# Ligand-induced cleavage and regulation of nuclear entry of Notch in *Drosophila melanogaster* embryos

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**Notch, a transmembrane protein found in a wide range of organisms, is a component of a pathway that mediates cell-fate decisions that involve intercellular communication. In this paper, we show that in *Drosophila melanogaster*, Notch (N) is processed in a ligand-dependent fashion to generate phosphorylated, soluble intracellular derivatives. Suppressor of Hairless [Su(H)] is predominantly associated with soluble intracellular N. It has been demonstrated by others that N has access to the nucleus, and we show that when tethered directly to DNA, the cytoplasmic domain of N can activate transcription. Conversely, a viral activator fused to Su(H) can substitute for at least some N functions during embryogenesis. We suggest that one function of soluble forms of N is to bind to Su(H), and in the nucleus, to act directly as a transcriptional transactivator of the latter protein. Although N has functional nuclear localization signals, the N/Su(H) complex accumulates in the cytoplasm and on membranes suggesting that its nuclear entry is regulated. Localization studies in cultured cells and embryos suggest that Su(H) plays a role in this regulation, with the relative levels of Delta, N and Su(H) determining whether a N/Su(H) complex enters the nucleus.**

[*Key Words*: Notch; processing; Delta; Suppressor of Hairless; nuclear entry; transcriptional transactivator]

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Notch (N) is a 2703-amino-acid transmembrane protein that is found in a wide range of organisms from *Caenorhabditis elegans* to humans. The *N* gene was originally identified in *Drosophila melanogaster* in which N is a component of a pathway involved in cell-fate decisions that involve intercellular communication. During *Drosophila* embryogenesis, this process, which is known as lateral inhibition, operates in all three germ layers and ensures that only one cell of several equipotential cells, termed an equivalence group, will adopt the primary cell fate. Because this pathway was first characterized in the neuroectoderm, in which the absence of any member of the pathway results in overproliferation of neuroblasts, the genes involved in lateral inhibition are known as neurogenic genes. *N*, and other members of the neurogenic gene family are also conserved during evolution (for review, see Artavanis-Tsakonas et al. 1995; Greenwald 1998).

Genetic mosaic analysis has demonstrated that N is cell autonomous, suggesting that it is a receptor (Hoppe 1986; Heitzler and Simpson 1991). Delta (Dl), also a transmembrane protein, has been identified as the ligand for N in its role in lateral inhibition (for review, see Artavanis-Tsakonas et al. 1995; Nye and Kopan 1995). Activation of N results in transcription of genes of the *Enhancer of split* [*E(spl)*]) complex. This transcriptional activation is mediated by Suppressor of Hairless (Su(H)), a DNA binding protein that has been demonstrated to bind to the cytoplasmic domain of N (Fortini and Artavanis-Tsakonas 1994; Jennings et al. 1994; Jarriault et al. 1995; Lecourtois and Schweisguth 1995; Tamura et al. 1995; Kato et al. 1997; Schroeter et al. 1998).

Previous work has shown that expression of N proteins deleted for the extracellular domain results in gain of function phenotypes indicative of ligand-independent activation (for review, see Artavanis-Tsakonas et al. 1995; Greenwald 1998). The cytoplasmic domain of N contains functional nuclear localization signals (Stifani et al. 1992; Lieber et al. 1993), and it has been proposed that on binding Dl, the transmembrane N protein is cleaved releasing the cytoplasmic domain that translocates to the nucleus, where it is tethered to DNA via Su(H) and behaves as a transcriptional transactivator (Lieber et al. 1993; Struhl et al. 1993; Jarriault et al. 1995; Kopan et al. 1996). In tissue culture, Schroeter et al. (1998) have mapped a site in the transmembrane domain of mouse Notch-1 at which it undergoes ligand-dependent proteolytic cleavage. Mutating this site reduces the activity of Notch-1 in cell culture. In addition, it has been found that in vivo the cytoplasmic domain of *Drosophila* N has access to the nucleus, although the biochemical nature of this access was not determined (Lecourtois and Schweisguth 1998; Struhl and Adachi 1998).

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In this paper, we present data indicating that in wild-type *Drosophila*, N is processed in a ligand-dependent manner to generate a cytoplasmic domain that, on the basis of size and solubility, does not span the membrane. This domain is phosphorylated and Su(H) preferentially associates with this form. The N/Su(H) complex is found associated with membranes and predominantly in the cytoplasm, indicating that there is a mechanism for regulating its subcellular location. Our data suggest that Su(H) can inhibit nuclear entry of the soluble N proteins, and that nuclear entry occurs in a fashion dependent on the relative abundance of soluble N and Su(H). In prior genetic studies of N nuclear activity, it was suggested that nuclear N participates in the transcriptional regulation of downstream target genes (Lecourtois and Schweisguth 1998; Struhl and Adachi 1998). We show that in the nucleus, N behaves as a transcriptional transactivator, and that a heterologous activator fused to Su(H) can substitute for activated N function in embryos.

## Results

### *Su(H) interacts with phosphorylated Notch proteins*

N and Su(H) proteins have been shown to physically interact (Fortini and Artavanis-Tsakonas 1994; Jarriault et al. 1995; Tamura et al. 1995; Kato et al. 1997; Schroeter et al. 1998). To characterize the nature of the associated N and Su(H) proteins in vivo, immunoprecipitations with antibodies against either N or Su(H) were performed on detergent extracts of *Drosophila* embryos. Following electrophoresis, N proteins in these immunoprecipitates were detected by Western blot. When anti-N and anti-Su(H) immunoprecipitations are probed with anti-N antibodies, only a small subset of the proteins immunoprecipitated by anti-N antibodies are found in the corresponding Su(H) immunoprecipitate [cf. Fig. 2A, below, lanes 1 and 2, anti-Su(H) immunoprecipitation, with Fig. 2C, lanes 1 and 2, anti-N immunoprecipitation]. This suggests that the interactions between N and Su(H) detected in this assay occurred in vivo and not during the course of the immunoprecipitation, as the latter might be expected to result in similar array of N proteins in both immunoprecipitates.

Anti-Su(H) immunoprecipitates contain two major size classes of N proteins, both of which are recognized by antibodies raised against 2 different regions of the intracellular domain of N (see Fig. 1 for antibodies used in this work); one the size of full length N and another, substantially enriched, which migrates as smear of proteins of ~114 kD (Fig. 2A, lane 1). The existence of a smear of bands at 114 kd suggests that these N proteins might be post-translationally modified, perhaps by phosphorylation. Figure 2A, lane 2, shows that treating the Su(H) immunoprecipitates with alkaline phosphatase results in the smear of proteins being resolved into three proteins of ~100 kD, which we have termed in order of decreasing molecular weight $N^{p100A}$, $N^{p100B}$, and $N^{p100C}$. $N^{p100A}$ and $N^{p100C}$ differ by ~4 kD. We have

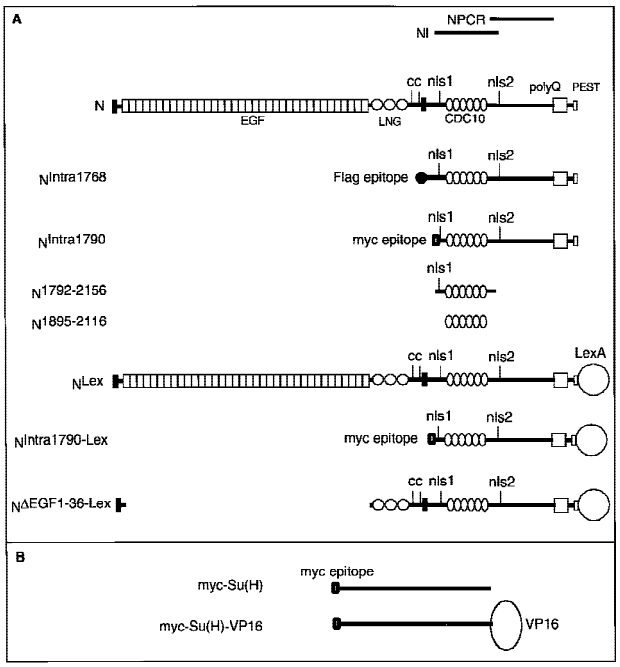


**Figure 1.** Constructs and antibodies. (*A*) A diagram of the N protein (N). (EGF) Epidermal growth factor-like repeats; (LNG) lin-12, Notch, Glp-1 repeats; (cc) evolutionary conserved cysteine residues; (CDC10) CDC10 or ankyrin repeats; (nls1, nls2) putative nuclear localization signals; (polyQ) polymeric glutamines; (PEST) PEST sequence thought to be involved in protein stability (Rogers et al. 1986). The bars above the diagram of N indicate the various regions used as antigens to create anti-N antibodies. Beneath the diagram of N are the N constructs used in this paper. (*B*) Su(H) constructs used in this paper.

collectively termed the phosphorylated forms of these proteins $N^{pp114}$. Treatment of the immunoprecipitates with alkaline phosphatase in the presence of phosphatase inhibitors reduces the effect of the phosphatase (Fig. 2A, lane 3). Because $N^{pp114}$ reacts with two different N antibodies, it is most likely that these proteins are N. As no alternatively spliced *N* transcripts have been observed, and no appropriately positioned methionine exists at which internal translation could initiate to give rise to a protein with the size and antigenic determinants of $N^{pp114}$ (Wharton et al. 1985; Kidd et al. 1986; Kopan et al. 1996), it is most likely that these smaller N proteins are the result of proteolytic cleavage.

The proteins used for the previous experiment were from an overnight collection of embryos. We wondered if production of the various N proteins associated with Su(H) is developmentally regulated. Figure 2B shows that whereas $N^{pp114}$ is present throughout embryogenesis with $N^{p100B}$ as its major component, the amount of $N^{p100A}$ appears to increase with age, and $N^{p100C}$ is only found late in embryogenesis. In addition, embryos that are young (0–4 hr) contain significantly more processed N protein that comigrates with hypophosphorylated $N^{p100B}$ (indicated by an asterisk) relative to $N^{pp114}$. Late in embryogenesis, proteins that comigrate with both hypophosphorylated $N^{p100B}$ and $N^{p100C}$ are found.

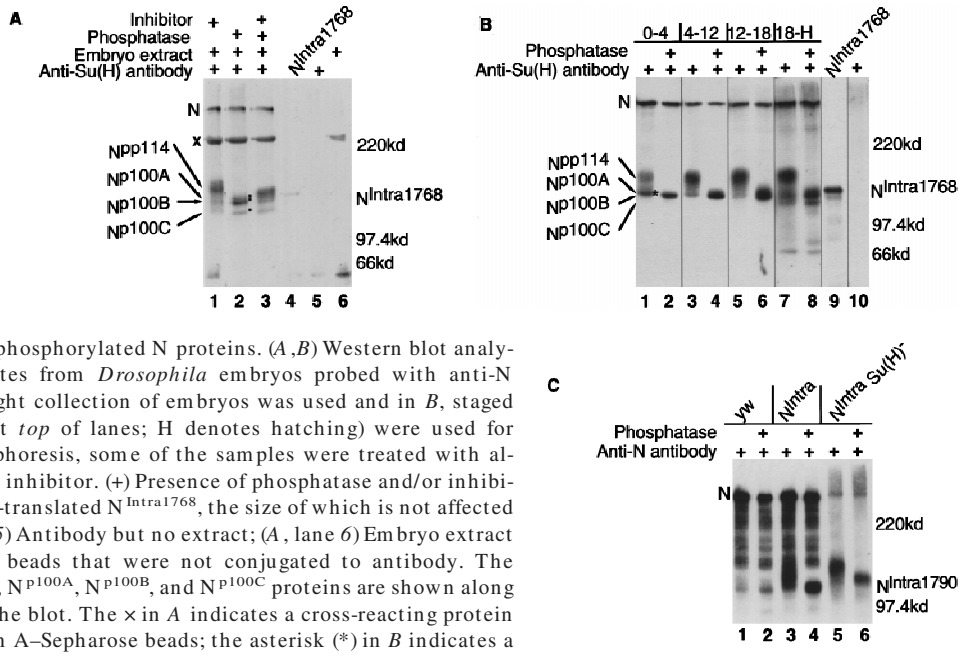


**Figure 2.** Su(H) is associated with phosphorylated N proteins. (*A*,*B*) Western blot analysis of anti-Su(H) immunoprecipitates from *Drosophila* embryos probed with anti-N (NPCR) antibody. For *A*, an overnight collection of embryos was used and in *B*, staged embryos (age indicated in hours at *top* of lanes; H denotes hatching) were used for detergent extracts. Prior to electrophoresis, some of the samples were treated with alkaline phosphatase with or without inhibitor. (+) Presence of phosphatase and/or inhibitor. (*A*, Lane *4* and *B*, lane *9*) In vitro-translated $N^{Intra1768}$, the size of which is not affected by phosphatase treatment; (*A*, lane *5*) Antibody but no extract; (*A*, lane *6*) Embryo extract recovered on protein A–Sepharose beads that were not conjugated to antibody. The locations of N (N), $N^{Intra1768}$, $N^{pp114}$, $N^{p100A}$, $N^{p100B}$, and $N^{p100C}$ proteins are shown along side each blot, and also by dots on the blot. The × in *A* indicates a cross-reacting protein nonspecifically bound to the protein A–Sepharose beads; the asterisk (*) in *B* indicates a hypophosphorylated N protein that comigrates with $N^{p100B}$. (*A*, all lanes with extract; *B*, lanes *3–6*) 500 µg of protein was used for each immunoprecipitation; the remaining lanes in *B* contain 2.5 mg(lanes *1*,*2*) and 1.75 mg (lanes *7*,*8*). (*C*) Western blot analysis of anti-N (NI) immunoprecipitates from *Drosophila* embryos probed with anti-N (NPCR) antibody. (Lanes *1*, *2*) Immunoprecipitates from *yw* embryos; (lanes *3*,*4*) immunoprecipitates of embryos resulting from a cross of *Su(H)* $hsN^{intra1790}$/*CyO* males to $Su(H)^{SF8}$ *FRT40A*/*CyO* virgins; (lanes *5*,*6*) immunoprecipitates of embryos resulting from a cross of *Su(H)* $hsN^{intra1790}$/*CyO* males to *hsflp12*/*yw*; $Su(H)^{SF8}$ *FRT40A*/$ovo^{D}$ *FRT40A* virgins. All $N^{Intra}$ expressing embryos are both zygotically and maternally *Su(H)* null. For all genotypes, 3- to 5 hr-old embryos were subjected to a 30 min heat shock at 37°C and allowed to recover for 15–30 min prior to collection. (+) Phosphatase treatment. The locations of N (N) and $N^{Intra}$ are indicated next to the blot.

The above experiment indicates that phosphorylated, processed N proteins interact with Su(H). To address whether phosphorylation is a consequence of Su(H) binding, we asked whether $N^{Intra1790}$ (Fig. 1) is phosphorylated in embryos that lack Su(H). As can be seen in Figure 2C, lanes 5 and 6, $N^{Intra1790}$ expressed in embryos that are both maternally and zygotically $Su(H)^{-}$ is phosphorylated, indicating that phosphorylation of processed N proteins is not dependent on Su(H) binding. Intriguingly, even after phosphatase treatment, $N^{Intra1790}$ immunoprecipitated from $Su(H)^{-}$ embryos migrates slower than $N^{Intra1790}$ immunoprecipitated from embryos that contain Su(H) (cf. lanes 4 and 6). This might be because $N^{Intra1790}$ bound to Su(H) has undergone additional proteolytic processing, or $N^{Intra1790}$ not bound to Su(H) has been subject to additional post-translational modification. However, in the $Su(H)^{-}$ extracts there is also a smear extending upward from endogenous N. This suggests that in $Su(H)^{-}$ embryos, $N^{Intra1790}$, as well as being phosphorylated, has undergone additional post-translational modification.

We have also analyzed Su(H) coimmunoprecipitated with anti-N antibodies (data not shown). The Su(H) associated with N comigrates with the Su(H) immunoprecipitated by anti-Su(H) antibodies. In both immunoprecipitates, there is no change in mobility on phosphatase treatment, suggesting that Su(H) is not extensively phosphorylated.

### *Cleavage of N is ligand dependent*

If N signaling is dependent on ligand-induced cleavage of N, then $N^{pp114}$ might result from the binding of a N ligand. The two experiments shown in Figure 3 demonstrate that the presence of ligand and the ability of N to bind ligand is required for the presence of $N^{pp114}$. All the known N ligands bind to the extracellular EGF-like repeats, deletion of which results in a nonfunctional protein (Rebay et al. 1991, 1993; Lieber et al. 1993). Extracts from embryos expressing a form of N that spans the membrane but lacks the EGF-like repeats and is tagged at the Carboxyl terminus with the DNA-binding domain of LexA [$N^{\Delta EGF1\text{-}36\text{–}LexA}$ (Fig. 1)] were immunoprecipitated with anti-N and anti-Su(H) antibodies, treated with phosphatase, and the Western blot probed with anti-LexA antibody. As can be seen in Figure 3A, lane 4, the anti-Su(H) immunoprecipitates from $N^{\Delta EGF1\text{-}36\text{–}LexA}$ embryos do not contain $N^{p100\text{–}LexA}$. Expression of $N^{\Delta EGF1\text{-}36\text{–}LexA}$ will not rescue the neurogenic phenotype of a zygotically $N^{-}$ embryo (data not shown). As a control for this experiment, immunoprecipitations from embryos expressing LexA-tagged N ($N^{LexA}$) were carried out. In these experiments, a LexA-tagged $N^{p100}$ protein is seen (Fig. 3A, lane 3, $N^{p100\text{–}LexA}$, indicated by an asterisk, presumably the hypophosphorylated form of $N^{pp114\text{–}LexA}$). Expression of $N^{LexA}$ will rescue the neurogenic phenotype of zygotically $N^{-}$ embryos (data not shown).

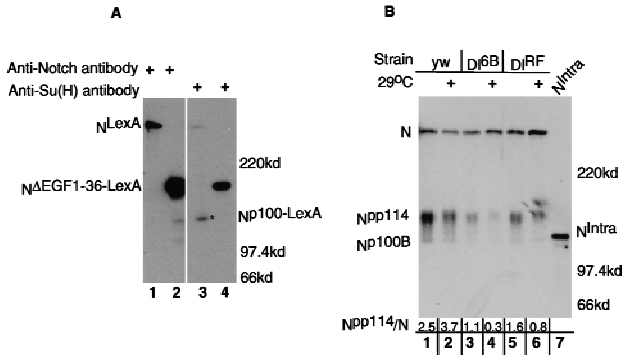


**Figure 3.** The presence of Notch ligand and the extracellular domain of N are required for the isolation of $N^{p100}$ bound to Su(H). (*A*) Detergent extracts from embryos expressing either $N^{LexA}$ (lanes *1*,*3*) or $N^{\Delta EGF1\text{-}36\text{–}LexA}$ [lanes *2*,*4* (see Fig. 1 for structures of $N^{LexA}$ and $N^{\Delta EGF1\text{-}36\text{–}LexA}$] were immunoprecipitated with antibodies against either N (NI) (lanes *1*,*2*) or Su(H) (lanes *3*,*4*) treated with phosphatase, and then probed with antibodies against LexA. Antibodies against N immunoprecipitate $N^{LexA}$ (lane *1*) and $N^{\Delta EGF1\text{-}36\text{–}LexA}$ (lane *2*); the smaller proteins seen in lane *2* are missing from lane *1* and, are most likely nonspecific breakdown products visible because of massive overexpression of $N^{\Delta EGF1\text{-}36\text{–}LexA}$ compared with $N^{LexA}$. $N^{LexA}$ and $N^{\Delta EGF1\text{-}36\text{–}LexA}$ are coimmunoprecipitated by anti-Su(H) antibodies as is a smaller, processed form of $N^{LexA}$ ($N^{p100\text{–}LEXA}$, lane *3*, *). No such processed protein is seen when the extracellular ligand binding domain of N is deleted (lane *4*). (*B*) *Dl* temperature-sensitive mutants reduce the level of processed N protein. *yw* (wt), $Dl^{6B}$/*TM6* (a strong) or $Dl^{RF}$/*TM6* (a weak temperature-sensitive *Dl* allele) males were mated to $Dl^{x}$/*TM6* (an amorphic *Dl* allele) females. All the embryos resulting from the above crosses were incubated at either room temperature (lanes *1*,*3*,*5*) or at the nonpermissive temperature, 29°C (lanes *2*,*4*,*6*). N proteins coimmunoprecipitated from detergent extracts by anti-Su(H) antibody were detected with the NPCR anti-N antibody. Beneath each lane the ratio of processed N to N (termed $N^{pp114}$/N) is shown. The strong temperature-sensitive *Dl* allele (lanes *3*,*4*) has a considerably greater effect on the level of processed N than the weaker allele (lanes *5*,*6*)

We then examined the effect of lowering the level of ligand by using temperature-sensitive mutants of the N ligand *Delta*. $Dl^{6B}$/*TM6* (a strong temperature-sensitive *Dl* allele) and $Dl^{RF}$/*TM6* (a weak temperature-sensitive *Dl* allele) males were mated to $Dl^{x}$/*TM6* (an amorphic *Dl* allele) females. All the embryos from this cross were incubated at either the permissive or nonpermissive temperature. Extracts of these embryos were immunoprecipitated with anti-Su(H) antibody. Figure 3B shows that at the nonpermissive temperature, temperature-sensitive *Dl* alleles reduce the level of processed compared with full-length N bound to Su(H). The ratios of processed to full-length N obtained by scanning this image are shown beneath each lane. The level of reduction produced by each temperature-sensitive *Dl* allele appears to correlate with the strength of its mutant phenotype. Incubation of the stronger temperature-sensitive allele, $Dl^{6B}$, at the nonpermissive temperature (Fig. 3, lanes 3,4) reduces the ratio of processed to full-length N from 1.1 to 0.3, whereas the weaker allele, $Dl^{RF}$ (lanes 5,6), only reduces the ratio from 1.6 to 0.8. In contrast, in extracts from wild-type embryos, the ratio increases from 2.5 at the permissive to 3.7 at the nonpermissive temperature (lanes 1,2). In extracts of embryos with neurogenic phenotypes produced by expressing antisense *m8* RNA or produced by $pcx^{1}$ parents (Perrimon et al. 1984), there is no decline in the level of processed relative to full-length N (data not shown).

The above experiments indicate that the presence of $N^{pp114}$ bound to Su(H) is correlated with N function and suggest that ligand binding is required for cleavage of N to generate $N^{pp114}$ that is bound to Su(H). However, in converse experiments in which the N ligand is overexpressed, a more complex picture emerges. In these experiments, heat shock GAL4 was used to induce expression of UAS Dl in otherwise wild-type embryos. Two hours after induction of Dl, immunoprecipitations with anti-N antibodies reveal that a N protein, the size of $N^{p100B}$ associated with Su(H), has become more abundant than in wild-type extracts or in uninduced UAS Dl embryos (Fig. 4A; cf. the samples of lanes 1, 3, and 5, which are unphosphatased immunoprecipitations). The increase in abundance of this protein is most obvious when comparing wild-type extracts with extracts from heat shock-induced UAS Dl embryos (Fig. 4A, lanes 1, 5). Because of the leakiness of the heat shock promoter, some of this protein can be seen in extracts from uninduced UAS Dl embryos (Fig. 4A, lane 3). The mobility of most of the protein produced by UAS Dl induction does not change significantly on phosphatase treatment, suggesting little or no phosphorylation (Fig. 4A, lanes 5, 6).

Although ligand binding is required for production of $N^{pp114}$ associated with Su(H) (see above), the pool of N protein coimmunoprecipitated by anti-Su(H) on overexpression of the ligand Dl did not appear to increase (data not shown), suggesting that most of this Dl-induced protein has not become associated with Su(H). We reasoned that we were more likely to see an increase in processed N associated with Su(H) if we induced a tagged form of N at the same time as Dl. In this way, rather than looking for a change superimposed on the steady state level N, we would be looking for a change only in the tagged N synthesized at the same time as Dl. Therefore, we compared the amount of processed $N^{LexA}$ bound to Su(H) in extracts of embryos in which $N^{LexA}$ alone was induced by *hairy* GAL4 with the amount of processed $N^{LexA}$ bound to Su(H) in extracts of embryos in which Dl was induced along with $N^{LexA}$. The results are shown in Figure 4B. In this experiment, coexpression of $N^{LexA}$ with Dl results in a 42% (the phosphatased samples of Fig. 4B, lanes 6, 8) or 52% (unphosphatased samples of Fig. 4B, lanes 5, 7) increase in the level of $N^{pp114\text{–}LexA}$ associated with Su(H) when compared with $N^{LexA}$ expressed in the absence of additional Dl. A histogram summarizing the results of four immunoprecipitations from three protein preparations is shown in Figure 4C. On average, there is a 1.75-fold increase in the amount of processed compared with full-length N associated with Su(H). In addition, as was the case with overexpression of Dl in wild-type flies, overexpression of Dl along with $N^{LexA}$ results in the pro-

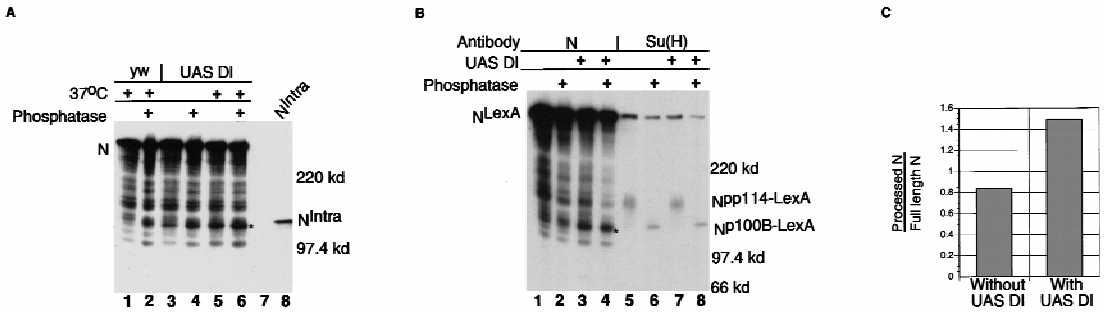


**Figure 4.** Ligand-induced cleavage of N. (*A*) Anti-N antibodies immunoprecipitate a N protein, the size of $N^{p100B}$ (*) from embryos exposed to ectopic Dl (lane *5*). Embryos aged from 2 to 4 hr were heat-shocked at 37°C for 30 min and allowed to recover at 30°C for 2 hr prior to detergent extraction. Extracts from either heat-shocked *yw* (lanes *1*,*2*) or heat-shocked *GAL4; UAS Dl* with (lanes *5*,*6*), or without heat shock (lanes *3*,*4*) were immunoprecipitated with anti-N NI antibody. Lane *7* lacks embryo extract; lane *8* contains $N^{Intra1768}$ in vitro translation products. After immunoprecipitation, lanes marked with a + were treated with alkaline phosphatase prior to electrophoresis. N proteins were detected with the anti-N antibody NPCR. (Labels) Locations of N (N) and $N^{Intra1768}$($N^{Intra}$). (*B*) Coexpression of $N^{LexA}$ and Dl results in an increase in the level of $N^{pp114-LexA}$. Detergent extracts from 4–8 hr $UASN^{lexA} \times hGAL4$ embryos (lanes *1*,*2*,*5*,*6*) or *UAS Dl30B*; $UAS\ N^{lexA} \times hGAL4$ embryos (lanes *3*,*4*,*7*,*8*) were immunoprecipitated with either anti-N antibody (NI) (lanes *1–4*) or anti-Su(H) antibody (lanes *5–8*). Samples in lanes *2*, *4*, *6*, and *8* were treated with alkaline phosphatase prior to electrophoresis. After blotting, proteins were detected with an anti-LexA monoclonal antibody. Coexpression of Dl and $N^{LexA}$ causes the appearance of a novel hypophosphorylated $N^{LexA}$ protein in the anti-N immunoprecipitations (*, cf. lanes *1* and *3*). In this experiment, there was an ~40%–50% increase in the amount of $N^{pp114-LexA}$ bound to Su(H) (cf. the ratios of $N^{pp114LexA}$ to $N^{LexA}$ in lane *5* with lane *7*, and in lane *6* with lane *8*). (*C*) A histogram comparing the relative amounts of processed vs. full-length N associated with Su(H) in the absence or presence of ectopic Dl. The average of the results of four immunoprecipitations are plotted. The fold increase in the amount of processed N compared with full-length N in the presence of UAS Dl for each of the four experiments was as follows: 1.7, 1.5, 2.0, and 1.8.

duction of a hypophosphorylated N protein the size of $N^{p100B}$ fused to LexA that is immunoprecipitated with anti-N antibody but does not associate with Su(H) (cf. the unphosphatased samples of Fig. 4B, lanes 1,3).

### *Intracellular location of the Su(H) bound N proteins*

The three dephosphorylated components of $N^{pp114}$ are small enough to be soluble. To see if this was the case, immunoprecipitations were carried out on subcellular fractions of *Drosophila* embryos. Equal fractions of each subcellular fraction were immunoprecipitated to allow the relative abundance of the proteins in each fraction to be determined. When the fractionation is carried out under hypotonic conditions (10 mM KCl), the majority of N proteins, full-length as well as $N^{pp114}$, immunoprecipitated by both N and Su(H) antibodies are in the membrane fraction (Fig. 5A, lanes 1,2,7,8). Some $N^{pp114}$ is found in the soluble fraction (Fig. 5A, lanes 3,4,9,10) and little or no N is detectable in the nuclear fraction (Fig. 5A, lanes 5, 6, 11, 12). In addition to $N^{pp114}$, the anti-N immunoprecipitate of the soluble fraction is also enriched for two proteins of ~99 kD and 86 kD, which are superimposed over an 86–114 kD smear (Fig. 5, A, lane 3, and B, lane 1). Phosphatase treatment reduces the smear to the hypophosphorylated components of $N^{pp114}$ and a protein of 86 kD (termed $N^{p86}$) (Fig. 5, A, lane 4 and B, lane 2) suggesting that $N^{pp99}$ is a phosphorylated form of $N^{p86}$. Despite being soluble, $N^{pp99}$ was not found associated with Su(H) (Fig. 5A, cf. lanes 3 and 4 with lanes 9 and 10).

In contrast, when subcellular fractionations are carried out under physiological salt conditions (100 mM KCl) the majority of $N^{pp114}$ associated with Su(H) is found in the soluble fraction (Fig. 5C, lane 2). Although there is a significant amount of $N^{pp114}$ still found associated with the membrane fraction (Fig. 5C, lane 1), incubation of the post-nuclear supernatant in increasing concentrations of KCl prior to centrifugation disrupts the association of $N^{pp114}$ with membranes (Fig. 5C, lanes 3,5). These results support the notion that $N^{pp114}$ is the soluble, phosphorylated intracellular domain of N. In conjunction with the results we have presented above, this suggests that on binding its ligand Dl, transmembrane N is cleaved, generating a soluble form that encompasses its cytoplasmic domain, is phosphorylated, and associates with Su(H).

As well as coimmunoprecipitating $N^{pp114}$, anti-Su(H) antibodies also precipitate a N protein the size of hypophosphorylated $N^{p100B}$ from the membrane fraction (indicated by an asterisk in Fig. 5C). The same salt conditions which disrupted the association of $N^{pp114}$ with membranes do not do so with this protein, suggesting a stronger association with the membrane.

### *Su(H) is capable of retaining the cytoplasmic domain of N in the cytoplasm*

The biochemical data presented above suggest that some processed N complexed with Su(H) protein is still associated with membranes even though, on the basis of size, it probably lacks a transmembrane domain. In addition, there are larger amounts of N, principally $N^{pp114}$, in the soluble fraction. This is surprising, as we and others have

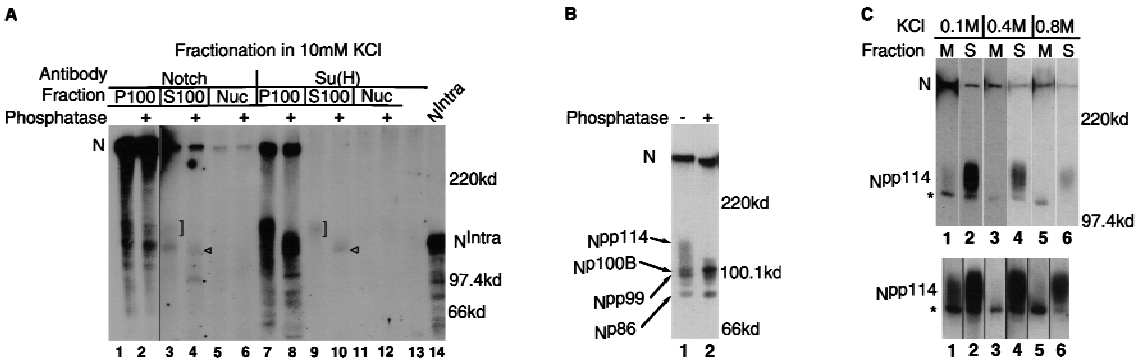


**Figure 5.** $N^{pp114}$ is a soluble protein. (*A*) Subcellular distribution of $N^{pp114}$. Subcellular fractions were prepared from *Drosophila* embryos that had been lysed under hypotonic conditions (10 mM KCl). (Nuc) The nuclear fraction; (P100) the membrane fraction; (S100) the soluble fraction. The position of $N^{pp114}$ is indicated by a square bracket, $N^{p100B}$ by an arrowhead, and $N^{p86}$ by a dot. Equal proportions of each fraction were immunoprecipitated with antibodies against either N (NI) (lanes *1–6*) or Su(H) (lanes *7–12*) and then detected with the anti-NPCR antibody. The autoradiograph has been overexposed to show the presence of processed N in the soluble fraction. Because the amount of N in the membrane fraction is so high, lanes *1* and *2* are from a shorter exposure. (*B*) Evidence for additional processing of the cytoplasmic domain of N. Soluble proteins extracted in 0.4 M KCl were immunoprecipitated with anti-NI antibody and detected with anti-NPCR antibody. As well as immunoprecipitating the same N proteins as those coimmunoprecipitated by anti-Su(H) antibodies, anti-N antibodies immunoprecipitate two novel proteins of ~99 kD ($N^{pp99}$) and 86 kD ($N^{p86}$) (lane *1*), treatment with alkaline phosphatase (lane *2*) reduces the amount of $N^{pp99}$ and increases the amount of $N^{p86}$, suggesting that $N^{pp99}$ is a phosphorylated form of $N^{p86}$. (*C*) Some $N^{pp114}$ is associated, but not stably, with membranes. Postnuclear supernatants were incubated with increasing amounts of KCl prior to fractionation by centrifugation into membrane bound (lanes marked M) or soluble proteins (lanes marked S). Fractionated proteins were then immunoprecipitated with anti-Su(H) antibody and detected with the anti-N NPCR antibody, a longer exposure of the relevant region of the resulting autoradiograph is shown at *bottom*. Some $N^{pp114}$ can be seen to be associated with membranes when extracted at 0.1 M KCl but is removed at higher salt concentrations. The protein that comigrates with $N^{p100B}$ and appears to be stably associated with membranes is marked by an asterisk.

shown previously that the intracellular domain of N has functional nuclear localization signals and can localize to nuclei (Stifani et al. 1992; Lieber et al. 1993).

Given our fractionation studies of $N^{pp114}$, subcellular localizations of $N^{Intra}$ were further examined. Whereas in S2 cells $N^{Intra1768}$ (Fig. 1) is totally nuclear (Fig. 6A), in embryos, a substantial fraction of $N^{Intra1768}$ is retained in the cytoplasm. This is illustrated in Figure 6B by use of an anti-Flag-antibody to recognize Flag-tagged $N^{Intra1768}$. Using an anti-N antibody, we found that it is in cells in which $N^{Intra1768}$ is expressed at higher levels that it is found in nuclei. In cells in which $N^{Intra1768}$ is expressed at lower levels, it is primarily cytoplasmic (Fig. 6C). This suggests that there is something in embryos that is retaining $N^{Intra1768}$ in the cytoplasm, and that this retention mechanism can be saturated by high levels of $N^{Intra1768}$. It has been shown that the cdc10 repeats of N can mediate homotypic N interactions (Roehl et al. 1996; Matsuno et al. 1997). However, in embryos that are both maternally and zygotically *N* null, there is still substantial cytoplasmic localization of $N^{Intra1768}$ (Fig. 6D).

An obvious candidate for a factor influencing subcellular localization of $N^{Intra}$ in the absence of transmembrane N is Su(H). In early embryos, Su(H) is present ubiquitously and localizes to both cytoplasm and nuclei (data not shown). It has been shown in wing discs that Su(H) is present in the cytoplasm, and that when $N^{Intra}$ is expressed to high levels, it is capable of dragging endogenous Su(H) into nuclei (Gho et al. 1996). Although this is true for high levels of $N^{Intra1768}$ (Fig. 7, cf. A with B and C), when low levels of $N^{Intra1768}$ are coexpressed with Su(H) in S2 cells, $N^{Intra1768}$ is retained in the cytoplasm (Fig. 7D–G). Thus, raising the relative level of Su(H) favors cytoplasmic localization of $N^{Intra}$. In accord with this observation, $N^{Intra1790}$ is predominantly nuclear in embryos with reduced levels of Su(H) (Fig. 6E).

### *The cytoplasmic domain of N behaves as an activator when bound to DNA*

The data we have presented above indicate that N is processed and associates with Su(H), and the entry of this complex into the nucleus appears to be dependent on the relative levels of processed N and Su(H). During the course of yeast two-hybrid experiments, it was found that the cytoplasmic domain of N was a strong activator. Figure 8A shows a comparison in yeast of the activating ability of the N cytoplasmic domain with that of the well-characterized transcriptional activator GAL4. It can be seen that the cytoplasmic domain of N has almost as much activator activity (85%) as GAL4. Smaller derivatives of the N cytoplasmic domain activate to a lesser degree. Thus, in a heterologous system, the cytoplasmic domain of N strongly activates transcription from a heterologous promoter. This suggests that at least one aspect of N function could be mediated by its ability to act as a transcriptional transactivator for Su(H). We tested this in two ways. First, we fused the DNA-binding domain of the bacterial repressor LexA to the cytoplasmic domain of N. In S2 cells, this $N^{LexA}$ fusion protein ($N^{Intra-LexA}$; Fig. 1), but not $N^{Intra1790}$, activates transcription from a LexA reporter (data not shown). In Fig-

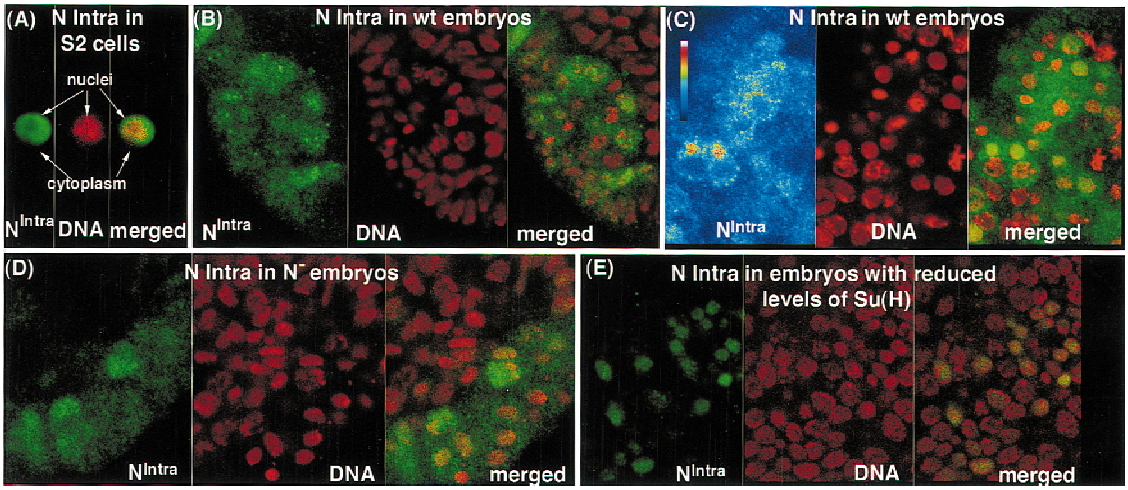


**Figure 6.** $N^{Intra}$ is retained in the cytoplasm in embryos. Confocal images showing the localization of $N^{Intra}$ in embryos and *Drosophila* S2 cells as detected by immunofluorescence. With the exception of the *left* panel in *C*, $N^{Intra}$ protein is represented in green and the nuclei in red. This panel is a pseudocolored representation to illustrate the relative amounts of $N^{Intra1768}$. The correspondence of the colors with the intensity of the signal is indicated by the pseudocolor bar, with the more intense signals being depicted by colors higher up the bar. (*A*) Heat shock-induced $N^{Intra1768}$ appears to be totally nuclear in S2 cells. UAS $N^{Intra1768}$ was cotransfected along with HS GAL4 and detected with a rabbit anti-N (NI) antibody. The nucleus was detected with SYTOX Green. (*B*,*C*) In embryos (*hGAL4*; *UAS* $N^{intra1768}$) there is retention of $N^{Intra1768}$ in the cytoplasm. (*B*,*C*, *right*) The merged images of the N signal in green and the nuclei in red. (*C*, *left*) $N^{Intra1768}$ is primarily nuclear in those cells in which it is most highly expressed. Mouse anti-NPCR antibody was used to detect $N^{Intra1768}$. (*B*) Mouse anti-Flag antibody was used to detect Flag-tagged $N^{Intra1768}$. The nuclei were detected by propidium iodide. (*D*) $N^{Intra1768}$ is still retained in the cytoplasm in embryos that are maternally and zygotically *N* null ($N^{264-47}$ *FRT/ovo*$^{D}$ *FRT*; *hGAL4/hsflp X FM7/Y*; $N^{intra1768}$. (*D*, *left*) Mouse NPCR antibody was used to detect $N^{Intra1768}$; (*D*, *right*) a merged image of N (green) and nuclei (red). (*E*) $N^{Intra1790}$ is predominantly nuclear in embryos with reduced levels of Su(H). Anti-myc antibody was used to detect myc-tagged $N^{intra1790}$ in embryos that are maternally *Su(H)*$^{-}$ (*hsflp/yw;Su(H)*$^{SF8}$ *FRT/ovo*$^{D}$ *FRT;hGAL4 X Su(H)/CyO*; *UASN*$^{Intra1790}$). In embryos that are maternally *Su(H)*$^{+}$ (*hGAL4 X Su(H)/CyO;UASN*$^{Intra1790}$) there is retention of $N^{Intra1790}$ in the cytoplasm (data not shown).

ure 8D, we show that in embryos, $N^{Intra-LexA}$ can activate transcription from a LexA–β-galactosidase reporter. The pattern of expression of $N^{Intra-LexA}$ is presented in Figure 8C, and coincides well with the pattern of induced β-galactosidase reporter. Figure 8B shows that $N^{Intra1790}$ alone, although expressed in the same pattern as $N^{Intra-LexA}$ (data not shown), cannot activate transcription of the LexA reporter. This experiment indicates that in vivo, when N is directly tethered to DNA, it behaves as a transcriptional activator and suggests that the role of Su(H) is to guide a transcriptional activator to DNA.

If N is functioning as a transcriptional transactivator, one would predict that a transcriptional activator directly coupled to Su(H) could substitute for at least some aspects of N function. To test this, we fused the viral activator VP16 to Su(H) (Fig. 1). In S2 cells this Su(H)–VP16 fusion, but not Su(H), activates transcription from an *m8* reporter (data not shown). In Figure 8E we show that this Su(H)–VP16 fusion but not Su(H) (data not shown) can activate *m8* transcription in an embryo that is both maternally and zygotically $N^{-}$. This experiment demonstrates that a role of N is to either directly or indirectly provide activator function to Su(H).

## Discussion

Previous work has led to the model that on ligand binding, N is cleaved, and the cytoplasmic domain enters the nucleus where, in concert with Su(H), it activates transcription of genes such as *m8*, a member of the *E(spl)* complex (Lieber et al. 1993; Jarriault et al. 1995; Kopan et al. 1996; Lecourtois and Schweisguth 1998; Schroeter et al. 1998; Struhl and Adachi 1998). In this study, it has been shown that (1) soluble cytoplasmic N proteins are produced in vivo in response to the N ligand, Dl (2) Su(H) is recovered in association with these soluble forms of N, and (3) intracellular forms of N appear to function as transcriptional activators in embryos when physically associated with Su(H).

We have used antibodies against Su(H) and N to examine the structure of the N proteins associated with Su(H). During most of *Drosophila* embryogenesis, two size classes of N proteins are coimmunoprecipitated by antibodies against Su(H). These include full-length N proteins and, to a greater extent, phosphoproteins of ~114-kD, $N^{pp114}$. Unlike mammalian systems in which N exists predominantly as a heterodimer, during *Drosophila* embryogenesis, the bulk of N exists as the full-length form (Results; Kidd et al. 1989; Blaumueller et al. 1997). When dephosphorylated, $N^{pp114}$ resolves into three proteins, $N^{p100A}$, $N^{p100B}$, and $N^{p100C}$ of ~100 kD. Through most of embryogenesis, the most abundant of these proteins is $N^{p100B}$, $N^{p100C}$ being found only late in development. The size difference between the two proteins might be because $N^{p100C}$ has been cleaved further into

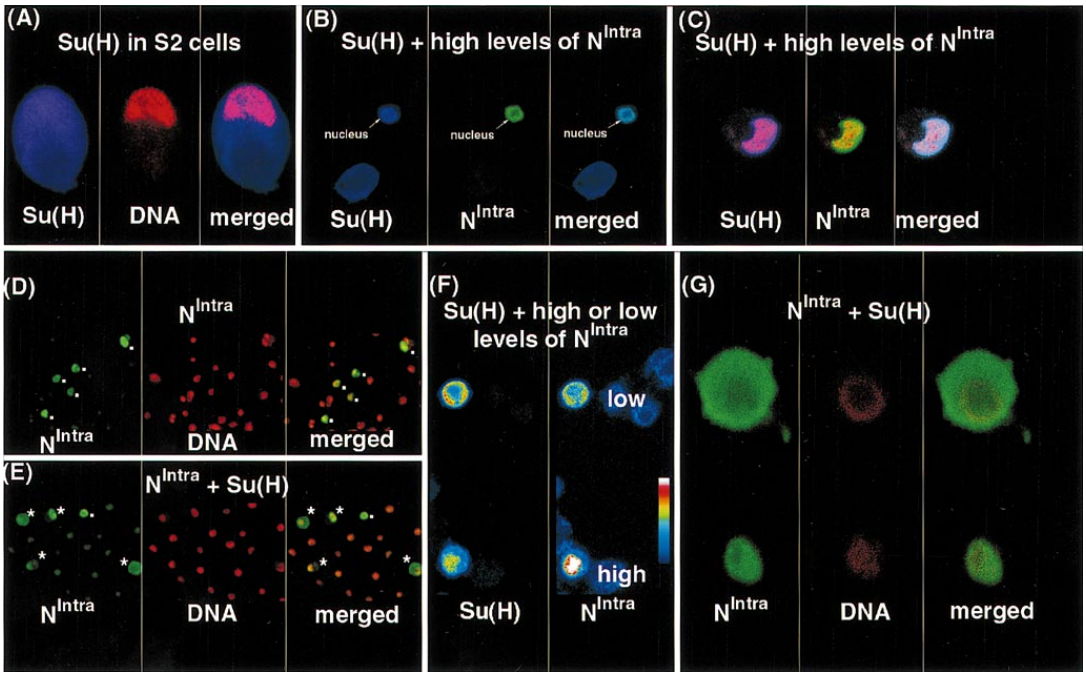


**Figure 7.** Su(H) can retain $N^{Intra}$ in the cytoplasm. Confocal images showing the localization of Su(H) and $N^{Intra1768}$. With the exception of *F*, which is a pseudocolored image, Su(H) is represented in blue, $N^{Intra}$ in green, and the nuclei in red. (*A*) Su(H) is expressed in both the cytoplasm and nuclei of transfected S2 cells. S2 cells were transfected with a construct expressing Su(H) under the control of an actin promoter. Su(H) is detected with rat anti-Su(H) antibody and the DNA with SYTOX Green. (*B*,*C*) Coexpression of high levels of $N^{Intra1768}$ along with Su(H) results in both being found in nuclei of S2 cells. (*B*) Two cells are depicted showing Su(H) expression in blue and N expression in green. In the lower cell, no $N^{Intra1768}$ is present and Su(H) is localized ubiquitously. In contrast, the upper cell expresses high levels of $N^{Intra1768}$ along with Su(H) and both are localized in the nucleus. A merged image of the first two panels is shown at *right*. The cell shown in *C* was probed with a DNA marker as well as with anti-N and Su(H) antibodies. Actin driven Su(H) was cotransfected with UAS $N^{Intra1768}$ and HS GAL4. Su(H) protein was detected by rat anti-Su(H) antibody, N protein was detected by a mouse anti-Flag antibody, and the DNA with SYTOX Green. (*D*,*E*,*G*) The difference in localization of $N^{Intra1768}$ promoted by expression of an excess of Su(H). $N^{Intra}$ is represented in green and the nuclei in red. (*D*) UAS $N^{Intra1768}$ and HS GAL4 were transfected into S2 cells. (*E*,*G*) A 20× mass excess of actin Su(H) was cotransfected along with UAS $N^{Intra1768}$ and HS GAL4. (*D*,*E*) Localization of $N^{Intra1768}$ in nuclei is indicated by dots and retention in the cytoplasm by asterisks. Two cells from those shown in *E* are depicted at higher magnification in *G*. As cytoplasmic localization of $N^{Intra1768}$ is never seen in cells lacking ectopic Su(H) (*D*), the upper cell in *G* must have received more Su(H) relative to $N^{Intra1768}$ than the lower one, resulting in $N^{Intra1768}$ being retained in the cytoplasm. $N^{Intra1768}$ was detected with a rabbit anti-N (NI) antibody and the nuclei with SYTOX Green. (*F*) When low levels of $N^{Intra1768}$ are expressed along with Su(H), $N^{Intra1768}$ is retained in the cytoplasm. A pseudocolored confocal image showing the relative levels on N at *right* and Su(H) at *left* is portrayed. The intensity of staining is depicted by the pseudocolor bar with the colors representing the more intensely stained regions being higher up the bar. In the lower cell, $N^{Intra1768}$ is expressed at relatively high levels and both $N^{Intra1768}$ and Su(H) are found in the nucleus. In the upper cell, $N^{Intra1768}$ is expressed at relatively low levels and both $N^{Intra1768}$ and Su(H) are found in the cytoplasm. When cells are doubly stained for DNA and $N^{Intra1768}$, the two stains converge only when levels of $N^{Intra1768}$ are high compared with Su(H) (data not shown). Su(H) was detected with a rat anti-Su(H) antibody and $N^{Intra1768}$ with a mouse anti-Flag antibody.

the intracellular domain than $N^{p100B}$, or the two proteins may both have the same amino termini, but $N^{p100C}$ might have been additionally cleaved at the carboxyl terminus. It is also possible that there is a precursor product relationship between the two. In any case, the occurrence of $N^{p100C}$ only late in embryogenesis suggests that production of these forms of N is under developmental control.

Throughout most of embryogenesis, the majority of processed N proteins that are associated with Su(H) show some level of phosphorylation. Full-length N has been shown previously to be phosphorylated on serines (Kidd et al. 1989). We do not know how the latter relates to the phosphorylation described here, although the presence of hypophosphorylated forms of N bound to Su(H) suggests that the two events are unrelated. How this phosphorylation is effected and how it influences N function is not known. There are two lines of evidence that suggest that phosphorylation is not an immediate consequence of ligand binding and cleavage. First, most if not all of $N^{Intra1790}$, none of which has been produced as a result of ligand binding and cleavage of N, is phosphorylated (Fig. 2C). Second, overexpression of Dl induces at least one processed form of N which is hypophosphorylated (Fig. 4). In addition, we have shown that phosphorylation of $N^{Intra1790}$ is not dependent on the presence of Su(H) (Fig. 2C). Because most, if not all, of $N^{Intra1790}$ is phosphorylated and there is an enrichment

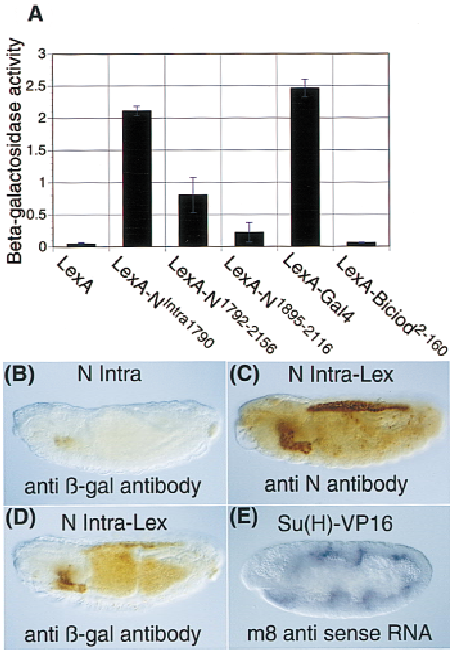


**Figure 8.** The cytoplasmic domain of N can behave as a transcriptional activator. (*A*) The cytoplasmic domain of N is a transcriptional activator in yeast. The transcriptional activation activity of various N constructs fused to the DNA-binding domain of LexA (Fig. 1) was determined by their ability to drive expression from a LexA–β-galactosidase reporter. The bar marked LexA is the β-galactosidase activity of yeast expressing the vector alone, LexA–Bicoid$^{2\text{-}160}$ is a negative control. The height of the bar is the average of three samples, standard deviations are shown by the error bars. (*B*) N$^{Intra1790}$ cannot activate transcription from LexA–β-galactosidase reporter. A *lexA–β-gal; UAS N$^{intra1790}$; HS GAL4* embryo is stained with anti β-galactosidase antibody. There is anti β-galactosidase reactivity in the secretory cells and the anal pads that results from leakiness of the reporter. (*C*) N$^{Intra1790\text{–}LexA}$ accumulates to its highest levels in the salivary glands, amnioserosa, and midgut. A *lexA–β-gal*; *UAS N$^{intra1790\text{–}lexA}$*; HS GAL4 embryo is stained with anti-NPCR antibody. (*D*) N$^{Intra1790\text{–}LexA}$ induces expression of β-gal from the LexA–β-gal reporter. A *lexA–β-gal; UAS N$^{intra1790\text{–}LexA}$*; *HS GAL4* embryo is stained with anti β-galactosidase antibody. β-Galactosidase accumulates in the salivary glands, amnioserosa and midgut, which correspond with regions that accumulate the highest levels of N$^{Intra1790\text{–}LexA}$ in *C*. (*B*,*C*,*D*) Embryos were fixed 2 hr after a 30-min heat shock. (*E*) A heterologous activator fused to Su(H) can substitute for N function and activate transcription of *m8*. *N$^{264\text{-}47}$ FRT/ovo$^{D}$ FRT*; *h GAL4/hsflp* × *FM7 lac-Z/Y*; *myc Su(H)–VP16* embryos are stained with an *m8* probe. *m8* expression is induced in seven stripes, in which *h* is expressed. Overexpression of Su(H) alone does not result in induction of m8 expression (data not shown). Anti β-galactosidase antibody was used to distinguish the *N* null embryos.

of N$^{pp114}$ in the soluble fraction (Fig. 5), perhaps phosphorylation is related to the release of cleaved intracellular N from the membrane. Alternatively, phosphorylation may promote nuclear translocation or association with Su(H), or both.

There is some salt extractable N$^{pp114}$ associated with Su(H) in the membrane fraction. Finding the intracellular domain of N, which contains functional nuclear localization signals either in the membrane or cytoplasmic fractions, indicates that the cell contains mechanisms to restrain the nuclear entry of N cleavage products. Because it has been demonstrated that the cdc10 repeats of N mediate homodimerization (Matsuno et al. 1995; Roehl et al. 1996), newly produced intracellular forms of N may be retained by full-length forms of N at the membrane. This association might be particularly favored if, as believed, the receptor is presented at the cell surface as a dimer (Foster 1975; Portin 1975; Kelley et al. 1987; de Celis and Garcia-Bellido 1994). It is also conceivable that N$^{pp114}$ is retained on the membrane by a complex of Su(H) and full-length N.

### *Su(H) may regulate nuclear entry of N*

With respect to cytoplasmic retention of Su(H)/N$^{pp114}$ complexes, regulation may come from Su(H) itself. We have shown that whereas coexpressing high levels of N$^{Intra}$ along with Su(H) in S2 cells results in both proteins translocating to nuclei, when low levels of N$^{Intra}$ are coexpressed along with Su(H) in S2 cells, there is retention of N$^{Intra}$ in the cytoplasm. This suggests that excess Su(H) can promote cytoplasmic localization of soluble, intracellular forms of N. Given that there are multiple binding sites for Su(H) in the cytoplasmic domain of N (Kato et al. 1997; Wettstein et al. 1997; S. Kidd, unpubl.), differences in subcellular localization could reflect the number of Su(H) molecules bound to N, with changes in stoichiometry resulting from increased levels of intracellular N in response to ligand. Because in vivo levels of Su(H) appear to be in excess to soluble N product, as there is sufficient Su(H) to bind to ectopically expressed N$^{Intra}$ and generate gain of function phenotypes (Lieber et al. 1993; Rebay et al. 1993; Struhl et al. 1993), the cytoplasmic retention we observe in *Su(H)*$^{+}$ embryos is expected from the S2 cell studies. Further supporting our view that Su(H) can retain soluble N in the cytoplasm in vivo, we found that lowering the dose of *Su(H)* promotes nuclear localization of N$^{Intra}$ in embryos (Fig. 6E). We also find that lowering the dose of *Su(H)* increases the severity of the phenotype produced by ectopic expression of gain-of-function N proteins in transgenic flies: Whereas complete loss of *Su(H)* abolishes the ability of the *E(spl)* complex to respond to activated N (Bailey and Posakony 1995), lowering the *Su(H)* dose by one-half increases the lethality as well as the bristle loss observed in transgenic flies carrying N$^{\Delta LNrpts}$ under control of a heat shock promoter (T. Lieber, unpubl.). A priori, one would have predicted that lowering the dose of a downstream component in the *N* pathway would decrease the severity of gain-of-function *N* mutations. Lastly, it is possible that the subcellular distribution of Su(H)/N$^{pp114}$ complexes is regulated by interaction with additional factors. For example, it has been shown that numb, a membrane-associated protein that is asymmetrically localized during division of sensory organ precursor cells in the peripheral nervous sys-

tem, is able to retain $N^{Intra}$ at the membrane and in the cytoplasm of S2 cells (Frise et al. 1996).

In the absence of Su(H), both $N^{Intra}$ and N appear to have undergone additional modification. In addition, many intermediately sized N proteins are missing (Fig. 2C). Lecourtois and Schweisguth, (1998) and Schroeter et al. (1998) have suggested that the processed form of N is less stable in the absence of Su(H). Many proteins are targeted to the proteosome by ubiquitinylation. Perhaps the modification of $N^{Intra}$ and N we see in the absence of Su(H) is ubiquitinylation. Interestingly, phosphorylation has also been shown to target proteins to the ubiquitinylation machinery (King et al. 1996).

In addition to being required for the production of $N^{pp114}$, the N ligand Dl, when overexpressed, promotes accumulation of a hypophosphorylated N protein that has approximately the same mobility as $N^{p100B}$ (Fig. 4A). Our fractionation studies also showed the presence of a hypophosphorylated protein of approximately the same size as $N^{p100B}$, in this case associated with Su(H). This protein is retained in the membrane fraction under salt conditions that remove $N^{pp114}$, suggesting that it is tightly associated with the membrane and may well span it (Fig. 5C). The extracellular domain of N has been shown previously to be cleaved at several positions (Blaumueller and Artavanis-Tsakonas 1997; Pan and Rubin 1997; Logeat et al. 1998). It has been proposed that the cleavage closest to the membrane is ligand dependent (Logeat et al. 1998). Such a cleavage product may correspond to the protein we described above.

### *Soluble N as a transcriptional transactivator*

The work of Lecourtois and Schweisguth (1997) and Struhl and Adachi (1998) has shown genetically that the cytoplasmic domain of N has access to the nucleus. The most likely explanation for their results is that *Drosophila* N is proteolytically cleaved at the site described by Schroeter et al. (1998) to produce the fragment of N, $N^{pp114}$, that we have described in this paper. We have shown that when tethered directly to DNA via a bacterial DNA-binding domain, the cytoplasmic domain of N can activate transcription both in yeast and in vivo. Conversely, a viral activator fused to Su(H) can substitute for the functions of N mediated by its ability to activate transcription of *m8*, a natural target of N signaling, in embryos. Whereas maximal activation in yeast is seen with the entire cytoplasmic domain, in agreement with the results of Roehl et al. (1996) a truncated form of the cytoplasmic domain ($N^{1792–2156}$; Fig. 1) encompassing the cdc10 repeats does weakly activate and has a gain-of-function phenotype in embryos (T. Lieber, unpubl.). Smaller versions of the cytoplasmic domain ($N^{1895–2156}$) spanning just the cdc10 repeats are even weaker activators and when expressed in wild-type embryos do not have a gain-of-function phenotype (T. Lieber, unpubl.). Our data suggest that the prime function of the sequences downstream of the cdc10 repeats is to provide transactivator activity. In accord with this, the cytoplasmic domain of N has many features that are found in transcriptional activators (Lieber et al. 1993). Although it is possible that N indirectly confers activating ability on Su(H), given the finding of appropriately processed N proteins, which contain functional nuclear localization signals preferentially associated with Su(H), the simplest interpretation of our results is that one function of N is to bind to Su(H) and in the nucleus to directly act as its transcriptional transactivator. Recently it has been suggested that N activates transcription by disrupting the formation of a repressor complex between Su(H) and a histone deacetylase complex (SMRT/HDAC-1) (Kao et al. 1998). Our data suggest that rather than simply disrupting the Su(H)/SMRT/HDAC-1 complex, $N^{pp114}$ plays a more active role of providing transactivator activity to Su(H).

One other class of membrane-bound transcription factors has been identified previously. The proteolysis of sterol regulatory element binding proteins (SREBPs) (for review, see Brown and Goldstein 1997) is regulated by sterols that accumulate in membranes. As N like molecules have been found in all multicellular organisms where they have been sought, N is an evolutionarily old protein. The existence of a transcription factor that spans the membrane with an extracellular domain capable of interacting with ligands and an intracellular domain that can enter nuclei and activate transcription would provide a simple means for transducing information from neighboring cells. Possibly, the only additional components required would be a protease capable of recognizing a conformational change induced in N on ligand binding resulting in its cleavage, and a second protease that would degrade the cytoplasmic domain in nuclei so that the signaling could be terminated.

### *Establishing a threshold for Notch signaling*

The binary epidermal versus neural cell fate choice mediated by the *N* signaling pathway involves regulating groups of initially equivalent cells that express both ligand and receptor. Schroeter et al. (1998) have shown that in vertebrate cell culture, extremely low levels of nuclear N are sufficient for function, and our studies of the wild-type *Drosophila* embryo are consistent with this finding in that no N is detected in the nucleus either biochemically or by immunofluorescence. However, relatively abundant cleaved N associated with Su(H) is detected in the cytoplasm. Why should there be such a disparity between levels of soluble N in the cytoplasm and nucleus, and why shouldn't such a potent nuclear N signal favor saltatory cell fate decisions, with all cells composing an equivalence group assuming the same secondary cell fate? Uniform expression of ligand and receptor among interacting cells might also be expected to favor a saltatory response.

Some of the puzzling aspects of N signaling are reminiscent of ultrasensitive systems such as the *Xenopus* oocyte system described by Ferrell and Machleder (1998), in which a continuously variable signal, progesterone, is converted into an all-or-none response, oocyte maturation. An ultrasensitive system exhibits little response to

low levels of stimulus but switches from off to on over a narrow range of stimulus concentration. We suggest that the cytoplasmic retention of $N^{pp114}$/Su(H) complexes described in the present study may similarly reflect a mechanism in which the response to low levels of signal is damped. In such a model for N signaling, only high levels of signal result in sufficient cytoplasmic accumulation of $N^{pp114}$/Su(H) complex to permit its nuclear entry.

In the *Xenopus* oocyte system, added ultrasensitivity is provided by a positive feedback loop. Earlier genetic studies have suggested that small differences in the expression of N and Dl may also be amplified by positive feedback to generate robust intercellular differences in the expression of these proteins among cells derived from an equivalence group (Seydoux and Greenwald 1989; Heitzler and Simpson 1991; for review, see Greenwald 1998). By affecting the nuclear entry of N/Su(H) complex, which functions as a transcriptional activator, Su(H) would also be an element of such a feedback mechanism. We suggest that the N signal is initiated and maintained according to the relative amounts of N, Dl, and Su(H). Together, these would determine the rate and duration of accumulation of N/Su(H) complex and the threshold at which it enters the nucleus.

## Materials and methods

### *Constructs*

$N^{Intra1768}$ was expressed by cloning into a derivative of pUAST (Brand and Perrimon 1993) which contains the cactus initiation codon fused to a Flag epitope (Kidd 1992). To express the cytoplasmic domain of N by coupled in vitro transcription/translation (Promega), a derivative of $N^{Intra1768}$ was made in which the region containing the last three introns of N was replaced with the corresponding segment of cDNA. $N^{Intra1790}$ contains the first two amino acids of cactus followed by a 14 amino acid myc epitope (Xu and Rubin 1993), which was then fused to amino acid 1790 of N. The $hsN^{Intra\ 1790}$ used in Fig. 2C is $N^{Intracellular\ domain}$ (Lieber et al. 1993).

$N^{LexA}$ has amino acids 1–87 of LexA fused to the carboxyl terminus of N. $N^{\Delta EGF1\text{-}36\text{–}LexA}$ is a derivative of the above lacking the 36 EGF repeats. $N^{Intra1790\text{–}LexA}$ has amino acids 1–87 of LexA fused to the carboxyl terminus of $N^{Intra1790}$. The LexA–β-galactosidase, reporter has eight LexA operator sites (Ebina et al. 1983) upstream of a heat shock minimal promoter. This was then inserted in place of the GAL4–UAS region of pUAST (Brand and Perrimon 1993).

Myc-tagged Su(H) contains the first two amino acids of cactus followed by a 14 amino acid myc epitope that was then fused to amino acid 10 of Su(H) (Schweisguth and Posakony 1992). Myc-tagged Su(H)–VP16 has amino acids 19–105 of VP16 (Campbell et al. 1984) fused to the carboxyl terminus of myc Su(H).

With the exception of LexA–β-gal, all constructs were subcloned into pUAST (Brand and Perrimon 1993) for transformation into flies.

Yeast expression experiments were carried out as described by (Gyuris et al. 1993).

### *Fly stocks*

The following fly stocks, *w ovoD1 FRT101*; *hsFLP*, *yw FRT101* (Chou et al. 1993), *hsFLP12*; *Sco/Cyo*, *ovoD1 FRT40A/Cyo* (Chou and Perrimon 1996), *h-GAL4*, *hs-GAL4* (Brand and Perrimon 1993) were obtained from A. Brand and N. Perrimon; *Su(H)* (FlyBase 1998) was obtained from the Bloomington Stock Center; $Su(H)^{SF8}$ *FRT40A/CyO* (Schweisguth and Posakony 1994) was obtained from F. Schweisguth; $Dl^{6B}$ and $Dl^{RF}$ (Parks and Muskavitch 1993) were obtained from M. Muskavitch, *UAS–Dl*, (Doherty et al. 1996) was obtained from Y. Jan. $N^{264\text{–}47}$ and $Dl^{x}$ are described by (FlyBase 1998).

### *Antibodies*

Antibodies were raised against histidine-tagged Su(H) as previously described (Kidd et al. 1986; Lieber et al. 1993). The remaining N antibodies (shown in Fig. 1) have been described previously (Lieber et al. 1993). Anti-LexA monoclonal antibody was from Clonetech. M5 anti-Flag antibody was from Kodak. c-Myc antibody was from Calbiochem. SYTOX Green used to label S2 cell nuclei was from Molecular Probes. Immunocytochemistry and immunofluorescence was as described previously (Lieber et al. 1993). Double labeling with RNA and antibody was as described by Azpiazu and Frasch (1993).

### *Immunoprecipitations*

Embryo extractions and immunopreciptations were essentially as described by Kidd (1992). Between 300 µg and 1 mg of protein were used for immunoprecipitation with anti-Su(H) antibodies, one-fifth of this amount was used with anti-N antibodies. Immunoprecipitions were carried out overnight with protein A–Sepharose and Gamma Bind (Pharmacia) to collect rabbit and rat and mouse antibodies, respectively. After washing, the immunoprecipitates were treated with alkaline phosphatase (Boehringer Mannheim) as described previously (Kidd 1992) and electrophoresed without further washes. After blotting, N in rabbit anti-N and rat anti-Su(H) immunoprecipitates was detected with mouse anti-N, Su(H) in mouse anti-N immunoprecipitates was detected with rat anti-Su(H). Horseradish peroxidase (HRP) conjugated secondary antibodies were from Jackson. HRP activity was detected by the ECL system (Amersham).

Scanned autoradiographs were quantitated on a Macintosh computer with the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

Two procedures were used to produce subcellular fractions of embryos. The first, used for Figure 5A, was based on procedures for producing extracts for gel shifts (Andrews and Faller 1991). The second procedure, used for Figure 5, B and C, was as follows: Dechorionated embryos were extensively homogenized in 10 mM HEPES (pH7.6), 100 mM KCl, 50 mM NaF, 2 mM EDTA, 2 mM EGTA, and 2 mM ammonium molybdate with protease inhibitors. After centrifuging the homogenate at 900*g* for 5 min, the resulting postnuclear supernatant was incubated on ice for 20 min either with no additional salt or an additional 0.4 or 0.8 M KCl, and then centrifuged for 2 hr at 100,000*g*. The supernatants were adjusted to 0.5% Triton X-100 and to ~400 mM KCl, and the pellets resuspended in the Triton lysis solution prior to immunoprecipitations.

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