between *scute, Notch* and *Delta. Development.* 109:733–742.

Cabrera, C.A., and M.C. Alonso. 1991. Transcriptional activation by heterodimers of the *achaete-scute* and *daughterless* gene products of *Drosophila*. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2965–2973.

Cagan, R.L., and D.F. Ready. 1989. *Notch* is required for successive cell decisions in the developing *Drosophila* retina. *Genes Dev.* 3:1099–1112.

Campos-Ortega, J.A., and V. Hartenstein. 1985. The embryonic development of *Drosophila melanogaster*. Springer-Verlag, New York. 227 pp.

Caudy, M., E.H. Grell, C. Dambly-Chaudiere, A. Ghysen, L.Y. Jan, and Y.N. Jan. 1988a. The maternal sex determination gene *daughterless* has zygotic activity necessary for the formation of peripheral neurons in *Drosophila*. *Genes Dev.* 2:843–852.

Caudy, M., H. Vassin, M. Brand, R. Tuma, L.Y. Jan, and Y.N. Jan. 1988b. *daughterless*, a *Drosophila* gene essential for both neurogenesis and sex determination, has sequence similarities to *myc* and the *achaete-scute* complex. *Cell.* 55:1061–1067.

Couso, J.P., and A. Martinez-Arias. 1994. *Notch* is required for *wingless* signaling in the epidermis of *Drosophila*. *Cell.* 79:259–272.

Dambly-Chaudiere, C., A. Ghysen, L.Y. Jan, and Y.N. Jan. 1988. The determination of sense organs in *Drosophila*: interaction of *scute* with *daughterless*. *Roux's Arch. Dev. Biol.* 197:419–423.

Diederich, R.J., K. Matsuno, H. Hing, and S. Artavanis-Tsakonas. 1994. Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. *Development.* 120:473–481.

Fehon, R.G., P.J. Kooh, I. Rebay, C.L. Regan, T. Xu, M. Muskavitch, and S. Artavanis-Tsakonas. 1990. Molecular interaction between the protein products of the neurogenic loci *Notch* and *Delta*, two EGF-homologous genes in *Drosophila*. *Cell.* 61:523–534.

Fehon, R.G., K. Johansen, I. Rebay, and S. Artavanis-Tsakonas. 1991. Complex cellular and subcellular regulation of *Notch* expression during embryonic and imaginal development of *Drosophila*: implications for *Notch* function. *J. Cell Biol.* 113:657–669.

Fortini, M.E., and S. Artavanis-Tsakonas. 1994. The suppressor of Hairless protein participates in Notch receptor signaling. *Cell.* 79:273–282.

Frise, E., J.A. Knoblich, S. Younger-Shepherd, L.Y. Jan, and Y.N. Jan. 1996. The *Drosophila* Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage. *Proc. Natl. Acad. Sci. USA.* 93:11925–11932.

Giniger, E. 1998. A role for Abl in Notch signaling. *Neuron.* 20:667–681.

Giniger, E., L.Y. Jan, and Y.N. Jan. 1993. Specifying the path of the intersegmental nerve of the *Drosophila* embryo: a role for *Delta* and *Notch*. *Development.* 117:431–440.

Guo, M., L.Y. Jan, and Y.N. Jan. 1996. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron.* 17:27–41.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 726 pp.

Hoppe, P.E., and R.J. Greenspan. 1990. The *Notch* locus of *Drosophila* is required in epidermal cells for epidermal development. *Development.* 109: 875–885.

Johansen, K.M., R.G. Fehon, and S. Artavanis-Tsakonas. 1989. The *Notch* gene product is a glycoprotein expressed on the cell surface of both epidermal and neuronal precursor cells during *Drosophila* development. *J. Cell Biol.* 109: 2427–2440.

Kidd, S., M.R. Kelly, and M.W. Young. 1986. Sequence of the Notch locus of *Drosophila melanogaster*: relationship of the encoded protein to mammalian clotting and growth factors. *Mol. Cell. Biol.* 6:3094–3108.

Kidd, S., M.K. Baylies, G.P. Gasic, and M.W. Young. 1989. Structure and distribution of the Notch protein in developing *Drosophila. Genes Dev.* 3:1113–1129.

Kidd, S., T. Lieber, and M.W. Young. 1998. Ligand induced cleavage and regulation of nuclear entry of Notch in *Drosophila melanogaster* embryos. *Genes Dev.* 12:3728–3740.

Kooh, P.J., R.G. Fehon, and M.A.T. Muskavitch. 1993. Implications of dynamic patterns of Delta and Notch expression for cellular interactions during *Drosophila* development. *Development.* 117:493–507.

Kramatschek, B., and J.A. Campos-Ortega. 1994. Neuroectodermal transcription of the *Drosophila* neurogenic genes *E(spl)* and *HLH-m5* is regulated by proneural genes. *Development.* 120:815–826.

Lieber, T., S. Kidd, E. Alcamo, V. Corbin, and M.W. Young. 1993. Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. *Genes Dev.* 7:1949–1965.

Logeat, F., C. Bessia, C. Brou, O. LeBail, S. Jarriault, N.G. Seidah, and A. Israel. 1998. The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc. Natl. Acad. Sci. USA.* 95:8108–8112.

Lyman, D., and M.W. Young. 1993. Further evidence for function of the *Drosophila* Notch protein as a transmembrane receptor. *Proc. Natl. Acad. Sci. USA.* 90:10395–10399.

Matsuno, K., R.J. Diederich, M.J. Go, C.M. Blaumueller, and S. Artavanis-Tsakonas. 1995. Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development.* 121:2633–2644.

Matsuno, K., M.J. Go, X. Sun, D.E. Eastman, and S. Artavanis-Tsakonas. 1997. Suppressor of Hairless-independent events in Notch signaling imply novel pathway elements. *Development.* 124:4265–4273.

Murre, C., P.S. McCaw, H. Vaessin, M. Caudy, L.Y. Jan, Y.N. Jan, C.V. Cabrera, J.N. Buskin, S.D. Hauschka, A.B. Lassar, et al. 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell.* 58:537–544.

Ordentlich, P., A. Lin, C.-P. Shen, C. Blaumueller, K. Matsuno, S. Artavanis-Tsakonas, and T. Kadesch. 1998. Notch inhibition of E47 supports the existence of a novel signaling pathway. *Mol. Cell. Biol.* 18:2230–2239.

Pan, D., and G. Rubin. 1997. Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during *Drosophila* and vertebrate neurogenesis. *Cell.* 90:271–280.

Rebay, I., R.J. Fleming, R.G. Fehon, L. Cherbas, P. Cherbas, and S. Artavanis-Tsakonas. 1991. Specific repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell.* 67: 687–699.

Roehl, H., and J. Kimble. 1993. Control of cell fate in *C. elegans* by a Glp-1 peptide consisting primarily of ankyrin repeats. *Nature.* 264:632–635.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schroeter, E.H., J.A. Kisslinger, and R. Kopan. 1998. Notch-1 signaling requires ligand-induced proteolytic release of intracellular domain. *Nature.* 393:382–386.

Schweisguth, F. 1995. *Suppressor of Hairless* is required for signal reception during lateral inhibition in the *Drosophila* pupal notum. *Development.* 121: 1875–1884.

Schweisguth, F., and J.W. Posakony. 1994. Antagonist activities of *Suppressor of Hairless* and *Hairless* control alternative cell fates in the *Drosophila* adult epidermis. *Development.* 120:1433–1441.

Shellenbarger, D.L., and J.D. Mohler. 1975. Temperature sensitive mutations of the Notch locus in *Drosophila melanogaster. Genetics.* 81:143–162.

Shellenbarger, D.L., and J.D. Mohler. 1978. Temperature-sensitive periods and autonomy of pleiotropic effects of $l(1)N^{ts1}$, a conditional Notch lethal in *Drosophila*. *Dev. Biol.* 62:432–446.

Singson, A., M.W. Leviten, A.G. Bang, X.H. Hua, and J.W. Posakony. 1994. Direct downstream targets of proneural activation in the imaginal disc include genes involved in lateral inhibitory signaling. *Genes Dev.* 8:2058–2071.

Skeath, J.B., and S.B. Carroll. 1992. Regulation of proneural gene expression and cell fate during neuroblast segregation in the *Drosophila* embryo. *Development.* 114:939–946.

Spana, E.P., and C.Q. Doe. 1996. Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron.* 17:21–26.

Struhl, G., and A. Adachi. 1998. Nuclear access and action of Notch in vivo. *Cell.* 93:649–660.

Struhl, G., and I. Greenwald. 1999. Presenilin is required for activity and nuclear access of Notch in *Drosophila. Nature.* 398:522–525.

Struhl, G., K. Fitzgerald, and I. Greenwald. 1993. Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell.* 74:331–345.

Tamura, K., Y. Taniguchi, S. Minoguchi, T. Sakai, T. Tun, T. Furukawa, and T. Honjo. 1995. Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-jκ/Su(H). *Curr. Biol.* 5:1416–1423.

van Doren, M., H.M. Ellis, and J.W. Posakony. 1991. The *Drosophila* extramacrochaete protein antagonizes sequence-specific DNA binding by daughterless/achaete-scute protein complexes. *Development.* 113:245–255.

Vaessin, H., M. Brand, L.Y. Jan, and Y.N. Jan. 1994. *daughterless* is essential for neuronal precursor differentiation but not for initiation of neuronal precursor formation in *Drosophila* embryo. *Development.* 120:935–945.

Wang, S., S. Younger-Shepherd, L.Y. Jan, and Y.N. Jan. 1997. Only a subset of the binary cell fate decisions mediated by Numb/Notch signaling in *Drosophila* sensory organ lineage requires *Suppressor of Hairless. Development.* 124:4435–4446.

Wesley, C.S. 1999. Notch and Wingless regulate expression of cuticle patterning genes. *Mol. Cell. Biol.* 19:5743–5758.

Ye, Y., N. Lukinova, and M.E. Fortini. 1999. Neurogenic phenotypes and altered Notch processing in *Drosophila Presenilin* mutants. *Nature.* 328:525–529.

Zecchini, V., K. Brennan, and A. Martinez-Arias. 1999. An activity of Notch regulates JNK signalling and affects dorsal closure in *Drosophila. Curr. Biol.* 9:460–469.