A Functional Analysis of *Notch* Mutations in Drosophila

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ABSTRACT

The *Notch* gene encodes a receptor protein that is involved in many processes during development. Its best understood role is during neurogenesis in a process called "lateral inhibition." However, it has been proposed that *Notch* also has a role in defining the proneural clusters in the first place. This raises the possibility that the Notch protein is acting as a multifunctional receptor. To test this hypothesis, we have carried out a genetic analysis of molecularly characterized *Notch* alleles to identify alleles that affect only one of the two proposed functions. Here we present evidence that *Notch* alleles can be identified that appear to affect the function of Notch during either lateral inhibition or the definition of proneural clusters. In addition our results indicate that there may be discrete regions of the Notch protein required for each function.

THE *Notch* gene of Drosophila encodes a large, single span, transmembrane protein (WHARTON *et al.* 1985; KIDD *et al.* 1986) that is required for many cell fate decisions during development (SHELLENBARGER and MOHLER 1978; HARTENSTEIN *et al.* 1992). The extracellular domain contains 36 tandemly arranged epidermal growth factor (EGF)-like repeats that are believed to be involved in the binding of extracellular ligands (FEHON *et al.* 1990; REBAY *et al.* 1991; LIEBER *et al.* 1992), and three cysteine rich repeats, called lin-12/Notch repeats. The intracellular domain comprises three regions: the RAM-23 domain adjacent to the membrane (TAMURA *et al.* 1995); six cdc10 repeats, which have also been implicated in protein-protein interactions (DIEDERICH *et al.* 1994; FORTINI and ARTAVANIS TSAKONAS 1994); and a large C terminal domain, part of which interacts with the Dishevelled protein (AXELROD *et al.* 1996).

The best understood role of *Notch* in development occurs during neurogenesis in a process known as "lateral inhibition" (HEITZLER and SIMPSON 1991). In both the embryo and the adult of Drosophila, development of the peripheral nervous system is initiated by the activation of the proneural genes of the *achaete scute complex* in small groups of cells, known as proneural clusters (CAMPUZANO and MODOLELL 1992). The expression of these genes endows all the cells within the cluster with the potential to become neural. However, only one cell within each cluster adopts the neural fate and, in the process, it emits a signal that extinguishes proneural gene expression in its neighbors, which causes them to adopt an alternative, epidermal fate. The single cell that maintains the expression of the proneural genes goes on to form a sensory organ precursor cell (CUBAS

et al. 1991), which will undergo differential divisions to form the components of the sensory organ (HARTENSTEIN and POSAKONY 1990).

The process by which the neural fate is restricted to a single cell is known as lateral inhibition and the Notch protein is the receptor for the inhibitory signal (HEITZLER and SIMPSON 1991). The signal itself is encoded by the *Delta* gene (HEITZLER and SIMPSON 1991) and is transduced to the nuclei of the receiving cells by the Suppressor of Hairless protein (FORTINI and ARTAVANIS TSAKONAS 1994; BAILEY and POSAKONY 1995; LECOURTOIS and SCHWEISGUTH 1995; SCHWEISGUTH 1995).

However, the Notch protein is required for the development of many cell types other than the peripheral nervous system. In some of these the function of *Notch* does not appear to be dependent on *Delta*, suggesting that it is involved in different signaling processes. For example, during the formation of the egg chamber it is suggested that *Notch* functions with the *egghead* and *brainiac* genes in a function that is distinct from lateral inhibition (GOODE *et al.* 1996). It has also been proposed that the Notch protein may also have a function during the definition of proneural clusters through a signaling event that is distinct from that of lateral inhibition (COUSO and MARTINEZ ARIAS 1994; HING *et al.* 1994). This hypothesis stems from a close genetic relationship between *Notch* and the segment polarity gene *wingless*, whose function is required for the activation of the proneural genes in many regions in the embryo (M. RUIZ GOMEZ and A. MARTINEZ ARIAS, unpublished results) and the adult (PHILLIPS and WHITTLE 1993; COUSO *et al.* 1994).

Most of the functional analysis of *Notch* during neurogenesis has focused on its role during lateral inhibition and thereby on its relationship with *Delta* and other neurogenic genes. This has led to the division of *Notch* alleles into two classes: hypermorphic mutations, which

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have fewer sense organs than wild type; and hypomorhic mutations, which have more (DE CELIS and GARCIA BELLIDO 1994; HEITZLER and SIMPSON 1993). However, if *Notch* has a function in defining proneural clusters, it should be possible to identify classes of mutations that attenuate this function.

Here we present genetic evidence for functionally different classes of *Notch* alleles that affect different steps in the development of the peripheral nervous system. In particular one class affects a function of *Notch* in the establishment of proneural clusters, while other classes interfere with the role of *Notch* during lateral inhibition. In addition the molecular characterization of these alleles allows us to map these functions to discrete and different parts of the protein.

MATERIALS AND METHODS

Genetic strains: The *Notch* alleles used in this study are summarized in Table 1. In addition to these strains, the *Notch* duplication $Dp(1;2)51b$ (LINDSLEY and ZIMM 1992), the *wingless* null alleles wg^{CX4} (BAKER 1987) and $wg^{S107.5}$ (S. BISHOP, L. OWEN and A. MARTINEZ ARIAS, unpublished results), the *Delta* null allele Dl^{FX3} (LINDSLEY and ZIMM 1992), and the wild-type strain Oregon R were used.

The *Notch* alleles used in this study can be defined as loss or gain of function by the criteria suggested by MULLER (1932). These criteria are based on the behavior of alleles in *trans* with a deficiency of the locus and in flies containing a duplication of the locus. Namely, the phenotype of a loss-of-function allele should be enhanced by a deficiency and suppressed by a duplication, while a gain-of-function allele should be suppressed by a deficiency and enhanced by a duplication. MULLER (1932) also defined two other types of allele, antimorphs and neomorphs. The phenotype of a neomorph should not be affected by either a duplication or a deficiency of the locus, while the behavior of an antimorph is similar to that of a loss-of-function allele. However, inherent in the definition of an antimorph is the fact that it is a special type of loss-of-function allele, a dominant negative allele.

Microchaete numbers: The notal microchaete of at least eight flies were counted under the dissecting microscope and the average number of microchaete on a heminotum was calculated.

Cuticle preparations: Flies were allowed to lay on an apple juice agar plate overnight at 25°. The embryos were allowed to age for a further 48 hr and collected. Then, the embryos were dechorinated in bleach and mounted in Hoyer's medium (ASHBURNER 1989).

Fly crosses: All fly crosses were carried out at 25° apart from crosses involving N^{60g11} and the intragenic complementation, which were carried out at 17°. Once the flies had emerged, they were collected and stored in SH solution (75% ethanol, 25% glycerol) until their notal microchaete were counted.

DNA sequencing: The complete DNA sequence of the protein coding regions of the six *Notch* alleles, N^{264-47}, N^{1081}, Ax^{M1}, $l(1)N^3$, N^{t14} and N^{xk11}, was determined. Genomic DNA was isolated from homozygous embryos and was sequenced by ABI PRISM Dye Terminator Cycle Sequencing (Perkin Elmer). The primers used for these reactions are available on request.

RESULTS

A revised genetic analysis of *Notch*: The *Notch* (*N*) locus contains a broad array of alleles (LINDSLEY and

ZIMM 1992). Here we have explored the genetic behavior of a collection of molecularly characterized mutations in *Notch* (see Table 1, Figures 1 and 4). To classify these alleles we have exploited the fact that the number of thoracic bristles reflects the activity of the Notch protein in various genetic backgrounds. Initially we examined the phenotype of the alleles in heterozygous conditions. We then classified the alleles as hypermorphs and hypomorphs by examining how the phenotype generated by the mutation is altered in hemizygous conditions and in triploids containing two copies of the wild-type *Notch* gene. Finally we have examined the behavior of the alleles in genetic backgrounds that either reduce the expression of the proneural genes (*wingless* heterozygous) or weaken the lateral inhibition signal (*Delta* heterozygous).

One possible caveat to this analysis lies in the fact that *Notch*, in addition to its role in lateral inhibition, is also involved in the assignation of individual fates to the progeny of the sensory organ precursor (SOP; HARTENSTEIN and POSAKONY 1990). Normally four different cell types are formed by the progeny of the SOP cell: the neurone, the glial cell, the bristle cell and the socket cell (HARTENSTEIN and POSAKONY 1989). In the absence of *Notch* function, all the progeny of the SOP cell become neurones and consequently there will not be a bristle cell. In such a situation, although a SOP cell has formed, it would not be noticed in our analysis because the bristle cell is absent. Therefore bristle number might not always reflect exactly the number of SOP cells formed in the epithelium, which would bias our analysis. To test this possibility we have stained flies heterozygous for *Notch* null alleles with the neural specific antibody 22C10. This allowed us to correlate the number of bristles with the number of neurones in a situation where it is most likely that the number of bristles and neurones are not equal. In our experiments we have never observed a significant difference between these numbers and therefore consider that, in our study, the number of bristles is a fair representation of the number of SOPs formed.

Notch mutations that increase bristle number: Two classes of mutations were identified that increase the number of bristles on the thorax of the adult fly. Although in many ways the behavior of these two classes is very similar, they can be distinguished on two grounds: their behavior when transheterozygous with a *Delta* null allele and their ability to complement other mutations of the *Notch* gene (see below).

Alleles that do not respond to reductions of Delta function: Only two of the alleles we tested fall into this class, $Df(1)N^{81k1}$ and N^{55e11}. Flies heterozygous for these mutations have a marked increase in the number of sensory bristles on the notum (Table 2). Alleles of this class behave as loss-of-function mutations because their phenotype is enhanced in hemizygous conditions and is rescued, practically to wild type, by a duplication of the

TABLE 1

Notch alleles used in this study

Allele	Lesion	Reference	$N^{60g11}/+$ Phenotype
$Df(1)N^{81k1}$	Deletion of the locus	GRIMWADE *et al.* (1985)	Neurogenic
N^{55e11}	Insertion closely associated with the first exon of the *Notch* gene	KIDD *et al.* (1983)	Neurogenic
N^{MI}	Point mutation in EGF repeat 12	DE CELIS *et al.* (1993)	Neurogenic
spl	Point mutation in EGF repeat 14	KELLEY *et al.* (1987) PREISS *et al.* (1988)	Neurogenic
N^{su42c}	Point mutation in the fifth cdc10 repeat	DIETRICH *et al.* (1993)	Neurogenic
N^{264-47}	A 4-bp deletion at nucleotide 11362 in EGF repeat 19 forming a TGA stop codon at codon 763	This work	Neurogenic
N^{pk11}	Lesion is not within the protein encoding DNA	This work	Neurogenic
N^{60g11}	Small deletion after the cdc10 repeats resulting in termination of the protein	LYMAN and YOUNG (1993)	Antineurogenic at 17°
Ax^{59d}	Point mutation in EGF repeat 24	KELLEY *et al.* (1987)	Wild type
Ax^{M1}	Point mutation at amino acid 999 in EGF repeat 25 resulting in the Cys to Tyr amino acid substitution	This work	Wild type
$l(1)N^{3}$	A 2-bp deletion at nucleotide 16682 resulting in a frameshift adding 46 unique amino acids and terminating at codon 2528	This work	Wild type
N^{1081}	Point mutation at amino acid 2490 in the intracellular domain resulting in a Gly to Cys amino acid substitution	This work	Wild type
$l(1)N^{B}$	Point mutation in the third lin-12/*Notch* repeat	LYMAN and YOUNG (1993)	Antineurogenic
N^{414}	Point mutations at amino acids 1577 and 1641 in and just after the third lin-12 repeat resulting in a Asp to Gly and Gln to Arg amino acid substitution, respectively	This work	Antineurogenic
N^{141}	Mutation not determined	This work	Wild type

Brief summary of the *Notch* alleles used in this study, indicating the class to which we have assigned the allele, the lesion associated with the allele, the reference in which the allele was molecularly characterized, and the general phenotype of the heterozygous adult female. Nucleotide positions according to KIDD *et al.* (1986).

Notch gene (Table 2). The phenotype of these alleles can also be modified if the efficiency of proneural cluster induction is altered; when the concentration of the Wingless protein is reduced, as in $Df(1)N^{81k1}/+$; $wg^{S107}/+$ flies, we observe a correction of the number of microchaete to wild type (Table 2). However the number of bristles is not affected if a single copy of the *Delta* gene is removed (Table 2; also see DE CELIS *et al.* 1991 for N^{264-39} allele). Finally these alleles are likely to be amorphs because $Df(1)N^{81k1}$ is a deletion of the entire *Notch* gene (GRIMWADE *et al.* 1985).

Alleles that do respond to reductions in Delta function: Five alleles that we tested fall into this class, NM^1, *spl*, N^{su42c}, N^{264-47} and N^{pk11}. Like members of the above class these alleles have a dominant phenotype that results in an increase in the number of thoracic bristles (Table 3), although the increase is not as marked; behave as hypomorphic mutations (Table 3); and their phenotype is corrected to wild type if a single copy of the *wingless* gene is removed (Table 3). However, unlike the above class, the number of sensory bristles increases still further when the process of lateral inhibition is also attenuated by reducing *Delta* function. In the case of N^{M1}, the interaction in transheterozygotes with Df^{FX3} results in lethality before pupation. The published genetic behavior of another mutation N^{Co} suggests that it may also belong to this class (LYMAN and YOUNG 1993).

Although the behavior of the alleles of this class is similar to that of $Df(1)N^{81k1}$ and N^{55e11}, they can be distinguished on two accounts: the phenotype of these mutants responds to changes in the dosage of *Delta* and these mutants are able to complement certain alleles of *Notch*, which $Df(1)N^{81k1}$ and N^{55e11} are unable to do. For example, Ax^{59d}/N^{M1} flies have a similar number of bristles to $Ax^{59d}/+$, while $Ax^{59d}/Df(1)N^{81k1}$ have very few bristles (Tables 4 and 5).

The mutations associated with the N^{M1}, *spl*, N^{su42c} and N^{Co} alleles have all been mapped previously and have been shown to be point mutations (KELLEY *et al.* 1987; PREISS *et al.* 1988; DE CELIS *et al.* 1993; LYMAN and YOUNG 1993; DIEDERICH *et al.* 1994). Two of them, N^{M1} and *spl*, lie within EGF repeats 12 and 14, respectively, in the extracellular domain, while the other two are in the intracellular domain: the N^{su42c} mutation disrupts the structure of the fifth cdc10 repeat and the N^{Co} mutation truncates the Notch protein just after the RAM-23 domain. We have mapped the mutation associated with the N^{264-47} allele; it produces a stop codon at amino acid 763, which will truncate the Notch protein within EGF repeat 19 (Table 1). Unfortunately we were unable to find the N^{pk11} mutation within the coding region and therefore we assume that its phenotype might be associated with a regulatory mutation or a mutation resulting in aberrant splicing of the *Notch* transcript.
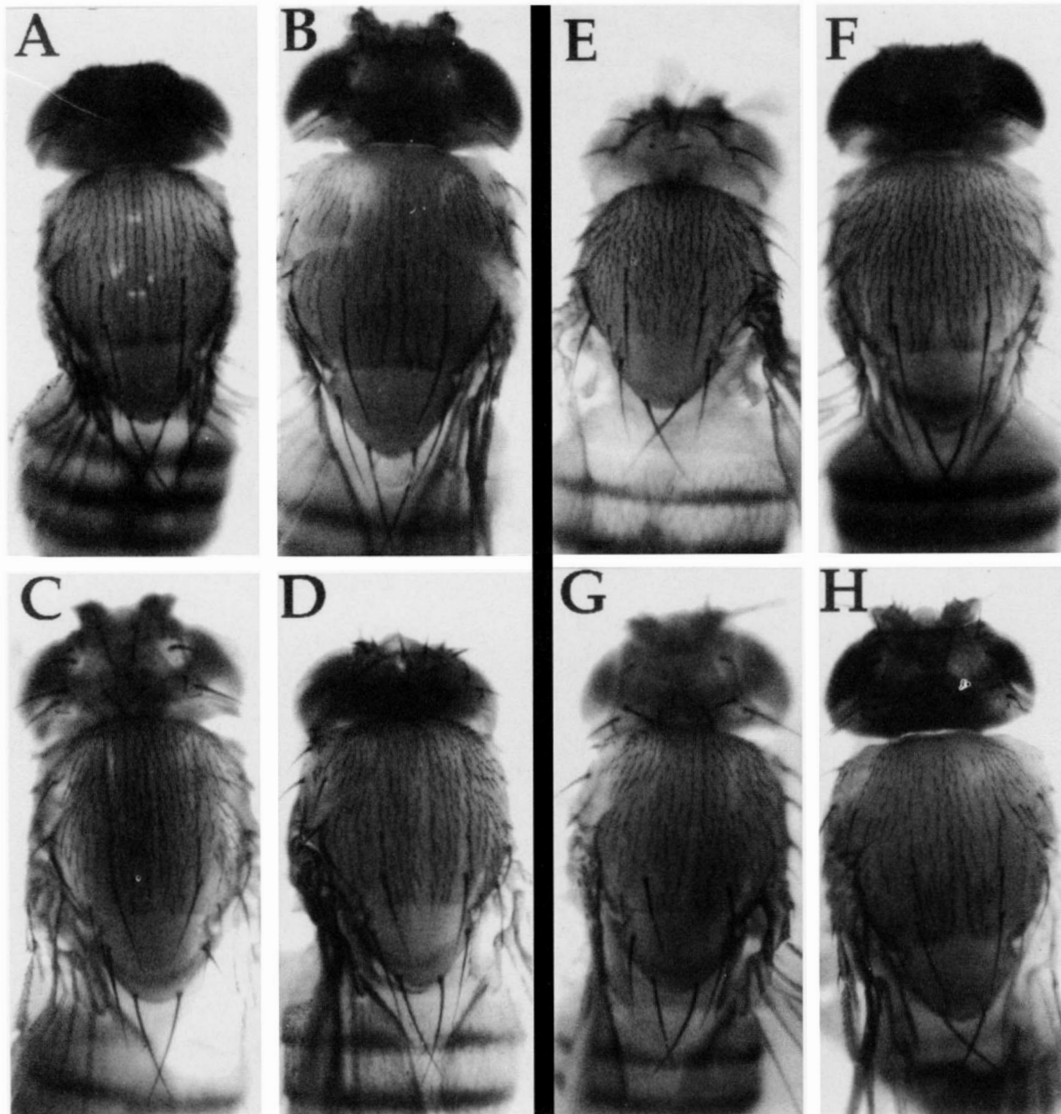
FIGURE 1.—Heterozygous thoracic phenotypes of *Notch* alleles used in this study: (A) *Oregon R*, (B) *l(1)N^B*/+, (C) *Df(1)N^{81k1}*/ +, (D) *N^{55e11}*/+, (E) *N^{Mll}*/+, (F) *N^{nu42c}*/+, (G) *Ax^{59d}*/+, and (H) *N^{60g11}*/+.

Notch alleles that reduce bristle number: We have identified two classes of alleles that reduce the number of sensory bristles. These two classes are easily distinguishable on genetic grounds because members of one class behave as hyper morphs while members of the other class behave as hypo morphs. They also demonstrate a difference in behavior to reductions in *Delta* function; the hypermorphic class does not respond to changes in *Delta* function, while the hypomorphic class is strongly affected.

Hypermorphic mutations of Notch that reduce bristle number: Three alleles we have studied, *l(1)N^B*, *N^{414}* and *N^{141}*, behave as hypermorphic mutations that reduce bristle number in flies heterozygous for the mutation (Table 6). Namely their phenotype is enhanced by a duplication of the *Notch* gene and suppressed by a deficiency. In the case of receptors, hypermorphic mutations can generate proteins that will signal more efficiently either by increasing the affinity of the receptor for the signal-

TABLE 2

Alleles that increase bristle number which are insensitive to reductions in Delta function

	OR	Df(1)N	Dp(1;2)51b	wg^{S107}	Dl^{FX3}
OR	130.7 ± 5.21	192 ± 7.73	146.6 ± 3.54	133.4 ± 2.80	193.3 ± 7.20
Df(1)N^{81k1}	192 ± 7.73	Lethal	141.1 ± 2.56	139.8 ± 4.79	200.5 ± 7.78
N^{55e11}	184.6 ± 3.82	Lethal	ND	146.8 ± 9.06	200.8 ± 9.98

Values are the number of thoracic microchaete on a heminotum ± SD. Lethal, no adult progeny are obtained of that genotype; ND, the number of thoracic bristles was not calculated for that genotype.

TABLE 3

Alleles that increase bristle number which are sensitive to reductions in Delta function

	OR	Df(1)N	Dp(1;2)51b	wg^{S107}	Df^{FX3}
N^{M1}	163.3 ± 10.3	Lethal	140.8 ± 5.23	131.8 ± 8.84	Lethal
spl	142.5 ± 4.29	198.3 ± 17.6	144.2 ± 8.12	133.3 ± 3.68	194.1 ± 5.28
N^{su42c}	167.0 ± 2.68	199.5 ± 8.20	139.4 ± 3.68	140.1 ± 3.71	181.9 ± 8.44
N^{264-47}	177.1 ± 3.84	Lethal	ND	141.1 ± 4.30	210.6 ± 6.65
N^{pk11}	173.6 ± 5.65	Lethal	148.3 ± 1.89	154.0 ± 4.53	211.1 ± 5.66

Values are the number of thoracic microchaete on a heminotum ± SD. Lethal, no adult progeny are obtained of that genotype; ND, the number of thoracic bristles was not calculated for that genotype.

ing ligand or by causing the receptor to activate the intracellular signaling cascade in the absence of the ligand. These two situations can be distinguished because the latter should be unaffected by reductions in the amount of the signaling ligand. The three alleles we have identified all appear to result in constitutively signaling Notch proteins because their phenotype is unaltered in Df^{FX3} transheterozygotes (Table 6). If proneural gene expression is attenuated in wg^{S107} transheterozygotes, the phenotype of these mutants is enhanced (Table 6). Physical mapping of the mutation associated with the N^{t14} allele indicates that it is in the lin-12/Notch repeats (Table 1) and is very close to the mutation in the $l(1)N^B$ allele (LYMAN and YOUNG 1993). We have not mapped the molecular lesion of the N^{t41} allele.

Hypomorphic mutations that reduce bristle number: One allele we tested, N^{60g11}, is a canonical member of this class. N^{60g11} transheterozygotes have fewer bristles than wild type. It is also a hypomorphic mutation, because it is enhanced by a deficiency of *Notch* and suppressed by a duplication of *Notch* (Table 4). The $N^{60g11}/+$ phenotype is also enhanced when proneural development is inhibited in a *wingless* heterozygote and suppressed when lateral inhibition is reduced in a heterozygous *Delta* background (Table 4).

Four other alleles we have tested, Ax^{59d}, Ax^{M1}, $l(1)N^B$ and N^{1081}, appear to be members of this class. Although these mutants do not have fewer bristles than wild type in heterozygotes, they behave in a very similar manner to N^{60g11} in wg^{S107} and Df^{FX3} transheterozygotes (Table

4). The inclusion of these alleles into this class does appear sensible when the molecular mapping of the mutations is considered; $l(1)N^B$ and N^{1081} both mutate the region of the Notch protein C terminal to the cdc10 repeats, which is deleted in the N^{60g11} allele (see Table 1 and LYMAN and YOUNG 1993). Also the mutations that are responsible for the Ax^{59d} and Ax^{M1} alleles destroy the structure of the EGF repeat they are in and therefore are likely to damage the structure of neighboring EGF repeats (see Table 1 and KELLEY *et al.* 1987). Consequently these mutations are likely to abolish the function of the Notch protein locally within the extracellular domain.

This class includes two clearly different kinds of mutations: Ax^{59d} and Ax^{M1} are pupal lethal, whereas the other three mutations are embryonic lethal. This difference is exemplified by the behavior of Ax^{59d} and N^{60g11} over a deficiency: $Ax^{59d}/Df(1)N^{81k1}$ flies die as pharate adults and display extreme *Abruptex* phenotypes, while $N^{60g11}/Df(1)N^{81k1}$ individuals die as embryos with holes in the ventral cuticle. The different behavior of these two alleles probably arises due to differing stabilities of the proteins they encode.

The two *Abruptex* alleles we describe here have been analyzed previously. Most recently they have been discussed as hypermorphic mutations (HEITZLER and SIMPSON 1993; DE CELIS and GARCIA BELLIDO 1994) notwithstanding the fact that one of them, Ax^{59d}, has been described in early work as a hypomorphic mutation (PORTIN 1975; KELLEY *et al.* 1987). In our study, we have found no evidence that either allele behaves as a

TABLE 4

Hypomorphic mutations that reduce bristle number

	OR	Df(1)N	Dp(1;2)51b	wg^{S107}	Df^{FX3}
N^{60g11}	93.7 ± 5.89	Lethal	116.8 ± 5.05	Lethal	139.4 ± 13.7
$l(1)N^3$	139.8 ± 5.33	ND	137.4 ± 2.69	116.6 ± 3.42	165.1 ± 4.99
N^{1081}	133.4 ± 2.14	ND	146.5 ± 6.84	110.8 ± 5.55	167.8 ± 7.42
Ax^{59d}	132.0 ± 3.89	8.5 ± 1.26	135.9 ± 6.39	113.9 ± 6.60	151.4 ± 3.82
Ax^{M1}	127.9 ± 2.25	ND	141.3 ± 1.71	111.0 ± 4.43	165.3 ± 6.59

Values are the number of thoracic microchaete on a heminotum ± SD. Lethal, no adult progeny are obtained of that genotype; ND, the number of thoracic bristles was not calculated for that genotype.

TABLE 5

Series of phenotypic strength of *Notch* alleles in transheterozygotes

	OregonR	*Ax^{59d}*
Df(1)N^{81k1}	192 ± 7.73	8.5 ± 1.26
N^{MI}	163.3 ± 10.3	116.8 ± 10.5
N^{ts1}	154.7 ± 6.20	15.3 ± 8.20
OregonR	130.7 ± 5.21	132.0 ± 3.89

Values are the number of thoracic microchaete on a heminotum ± SD.

hypermorph and since the phenotype of the *Ax^{59d}* allele is greatly enhanced when placed in *trans* with a deficiency of the *Notch* locus, our data suggest that these mutations are hypomorphic.

Other *Abruptex* mutations may also belong to this class. However due to the different behavior of the *Abruptex* alleles with a deficiency of the *Notch* locus (POR-TIN 1975) it will be necessary to assess each one individually. It is also likely that the *nd^2* allele falls into this class. This allele is temperature sensitive. At 17° homozygous *nd^2* flies display a phenotype similar to *N^{60g11}* heterozygotes; *nd^2* does not complement *N^{60g11}* (R. TATESON, J. P. COUSO and A. MARTINEZ ARIAS, unpublished results).

The distinction between the *l(1)N^B* and *Ax^{59d}* classes of alleles is also supported by data that indicate that the phenotype of *l(1)N^B* is dependent upon Su(H) function while the phenotype of *Ax^{59d}* is not dependent upon Su(H) function (R. TATESON, K. BRENNAN and A. MARTINEZ ARIAS, unpublished results). This work also indicates that the expression of the proneural genes is reduced in *Ax^{59d}*/*Y* flies, suggesting that alleles of the *Ax^{59d}* class disrupt the initiation of bristle development by diminishing the expression of the proneural genes.

Functional complementation: Although the two classes of *Notch* alleles that reduce the number of bristles are clearly different, it is possible that the two classes that increase bristle number simply reflect loss of *Notch* function to different degrees. Namely the class that does not respond to changes in *Delta* function are amorphs while the class that does respond to changes in *Delta* function are hypomorphs. This would lead to a series in phenotypic strength to be drawn, with weak hypomorphs having the weakest phenotype and

amorphs having the strongest phenotype in heterozygous conditions. Such a series can be drawn with *Df(1)N^{81k1}*/+ having the strongest phenotype, *N^{MI}*/+ having an intermediate phenotype, *N^{ts1}*/+ (a temperature-sensitive hypomorph; LINDSLEY and ZIMM 1992) having a weak phenotype and +/+ having a wild-type phenotype (Table 5). If this series truly reflects the varying degrees of loss of function of the different alleles, it should be maintained when the alleles are placed in *trans* to another mutation of the *Notch* gene, such as *Ax^{59d}*. However, this does not appear to be the case; *Df(1)N^{81k1}*/*Ax^{59d}*, *N^{ts1}*/*Ax^{59d}* and +/*Ax^{59d}* can be arranged in the expected series but *N^{MI}*/*Ax^{59d}* has a phenotype very similar to +/*Ax^{59d}*, which misplaces it in the series (Table 5). The ability of the *N^{MI}* allele to complement the *Ax^{59d}* allele is unexpected and suggests that the *N^{MI}* allele is not simply a hypomorphic allele. It has previously been proposed that the *N^{MI}* allele may be an antimorph (DE CELIS *et al.* 1993) However this also fails to explain the position of *N^{MI}* in either series because an antimorph is expected to have a stronger phenotype than an amorph as it encodes a protein that is not only unable to function normally but inhibits the function of the wild-type protein.

An alternative explanation for the complementation of *N^{MI}* and *Ax^{59d}* is that there are two functions of the Notch protein, one function that is required for the initiation of sensory bristle development, which is disrupted by alleles such as *Ax^{59d}*, and a second function in lateral inhibition, which is disrupted by alleles such as *N^{MI}*. Deletions of the *Notch* locus, such as *Df(1)N^{81k1}*, and alleles that reduce Notch function generally, such as *N^{ts1}*, will affect both functions of Notch. Therefore in *Df(1)N^{81k1}*/*Ax^{59d}* and *N^{ts1}*/*Ax^{59d}* flies the definition of sensory bristles will be extremely poor, which will prevent the development of nearly all the bristles regardless of the effects on lateral inhibition. However, in *N^{MI}*/*Ax^{59d}* flies, although the initiation of bristle development is affected, it does still occur and any defects in this process will be compensated for by the partial failure of lateral inhibition.

One prediction of this model is that if a point mutation affecting bristle definition is combined in *cis* with a point mutation that affects lateral inhibition function the resulting chromosome should behave as a strong hypomorphic or amorphic allele. The *splAx^{59d}* chromo-

TABLE 6

Hypermorphic mutations that reduce bristle number

	OR	*Df(1)N*	*Dp(1;2)51b*	*wg^{S107}*	*Dl^{FX3}*
l(1)N^B	102.5 ± 2.61	ND	94.1 ± 9.70	58.4 ± 7.98	75.5 ± 7.36
N^{t14}	93.7 ± 14.4	Lethal	87.9 ± 3.95	55.3 ± 5.34	82.0 ± 7.41
N^{t41}	139.3 ± 4.57	Lethal	120.0 ± 7.31	91.1 ± 8.82	143.5 ± 14.4

Values are the number of thoracic microchaete on a heminotum ± SD. Lethal, no adult progeny are obtained of that genotype; ND, the number of thoracic bristles was not calculated for that genotype.
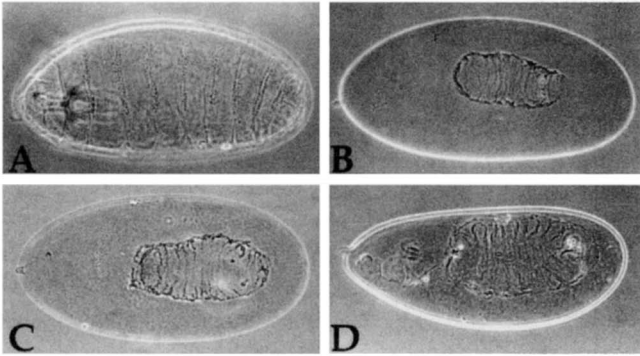
FIGURE 2.—Homozygous cuticle phenotypes of different strength *Notch* alleles: (A) *Oregon R*; (B) N^{264-47}, a strong *Notch* allele; (C) N^{60g11}, a weak *Notch* allele; and (D) $splAx^{59d}$, a weak *Notch* allele. All cuticles are at the same magnification.

some is an example of such a double mutant. Although both Ax^{59d}/Y and spl/Y survive until adulthood, the *cis* double mutant $splAx^{59d}/Y$ is embryonic lethal and displays a weak *Notch* mutant phenotype (Figure 2). Although we cannot rule out the possibility that the two mutations in *cis* produce an unstable Notch protein leading to embryos that lack the Notch protein, the result is also consistent with our hypothesis.

On the other hand, if there are indeed two independent functions, one would expect enhancement of the phenotype when two members of the same class are placed in *trans*, but complementation when two members of different classes are placed in *trans*. This appears to be the case. For example, the $N^{M1}/+$ phenotype is strengthened when placed in *trans* with *spl*, another allele in the same class, in N^{M1}/spl flies (Figure 3 and Table 7). However the phenotype is suppressed if the N^{M1} allele is in *trans* with an allele that affects bristle definition; for example, N^{M1}/Ax^{59d} flies have an essentially wild-type appearance (Figure 3 and Table 7).

The same pattern of complementation is displayed by members of the class that affects bristle definition. For example, Ax^{59d}/N^{60g11} flies display an extreme phenotype with almost complete loss of bristles on the notum and the wing margin (Figure 3 and Table 7). However the phenotype of these alleles is suppressed if they are in *trans* with an allele that attenuates lateral inhibition; for example, Ax^{59d}/spl and Ax^{M1}/N^{su42c} flies are practically wild type (Table 7 and data not shown).

This complementation is only observed between alleles that only affect one function and is not observed with null alleles. Null and hypomorphic alleles, which affect both functions, only increase the severity of the phenotype of the alleles that affect one function; for example, the phenotype of $Ax^{59d}/Df(1)N^{81k1}$ is much stronger than $Ax^{59d}/+$ phenotype (Table 4).

DISCUSSION

Our work has highlighted the existence of four classes of functionally different *Notch* alleles: three hypo-

morphic classes, two of which increase bristle number and one of which reduces the number of bristles compared to wild type, and one hypermorphic class. The genetics of *Notch* is usually interpreted on the basis of hypermorphic and hypomorphic mutations for the well-characterized role of *Notch* in lateral inhibition. However, the simple existence of four classes of *Notch* alleles makes it unlikely that there is only one function of *Notch*. The argument in favor of at least a second function of *Notch* becomes more compelling when the phenotype and behavior of the classes are considered. For example, it is impossible for a protein with a single function to be mutated to produce hypermorphic and hypomorphic molecules that produce the same phenotype, such as the $l(1)N^{B}$ and N^{60g11} classes. Similarly a single-function protein cannot be mutated to produce hypomorphic mutations that have opposing phenotypes, such as the N^{60g11} and N^{M1} classes. However these results can be explained if the Notch protein has two functions that can be mutated independently and if these two functions have opposing effects on bristle development, one required for the initiation of bristle development and one during lateral inhibition. In this situation hypermorphic mutations that increase the efficacy of lateral inhibition would cause a similar loss of bristle phenotype as hypomorphic mutations that affect the initiation of bristle development. Similarly hypomorphic mutations that only affect either lateral inhibition or the initiation of bristle development would have opposing phenotypes.

The presence of two functions within the Notch protein would explain the different phenotypes of $Df(1)N^{81k1}/Ax^{59d}$ and N^{M1}/Ax^{59d}. Both $Df(1)N^{81k1}/+$ and $N^{M1}/+$ flies have more thoracic bristle than wild type. However $Df(1)N^{81k1}/Ax^{59d}$ adults have very few bristles, while N^{M1}/Ax^{59d} flies have almost wild-type numbers of bristles. This difference can be explained if the Notch protein is involved in both defining proneural clusters and lateral inhibition. Deletions of the *Notch* locus, such as $Df(1)N^{81k1}$, will affect both functions of Notch, while mutations like N^{M1} only affect lateral inhibition and mutations like Ax^{59d} only affect the definition of proneural clusters. This hypothesis would explain the complementation that we observe between N^{M1} and Ax^{59d}, because the failure to initiate bristle development correctly due to the Ax^{59d} mutation will be compensated for by the partial failure of lateral inhibition, due to the N^{M1} mutation, allowing more cells than normal to become neural in each proneural cluster.

Consequently this analysis demonstrates the presence of at least two functions within the Notch protein, one required for the definition of bristles and one during lateral inhibition, which can be selectively attenuated; lateral inhibition is principally affected by point mutants like N^{M1} or $l(1)N^{B}$ while Ax^{59d} or N^{60g11} affect a proneural function of *Notch*.

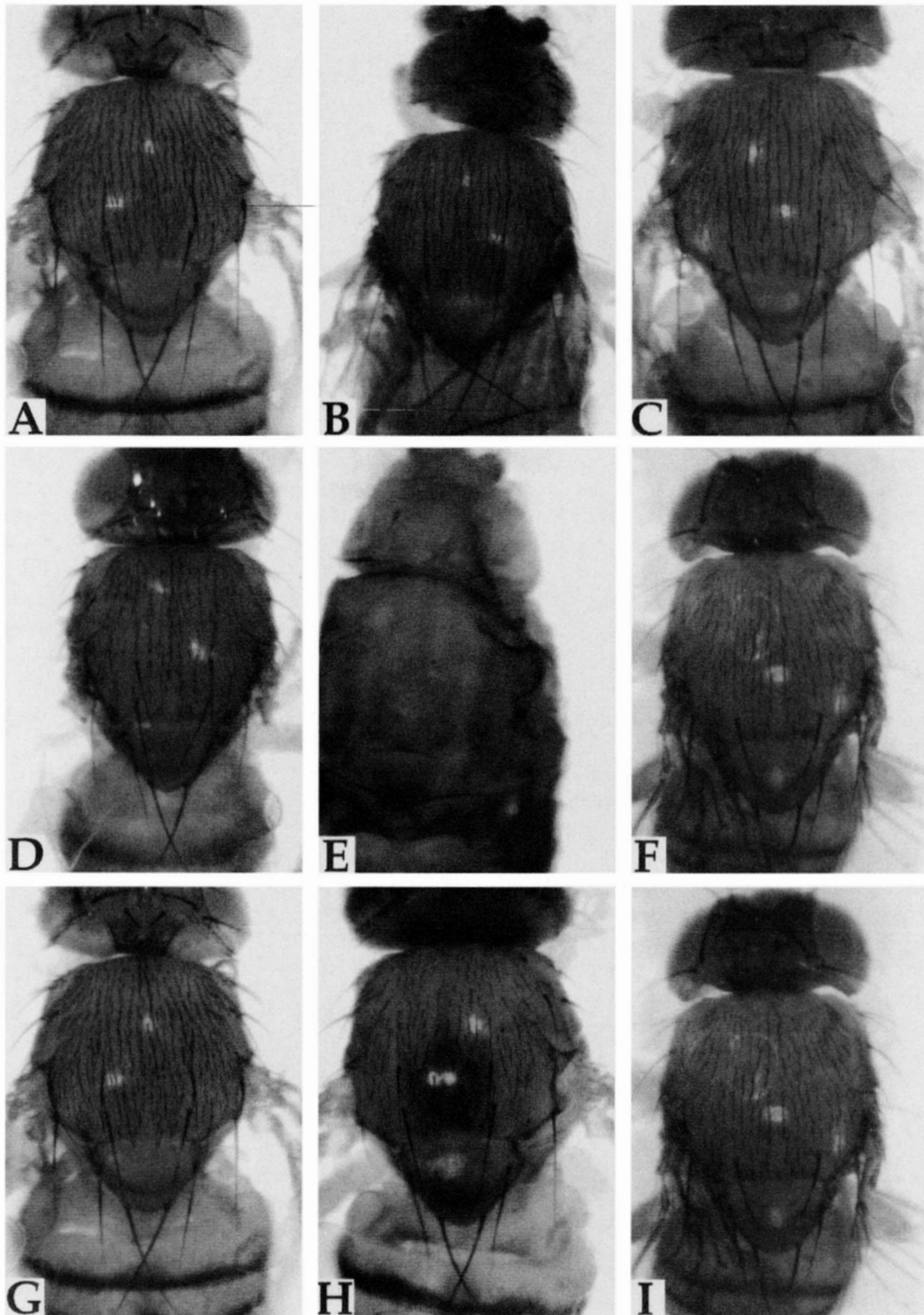Mutations that delete the whole of the *Notch* locus

FIGURE 3.—Thoracic phenotypes of intragenic complementation tests: (A) $N^{MI}/+$, (B) N^{MI}/spl, (C) $spl/+$, (D) $N^{60g11}/+$, (E) N^{60g11}/Ax^{59d}, (F) $Ax^{59d}/+$, (G) $N^{MI}/+$, (H) N^{MI}/Ax^{59d}, and (I) $Ax^{59d}/+$.

should affect both functions, which would suggest that complementation should occur. Therefore flies heterozygous for a *Notch* deletion would be expected to have little or no phenotype. However flies heterozygous for such mutations have vastly more thoracic bristles than wild type. This can be accounted for if one of the two

functions of *Notch* has a greater requirement for *Notch* than the other; this function would be more sensitive to the reduction of *Notch* function and thus lead to a "domineering" phenotype. The phenotype of null *Notch* mutants in heterozygotes suggests that the lateral inhibition function is more sensitive because there is

TABLE 7

Number of thoracic microchaete of transheterozygous combinations of *Notch* alleles

	spl	N^{60g11}	Ax^{59d}
N^{M1}	187.6 ± 3.90	ND	116.8 ± 10.5
spl		103.7 ± 6.90	124.5 ± 5.94
N^{60g11}			2.75 ± 2.78

Values are the number of thoracic microchaete on a heminotum ± SD. ND, the number of thoracic bristles was not calculated for that genotype.

an increase in the bristle number in these flies. This interpretation is also supported from experiments with a dominant negative Notch protein that consists of the extracellular and transmembrane domains only (R. TATESON, K. BRENNAN, V. ZECCHINI and A. MARTINEZ ARIAS, unpublished results). Expression of these molecules will block the signaling functions of the Notch receptor by sequestering signaling ligands. Low levels of this protein easily lead to an increase in bristle number due to a failure in lateral inhibition. However only high levels of this protein lead to a reduction in the number of bristles due to the failure to initiate neural development.

Mapping functional domains within the Notch protein: Since most of the point mutations used in this study have been physically mapped, it is possible to correlate the functions highlighed by our analysis with specific regions of the Notch protein (Figure 4).

Extracellular mutations: N^{M1} and *spl* both lie in the extracellular domain and map to EGF repeats 12 and 14, respectively (DE CELIS *et al.* 1993; KELLEY *et al.* 1987; PREISS *et al.* 1988). Since these two mutations are close together in the protein and affect the same function of *Notch*, they may highlight the region of the protein that interacts with Delta during lateral inhibition. This re-

gion has also been identified as a Delta binding site in aggregation assays (FEHON *et al.* 1990; REBAY *et al.* 1991; LIEBER *et al.* 1992) and therefore our observations corroborate the importance of the region spanning EGF repeats 10–14 in lateral inhibition.

The Ax^{59d} and Ax^{M1} mutations have been mapped to EGF repeats 24 and 25, respectively, within the region in which all molecularly mapped *Abruptex* mutations lie (EGF repeats 24–29; KELLEY *et al.* 1987). Our results indicate that these *Abruptex* alleles affect a function of *Notch* associated with the induction of proneural clusters. Once more the physical proximity of these mutations and their similar phenotypes indicate that they may define a region of the Notch protein that is important in the interaction with an extracellular ligand during the definition of proneural clusters and neural precursors. This region is clearly different from the Delta binding domain and suggests an organization of the EGF repeats into at least two functional domains.

The two hypermorphic mutations molecularly characterized map to the lin-12/Notch repeats. Similar gain-of-function mutations in the *Caenorhabditis elegans* homologues of *Notch*, *lin-12* and *glp-1* cluster in the equivalent region of the protein (GREENWALD and SEYDOUX 1990; BERRY *et al.* 1997) and deletion of these repeats results in constitutive *Notch* signaling (LIEBER *et al.* 1993). Altogether these results indicate that this domain regulates, in some manner, the ability of the Notch receptor to signal during lateral inhibition. One possibility is that it regulates the structural changes that transmit the binding of Delta to the intracellular effector domains. This is supported by the observation that removal of the RAM-23 and cdc10 domains destroys the gain-of-function character of a protein that already lacks the lin-12/Notch repeats (LIEBER *et al.* 1993).

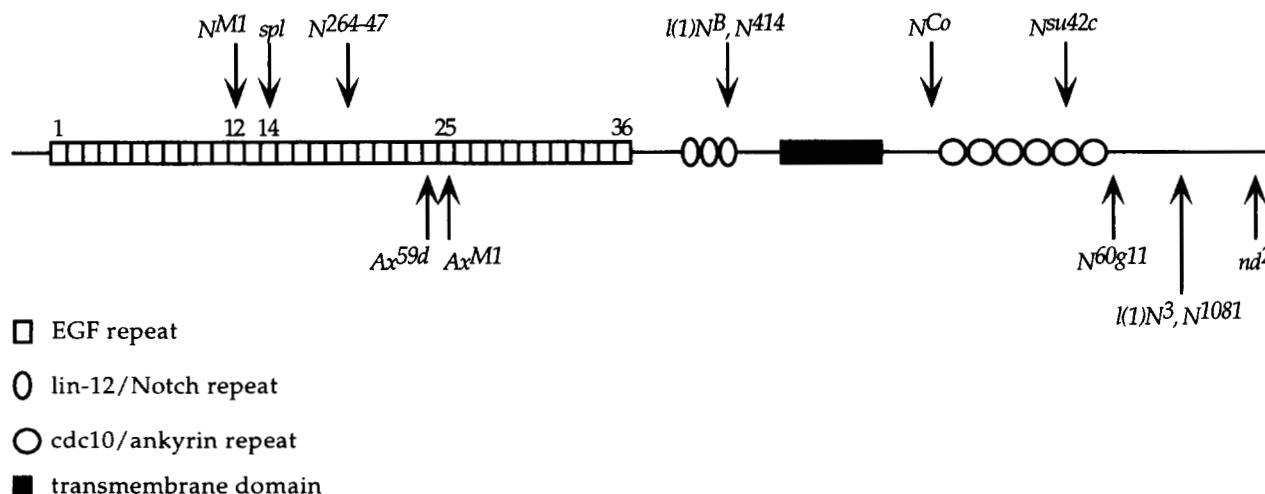*Intracellular mutations:* The cdc10 repeats have been

FIGURE 4.—A pictorial representation of the Notch molecule indicating the position of molecularly mapped mutations. Mutations that affect lateral inhibition are above the molecule and mutations that affect the function of Notch during the definition of proneural clusters are below the molecule.

implicated in lateral inhibition in previous experiments (LIEBER *et al.* 1993; REBAY *et al.* 1993; STRUHL *et al.* 1993). Embryos lacking *Notch* function have a neurogenic phenotype due to the failure to implement lateral inhibition during embryonic neurogenesis. This neurogenic phenotype can be rescued by the overexpression of the full-length Notch molecule and by the overexpression of Notch molecules that lack certain regions of the full-length protein. However the phenotype cannot be rescued if the deleted Notch molecule lacks the cdc10 repeats (LIEBER *et al.* 1993).

Two alleles that behave as mutations that affect lateral inhibition map to this region; N^{su42c}, a missense mutation in the cdc10 repeat 5 (DIEDERICH *et al.* 1994) and the N^{Co} mutation, which removes most of the intracellular domain (LYMAN and YOUNG 1993). The position of both mutations highlights the importance of the cdc10 repeats in the process of lateral inhibition. This is supported further by a comparison of the phenotypes of N^{Co} and N^{60g11}. Both are point mutations that result in the truncation of the intracellular domain of Notch (LYMAN and YOUNG 1993) but, whereas the N^{Co} mutation results in the truncation of almost the entire intracellular domain of Notch, the N^{60g11} mutation only removes the intracellular domain C terminal of the cdc10 repeats. While the N^{60g11} protein is capable of correctly participating in lateral inhibition, the N^{Co} protein is unable to do so. The difference between the two proteins is the inclusion of the cdc10 repeats in the N^{60g11} protein, suggesting that it is this domain of the Notch protein that is responsible for linking it to the intracellular signaling cascade of lateral inhibition.

The N^{60g11} allele identifies a class of mutants that disrupt the initiation of bristle development. The $l(1)N^3$ and N^{1081} alleles are also members of this class. The three mutations map C terminal to the cdc10 repeats: N^{60g11} and $l(1)N^3$ result in truncated proteins and N^{1081} encodes a missense mutation in this region. It has been suggested that truncations of this kind might lead to the activation of the lateral inhibition function (LYMAN and YOUNG 1993; AXELROD *et al.* 1996). However this would imply that these alleles are gain-of-function mutations, which our analysis clearly indicates that they are not. This region of the Notch protein has been shown to interact with the Dishevelled protein, a component of the intracellular Wingless signaling cascade (KLING-ENSMITH *et al.* 1994; AXELROD *et al.* 1996). Therefore one possible function for this region of Notch is to play a role in the initiation of proneural gene expression by interactions with elements of the Wingless signaling pathway.

This analysis suggests an arrangement of the Notch receptor into at least two functional modules, each containing one extracellular and one intracellular component. One module is involved in lateral inhibition. It contains an extracellular region, centered around EGF repeats 10–12, that binds Delta, and an intracellular

region, the RAM-23 domain and the cdc10 repeats, involved in the implementation of the Delta signal. The second functional module contains a region, centered around EGF repeats 24–26, that might be involved in binding an extracellular ligand, and another, which probably lies in the region C terminal to the cdc10 repeats, involved in linking the Notch receptor to an intracellular signaling cascade other than Suppressor of Hairless. The presence of an intracellular and extracellular region involved in the initiation of bristle development suggests that *Notch* may transduce an extracellular signal into the receiving cell as it does during lateral inhibition. In addition it is likely that the lin-12 repeats play a role in the transmission of the extracellular events to the intracellular effectors.

The organization outlined above is interesting in the light of the structure of some of the vertebrate *Notch* genes. Two of these genes, *Notch1* and *Notch2*, are very similar to Drosophila *Notch* but the other two, *Notch3* and *Notch4*, contain deletions of specific EGF repeats and intracellular regions (LARDELLI *et al.* 1994; UYTTEN-DAELE *et al.* 1996). The case of *Notch4* is particularly interesting since this molecule lacks most of the domain C terminal to the cdc10 repeats, and EGF repeats equivalent to EGF repeats 14–27 of the Drosophila molecule have been replaced by eight EGF repeats that are unique to *Notch4* (UYTTENDAELE *et al.* 1996). In our analysis these two regions are associated with the initiation of bristle development and raise the possibility that, in vertebrates, there are molecules specialized for one or the other function of *Notch* that we have described here. In this case *Notch4* might encode a molecule dedicated to lateral inhibition.

The roles of Notch during neurogenesis: The notion that *Notch* encodes a multifunctional receptor has been discussed in various manners within the context of the assignation of cell fates during development. In one specific model it has been suggested that during neurogenesis Notch is involved in at least two different signaling events (COUSO and MARTINEZ ARIAS 1994). In this hypothesis, *Notch* is required initially for the expression of the proneural genes, which initiates bristle development, and then it is required during lateral inhibition to restrict the expression of the proneural genes to one or two cells of the proneural cluster, which become sensory organ precursors. In addition, it was suggested that each of these functions is triggered by a different ligand that competes for the Notch protein.

There are two ways in which we can envision the function of *Notch* during the initiation of proneural gene expression. It might be that the expression of the *achaete scute complex* genes is initiated by the ligand-mediated release of a repression that is enacted by the Notch protein. In this case, mutations like Ax^{59d} and Ax^{M1} would reduce the affinity of Notch for the ligand that releases the repression, while mutations like N^{60g11} would reduce the ability of Notch to implement the

repressive signal. Alternatively *Notch* might play a direct role in the initiation of proneural gene expression by acting as a receptor for a protein that stimulates the expression of these genes. In this case, the *Abruptex* mutations would impair the binding of the signaling ligand to the Notch protein and the N^{60g11} mutation would disrupt the linkage of Notch to the intracellular signaling cascade. Finally it is also possible that it is a combination of these two mechanisms that occurs *in vivo*. However our analysis is unable to distinguish between these possibilities.

Once the expression of the proneural genes is initiated, lateral inhibition occurs to restrict the expression of these genes to one or two cells in each proneural cluster. This occurs through the well-documented mechanism described above. Mutations in the N^{M1} class of alleles disrupt the lateral inhibition signal, either by reducing the affinity of the Notch receptor for Delta or by preventing the interaction of Notch with intracellular effector of lateral inhibition, Supressor of Hairless, while mutations like $l(1)N^B$ increase the efficacy of lateral inhibition.

Our results provide evidence for these two functions and, in addition, indicate that there might be discrete regions of the Notch protein involved in each of them.

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