Modifications of the Notch Function by *Abruptex* Mutations in *Drosophila melanogaster*

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Manuscript received February 22, 1993
Accepted for publication September 15, 1993

ABSTRACT

The function of the *Notch* gene is required in cell interactions defining alternative cell fates in several developmental processes. The *Notch* gene encodes a transmembrane protein with 36 epidermal growth factor (EGF)-like repeats in its extracellular domain. This protein functions as a receptor that interacts with other transmembrane proteins, such as Serrate and Delta, which also have EGF repeats in their extracellular domain. The *Abruptex* mutations of the Notch locus are associated with amino acid substitutions in the EGF repeats 24–29 of the Notch protein. We have studied, in genetic combinations, the modifications of Notch function caused by *Abruptex* mutations. These mutations lead to phenotypes which are opposite to those caused by *Notch* deletions. The Abruptex phenotypes are modified by the presence of mutations in other loci, in particular in the genes *Serrate* and *Delta* as well as *Hairless*, and *groucho*. The results suggest that all *Abruptex* mutations cause stronger than normal Notch activation by the Delta protein. Some *Abruptex* alleles also display an insufficiency of N function. *Abruptex* alleles which produce stronger enhancement of Notch activation also display stronger Notch insufficiency. This insufficiency could be due to reduced ability of Abruptex proteins to interact with Notch ligands and/or to form functional Notch dimers.

THE control of size, shape and patterned cell differentiation in animal morphogenesis is mediated by cell-cell communication (reviewed in GURDON 1992; GREENWALD and RUBIN 1992; GARCIA-BELLIDO and DE CELIS 1992). In these processes growth factors and their receptors are connecting elements between cells. In Drosophila the *Notch* (*N*) and *Delta* (*Dl*) genes, whose products are involved in such interactions, have been extensively studied. These genes are involved in many of the processes involving cell fate choice during development (reviewed in CAMPOS-ORTEGA and KNUST 1990a; ARTAVANIS-TSAKONAS, DELIDAKIS and FEHON 1991).

Both *N* and *Dl* code for transmembrane proteins with several epidermal growth factor (EGF)-like repeats in their extracellular domain (WHARTON *et al.* 1985; KIDD, KELLEY and YOUNG 1986; VÄSSIN *et al.* 1987; KOPCZYNSKI *et al.* 1988). Whereas the Dl intracellular domain is short (VÄSSIN *et al.* 1987; KOPCZYNSKI *et al.* 1988) that of N is large and contains several cdc10 repeats (BREEDEN and NASMYTH 1987) which could be involved in protein-protein interactions (BENNET 1992). Biochemical analysis of the N protein indicates that N polypeptides exist in the cell membrane as dimers (KIDD *et al.* 1989). Developmental and genetic tests suggest that N act as a receptor of the Dl protein, the latter acting as one of its ligands (reviewed in ARTAVANIS-TSAKONAS, DELIDAKIS and FEHON 1991). *In vitro* assays show direct protein interactions between N and Dl proteins (FEHON *et al.* 1990; LIEBER *et al.* 1992).

The *N* gene has closely related homologs in *Caenorhabditis elegans* (YORCHEN, WESTON and GREENWALD 1988), Xenopus (COFFMAN, HARRIS and KINTNER 1990), mouse (FRANCO DEL AMO *et al.* 1992) and humans (ELLISEN *et al.* 1991; STIFANI *et al.* 1992), suggesting that N function has been widely conserved through evolution.

In the *N* locus of Drosophila several classes of mutations have been identified: recessive alleles with specific pattern effects (WELSHONS and VON HALLE 1962; WELSHONS 1965), lethal *N* alleles with highly pleiotropic effects (WELSHONS 1965; SHELLENBARGER and MOHLER 1978) and *Abruptex* (*Ax*) alleles (WELSHONS 1971). *Ax* alleles have attracted the attention of developmental geneticists for a long time. They are dominant mutations with adult phenotypes including the absence of bristles in several regions of the fly and lack of wing veins (WELSHONS 1971; FOSTER 1975; PORTIN 1981). Ax phenotypes in these two developmental processes can be considered as opposite to those of *N* alleles, the later causing extra chaetae and thicker veins (PALKA, SCHUBIGER and SCHWANINGER 1990; DE CELIS, MARI-BEFFA and GARCIA-BELLIDO 1991b; J. F. DE CELIS and A. GARCIA-BELLIDO, unpublished results).

The results of genetic combinations between *Ax* and *N* alleles has led to the distinction of three classes of

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Ax alleles, viable enhancers of N (*E(N)*), viable suppressors of N (*Su(N)*) and lethals alleles (*l(1)Ax*) (PORTIN 1975; FOSTER 1975). The genetic behavior of these classes of mutations has led to the interpretation that *Ax* mutations correspond to hypomorphic (*E(N)*), hypermorphic (*Su(N)*) and antimorphic (*l(1)Ax*) variants of *N* (PORTIN 1977a; PORTIN 1981). Further complexity appears when considering the results of combinations between different *Ax* alleles. Thus, combinations between some viable *Ax* alleles can be lethal, a phenomenon called negative complementation (FOSTER 1975; PORTIN 1975). The analyses of negative complementation in temperature shift experiments and in gynandromorph mosaics suggest that the lethality of *E(N)/Su(N)* combinations results from direct interactions between the Ax products (PORTIN and SIREN 1976; PORTIN 1977b). However, despite their different genetic behavior, all *Ax* alleles cause similar phenotypes, varying in degrees of expressivity. Moreover, all molecularly characterized *Ax* alleles contain amino acid substitutions in the 24–29 EGF-like repeats of the N protein (KELLEY *et al.*, 1987; HARTLEY, XU and ARTAVANIS-TSAKONAS 1987).

The aim of the present paper is to characterize the complex complementation patterns of the different *Ax* mutations in the context of a possible mechanism of *N* function. The phenotypes of *Ax* mutants in *Serrate*, *Delta*, *Hairless* and *groucho* mutant backgrounds are also described. The results suggest that all *Ax* mutations are antimorphic variants of the *N* gene that cause ligand-dependent hyperactivation of the N protein. Different *Ax* alleles may cause differential modifications in the binding parameters of N proteins with their ligands.

MATERIALS AND METHODS

Genetic strains: The following *N* mutations were used: the *N* null allele N^{55e11} (KIDD, LOCKET and YOUNG 1983), the *Ax* viable alleles Ax^{16172}, Ax^{E2}, Ax^{71d}, Ax^{28} Ax^{9B2}, Ax^{M2} and Ax^{M4} (FOSTER 1975; PORTIN 1975; KELLEY *et al.* 1987, DE CELIS, MARI-BEFFA and GARCIA-BELLIDO 1991b), the lethal alleles Ax^{M1} and Ax^{M3} (DE CELIS, MARI-BEFFA and GARCIA-BELLIDO 1991b), and the recessive alleles fa^{nd} and *spl* (WELSHONS 1965). The *N* gene duplications $Dp(1;2)w^{+51b7}$ and $Dp(1;3)w^{+67k}$ (LINDSLEY and ZIMM 1992) were also used. The variants in the *Dl* locus used were: the *Dl* lethal allele Dl^{M1} (DE CELIS, MARI-BEFFA and GARCIA-BELLIDO 1991b) and the Dl^{+} duplication $Dp(3;3)bxd^{110}$ (LINDSLEY and ZIMM 1992). The variants in other loci used were: the *Ser* null allele Ser^{RX106} (THOMAS, SPEICHER and KNUST 1991), the *groucho* lethal allele gro^{E73} (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988), and the *H* mutant allele H^{2} (MAIER *et al.* 1992).

Characterization of phenotypes: Three quantitative parameters of Ax phenotypes were measured: the number of micro- and macrochaetae present (in at least 20 heminota) and the remaining vein stretches as percentages of total wing vein length (in at least 10 wings). Chaetae phenotypes were scored under the dissecting microscope. Wings were removed and mounted for light microscope examination.

TABLE 1

Phenotypes of different *Ax* alleles in hemizygous males

Ax allele	Class	Mc	mc	VL
Ax^{M1}	*l-Ax*	2.1 ± 1.4	4 ± 2	0.15
Ax^{M3}	*l-Ax*	2.5 ± 1.2	12 ± 4	0.15
Ax^{16172}	*E(N)*	6.6 ± 1.0	61 ± 7	0.73
Ax^{71d}	*E(N)*	7.3 ± 0.6	91 ± 4	0.76
Ax^{E2}	*E(N)*	9.4 ± 0.9	76 ± 3	0.87
Ax^{9B2}	*Su(N)*	7.8 ± 0.8	119 ± 10	0.96
Ax^{28}	*Su(N)*	9.2 ± 0.7	110 ± 10	0.96
N^{+}	Control	11	120	1.00

Phenotypes of macrochaetae (Mc) and microchaetae (mc) of the notum and average length of wing veins (VL), for different *Ax* alleles (in hemizygosis). VL: average length of extant longitudinal veins presented as fraction of the total wing vein length of controls.

Longitudinal veins are numbered from anterior to posterior as I (costal), II (radius), III (medial), IV (cubitus) and V (anal). The length of present veins was estimated in drawings using a camera lucida. As controls we used *Vallecas* wild type flies.

Embryo preparations: Cuticle preparations of the embryonic lethal combinations were mounted for microscopic examination (LEHMANN *et al.* 1983).

Genetic crosses: All crosses were done at 25°. In some cases the cultures were transferred after 2 days of egg laying at 25° to either 17° or 29° until adult eclosion.

RESULTS

The *Ax* alleles: Nine *Ax* alleles were used: seven viable (*Ax*) and two late pupal lethal (*l(1)Ax*). The phenotypes of hemizygous males include a reduction in the number of macro- and microchaetae and in the length of wing veins. Table 1 shows quantitative descriptions of these traits for the different alleles studied. Allelic variations in these features show a high correlation: macrochaetae/microchaetae $R = 0.91$; macrochaetae/veins $R = 0.97$ and microchaetae/veins $R = 0.9$. The notal macrochaetae are lost in a common positional seriation in increasingly severe mutant phenotypes (Figure 1). The effects on vein differentiation can also be ordered in a common topographic seriation (Figure 2). The progressive and serial effects on chaetae and vein differentiation may correspond to different levels of N function. The fact that *Ax* and *N* mutations cause opposite phenotypes in chaetae and veins has led to the proposition that Ax products have either an altered (KELLEY *et al.* 1987; PALKA, SHUBIGER and SCHWANINGER 1990) or an increased (DE CELIS, MARI-BEFFA and GARCIA-BELLIDO 1991a) *N* function; propositions already put forward in the early studies of FOSTER (1975) and PORTIN (1975). What follows aims to investigate the nature of the *Ax* perturbations and their relationship with the *N* wild type function in different genetic combinations.

Dosage analysis of *Ax* alleles: It is known that both lethal and adult phenotypes of different *Ax* alleles are rescued in the presence of one N^{+} extra copy (WEL-

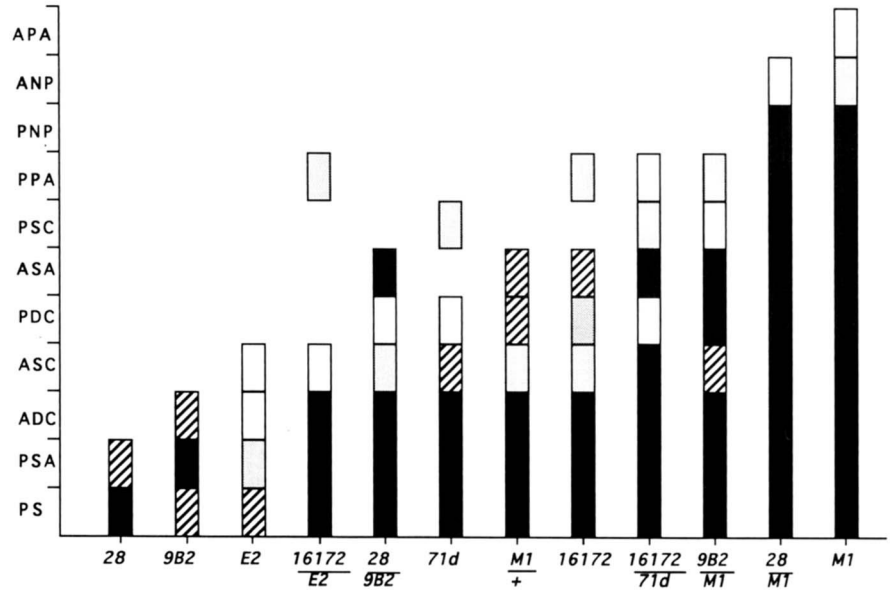


FIGURE 1.—Pattern seriation of effects on the notum macrochaetae of different *Ax* alleles (males) or *Ax* heteroallelic combinations (females). Ordinates frequencies of absence of chaetae in 5–25% □, 26–50% ▨, 51–75% □ and 76–100% ■. Abbreviations of different macrochaetae: presutural, PS; anterior and posterior dorsocentral, ADC and PDC; anterior and posterior notopleural, ANP and PNP; anterior and posterior supraalar, ASA and PSA; anterior and posterior postalar, APA and PPA; anterior and posterior scutelar, ASC and PSC.

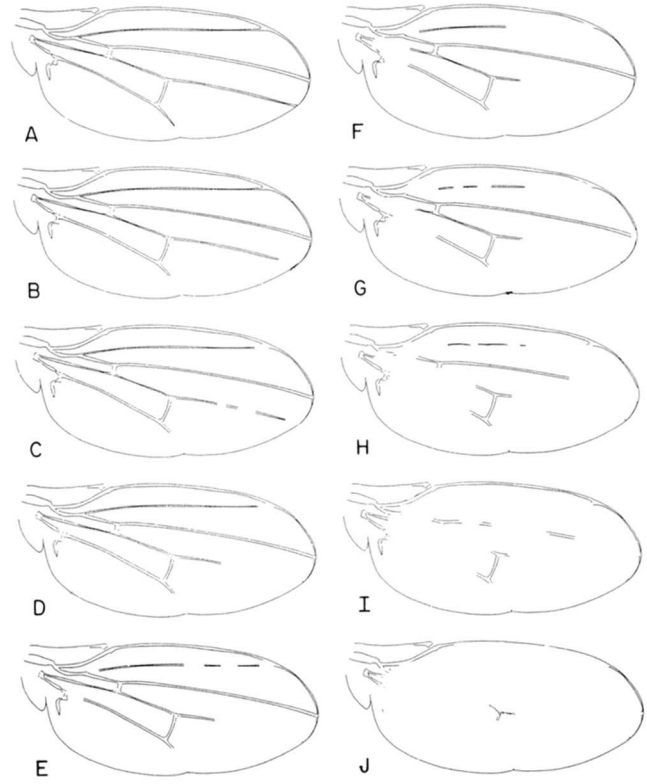


FIGURE 2.—Pattern seriation of effects on wing vein differentiation in schematic drawing of representative wing vein phenotypes of different *Ax* alleles and *Ax* heteroallelic combinations. (**A**) Ax^{28}/+. (**B**) Ax^{E2}. (**C**) Ax^{71d}. (**D**) Ax^{M1}/+. (**E**) Ax^{16172}. (**F**) and (**G**) Ax^{28}/Ax^{16172}. (**H**) Ax^{28}/Ax^{M3}. (**I**) Ax^{M1}. (**J**) Ax^{71d}/Ax^{M1}.

SHONS 1971; PORTIN 1981). We have repeated and extended the genetic analysis and phenotypic description of the *Ax* alleles in combinations with different number of copies of N^+. Females homozygous for viable *Abruptex* alleles have a phenotype similar to that of hemizygous males. Hemizygous *Ax* females (*i.e.*, heterozygous with *N* null alleles) have a weaker Ax phenotype than hemizygous *Ax* males (Figure 3). In these combinations different *Ax* alleles, either do not modify (Ax^{71d}) or show a suppression (*Su(N)*: Ax^{M5}, Ax^{28} and Ax^{9B2}) or an exaggeration (*E(N)*: Ax^{M2}, Ax^{16172}, Ax^{E2}) of the N haplo-insufficient phenotype of loss of wing margin (see also FOSTER 1975; PORTIN 1975). *l(1)Ax* alleles are late pupal lethal in hemizygous males, the scapers displaying extreme Ax phenotypes, including loss of most micro- and macrochaetae (Table 1), and both the absence and ectopic formation of wing margin bristles. In hemizygous females, *l(1)Ax* alleles are embryonic lethal with weak N phenotypes including fusion of dentical belts and ventral epidermal holes (not shown) characteristic of neurogenic phenotypes (LEHMANN *et al.* 1983). In this respect these *l(1)Ax* alleles can be considered as strong *E(N)* alleles.

Females heterozygous for *Ax* and a wild type *N* gene have a reduced Ax phenotype, and increasing the number of N^+ copies further reduces the Ax phenotype (Figure 3). These extra N^+ copies cause a "Confluens" (Co) phenotype consisting on thickening of veins in particular vein II, cv-p and terminal deltas. This Co phenotype is also reduced in combinations with *Ax* alleles; the stronger lethal alleles suppressing it altogether (Figure 4b). The titration between Ax and N^+ products is evident in combinations of any *Ax* allele and increasing doses of N^+ (see Ax^{M3} in Figure 4, a and b). Thus, for example, Ax^{M3}/N^+ is similar in phenotype to Ax^{M3}/Ax^{M3}; N^+/N^+ (not shown). This titration is indicative of the antimorphic behavior of all *Ax* alleles.

The *spl* mutation: We have also included in our analysis the *spl* mutation, associated with one amino acid substitution in the 14th EGF-like repeat of the N protein (HARTLEY, XU and ARTAVANIS-TSAKONAS 1987). The *spl* allele affects eye development with a

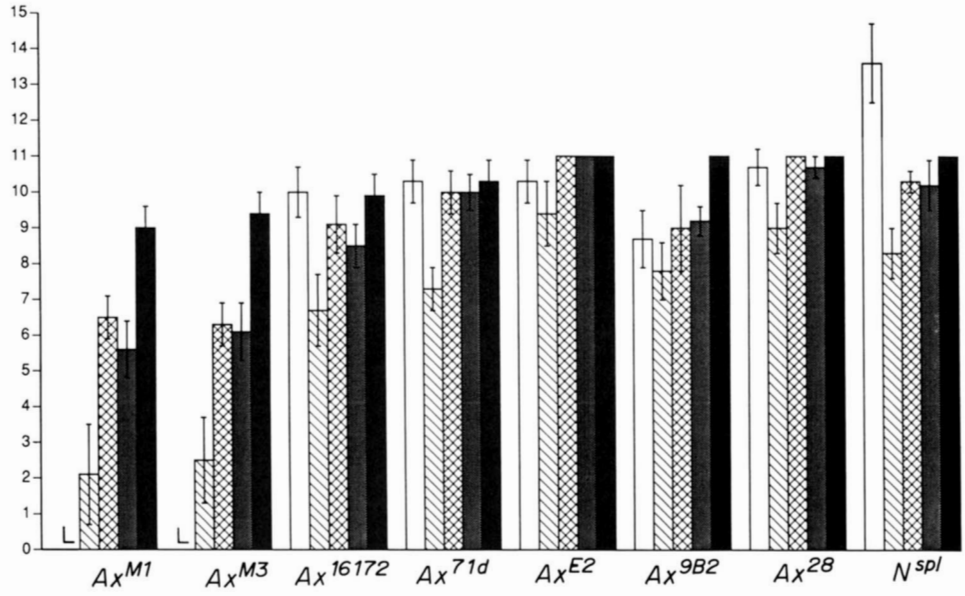
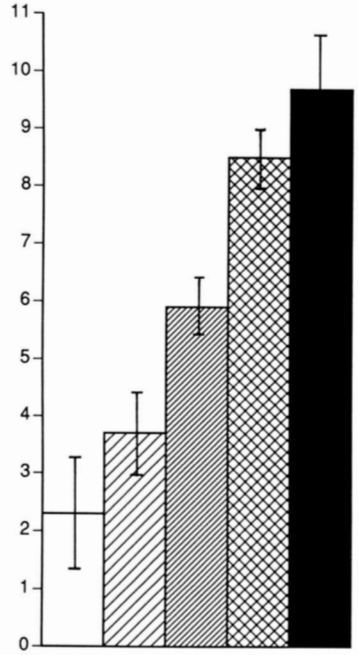
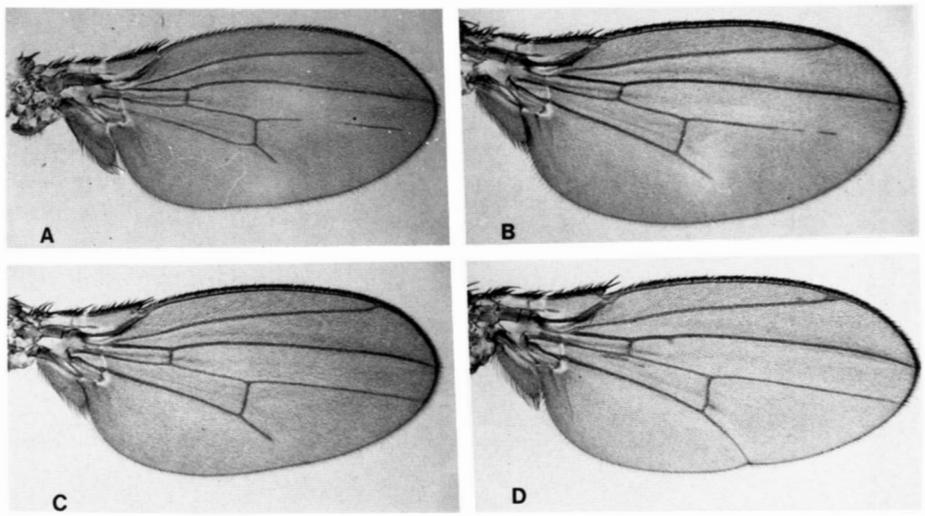


FIGURE 3.—Phenotypes (number of macrochaetae present) of combinations between *Ax* (or *spl*) alleles and variable number of copies of the N gen (in abscissas): □ Ax/N^{55e11}, ▨ Ax/Y ▩ $Ax;Dp(1;2)w^{+51b7},N^{+}/+$, □ $Ax/+$, ■ $Ax/+;Dp(1;2)w^{+51b7},N^{+}/+$. Bars, standard deviations; L, embryonic lethal combinations.





a **b**

FIGURE 4.—Phenotypes of combinations between Ax^{M3} and different doses of the *N* gene. (**a**) Mean number of macrochaetae present (in ordinates and in absciccas) in that order: Ax^{M3}, $Ax^{M3}/Ax^{M3};Dp(1;2)w^{+51b7}/+$, $Ax^{M3}/+$, $Ax^{M3}/+$; $Dp(1;2)w^{+51b7}/+$, $Ax^{M3}/+;Dp(1;2)w^{+51b7}/+;Dp(1;3)w^{+67k}/+$. Bars, standard deviations. (**b**) Wing phenotypes of the combinations: (**A**) $Ax^{M3}/Ax^{M3};Dp(1;2)w^{+51b7}/+$. (**B**) $Ax^{M3}/+$. (**C**) $Ax^{M3}/+;Dp(1;2)w^{+51b7}/+$. (**D**) $Ax^{M3}/+;Dp(1;2)w^{+51b7}/+;Dp(1;3)w^{+67k}/+$. All wings at the same magnification.

phenotype opposite to that of *N* null alleles (CAMPOS-ORTEGA and KNUST 1990b; BAKER, MODZLIK and RUBIN 1990). *spl* also causes loss or abnormal differentiation of micro- and macrochaetae in the notum. The affected macrochaetae are the most sensitive ones in *Ax* mutants (Figure 1). The *spl* allele in combination with a *N* null allele causes extra macro- and microchaetae (DE CELIS, MARI-BEFFA and GARCIA-BELLIDO 1991b) (Figure 2), and can in this respect be classified as an *E(N)* allele. In the presence of a N^{+} duplication the spl phenotype is reduced but not completely suppressed (Figure 3), indicating its antimorphic nature.

The antimorphic function of *Ax* alleles: The antimorphic behavior of *Ax* (and *spl*) alleles is consistent with abnormal gene products that interact and compete with those of the normal allele in multimeric complexes. Biochemical analysis has shown that N products exist as dimers in the cell membrane (KIDD *et al.* 1989). Furthermore, *in vitro* cell adhesion assays have shown that N also interacts with at least Dl and

TABLE 2

Phenotypes of *Ax* double heterozygous females

	Ax^{16172}	Ax^{71d}	Ax^{E2}	Ax^{9B2}	Ax^{28}
Ax^{M1}	L	1.2/50	1.3/50	3.8/96	2.3/51
Ax^{16172}		6.3/100	7.8/70	1.5/58	3.6/71
Ax^{71d}			7.8/115	3.7/105	4/80
Ax^{E2}				3.5/70	8.3/91
Ax^{9B2}					6.8/140

Phenotypes of extant macrochaetae (left figures) and microchaetae (right figures) of the notum of different *Ax* heteroallelic combinations. L: early lethal genotypes, without adult escapers.

Ser proteins of neighboring cells (REBAY *et al.* 1991), and with Dl products of the same cell (FEHON *et al.* 1990). In order to study the genetic basis of the *Ax* antimorphism we have searched for specific interactions of different Ax products, both among themselves and with genetic variants of other genes which are known to interact with *N*.

Heteroallelic combinations of Ax alleles: Combinations between *E(N)* and *Su(N) Ax* viable alleles give lethality, a phenomenon called "negative complementation" (FOSTER 1975; PORTIN 1975). We have repeated and extended these complementation analyses using new *Ax* alleles and making use of the fact that the lethal *Ax* alleles Ax^{M1} and Ax^{M3} are temperature sensitive.

The chaetae phenotypes of the different *Ax* heteroallelic combinations are presented in Table 2. If the different alleles were to correspond to quantitative variations of the same N misfunction (as indicated by their serial effects) we would expect additive phenotypes in *Ax* heteroallelic combinations. In fact, combinations of *l(1)Ax* alleles with viable *Ax* alleles are lethal, and the rare escapers have weaker phenotypes in notal microchaetae, wing margin chaetae and vein differentiation than the *l(1)Ax* escapers. Surprisingly, the lethal phase of these combinations is earlier than that of *l(1)Ax* hemizygotes, imaginal discs are larger than wild type and appear deformed; and the rare pharate adults show failures in wing and leg evagination and general morphological abnormalities that are never found in *l(1)Ax* pharate adults. These novel phenotypes indicate negative complementation between viable *Ax* and *l(1)Ax* alleles and show that a given combination shows either additive or negative complementation depending on the phenotype considered.

Combinations between different *E(N)* and different *Su(N)* alleles yields additive phenotypes, similar to those of the stronger *Ax* allele in the combination (Table 2). Combinations between *E(N)* and *Su(N)* alleles cause, in most cases pupal lethality (PORTIN 1975; FOSTER 1975). The phenotypes of these lethal combinations are stronger than that of the *Ax* alleles alone (FOSTER 1975) (Table 2) and the pharate adults show similar but weaker adult (Figure 5B) and imaginal disc phenotypes to that of *l(1)Ax/Ax* combinations.

These complementation patterns are not completely consistent. Thus, Ax^{9B2}/Ax^{M1} and Ax^{9B2}/Ax^{M3} (*Su(N)/l(1)Ax*) flies are viable and show only an enhancement of the *l(1)Ax* heterozygous phenotypes (Table 2, Figure 5E). Ax^{28}/Ax^{E2} (*Su(N)/E(N)*) flies are also viable and have stronger Ax phenotypes than the individual *Ax* alleles in homozygosis (Table 2, see also PORTIN and SIREN 1976). Further exceptions are shown in combinations involving Ax^{M2} (*E(N)*) and Ax^{M5} (*Su(N)*). These two alleles are viable in all heteroallelic combinations with either *l(1)Ax*, *Su(N)* or *E(N)* and their phenotypes are additive (Figure 6). The phenotypes of every *Ax* heteroallelic combination in macrochaetae, microchaetae and vein differentiation show weak correlations: macro-/microchaetae $R = 0.58$ ($n = 20$); microchaetae/veins $R = 0.69$ ($n = 13$) and macrochaetae/veins $R = 0.84$ ($n = 13$). Furthermore, these pattern phenotypes do not show correlation with imaginal disc and adult abnormalities in several *Ax* heteroallelic combinations. This differential behavior of a given heteroallelic combination in different developmental processes is indicative of partial positive and partial negative complementation between alleles, depending on the phenotype considered. For individual patterns, however, there is a congruent topographical seriation independently of the *Ax* allele or heteroallelic combination considered (Figures 1 and 2).

Combinations of Ax viable alleles with temperature-sensitive Ax lethal alleles: A second approach to analyze the Ax antimorphic function makes use of the temperature-sensitive phenotype of the *Ax* alleles, Ax^{M1} and Ax^{M3}. These *Ax* lethal alleles fail to complement *N* recessive mutations (such as *nd* and fa^{nd}) at 29° (data not shown). It is interesting to note that the previously known *Ax* lethal alleles, Ax^{59b} and Ax^{59d}, are also temperature sensitive (PORTIN 1977a). Thus, all known *l(1)Ax* alleles have a strong Ax phenotype at 17° and 25° and a N phenotype at 29° (Figures 6 and 7) (PORTIN 1977a). The N phenotypes at high temperatures may be due to partial or complete inactivation of the Ax protein. We have searched for correlated changes in the phenotypes of different heteroallelic combinations with Ax^{M1} and Ax^{M3} at different temperatures. The phenotypes of these combinations will depend on whether or not the temperature-sensitive product of the *l(1)Ax* allele can interact with that of the viable *Ax* allele and, in addition, to any direct effects of temperature on the activity of the Ax viable product.

In all combinations tested, the Ax phenotype of the heteroallelic combination is reduced at 29° compared with 25° (Figures 5 and 7). However, combinations involving either Ax^{E2} or Ax^{71d} (both *E(N)*) have stronger Ax phenotypes at 29° than the particular viable allele in Ax/N^{-} combinations at 29°. These Ax

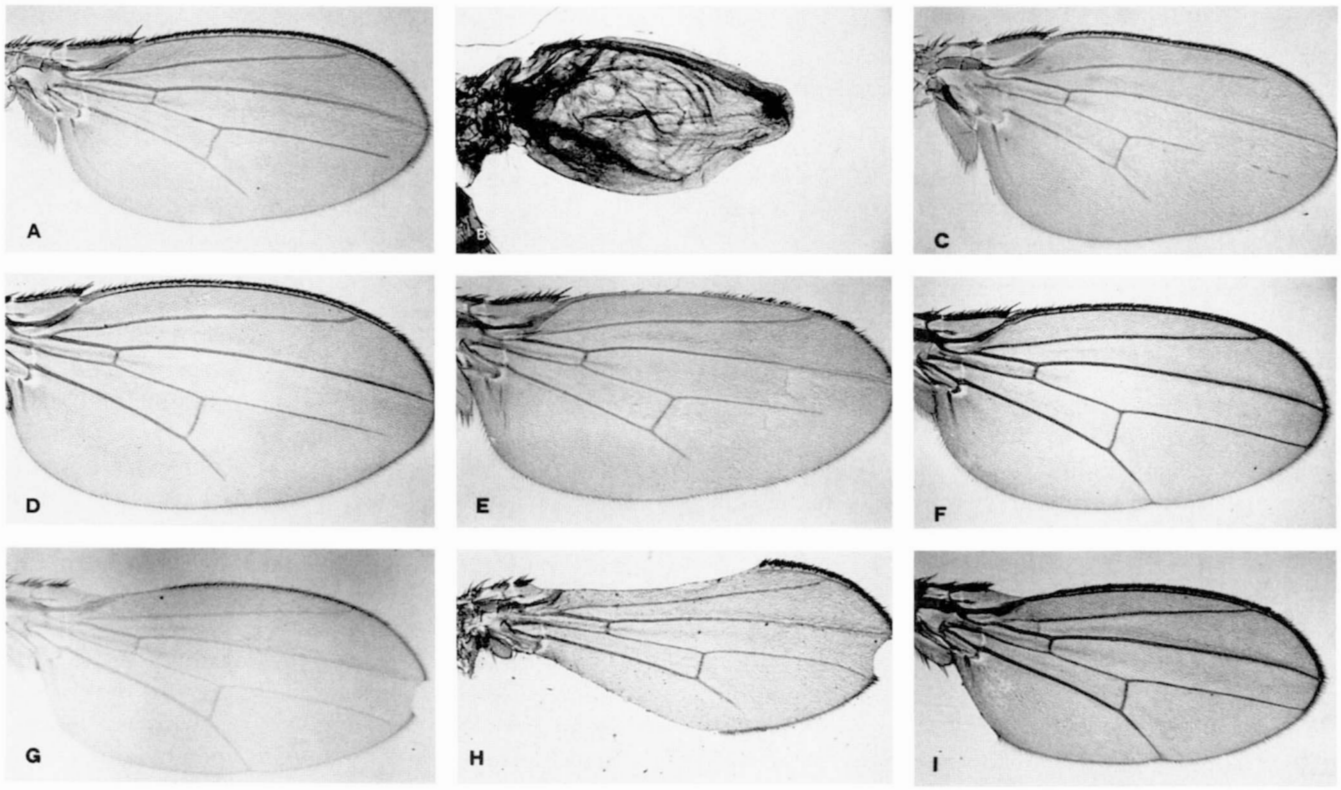


FIGURE 5.—Wing phenotypes of different Ax heteroallelic combinations: (**A**) Ax^{28} at 25°, (**B**) Ax^{28}/Ax^{16172} at 25°, (**C**) Ax^{16172} at 25°, (**D**) Ax^{9B2} at 25°, (**E**) Ax^{9B2}/Ax^{M1} at 25°, (**F**) Ax^{9B2}/N^{55e11} at 25°, (**G**) $Ax^{M1}/+$ at 29°, (**H**) Ax^{9B2}/Ax^{M1} at 29°, (**I**) Ax^{9B2}/N^{55e11} at 29°. All wings at the same magnification.

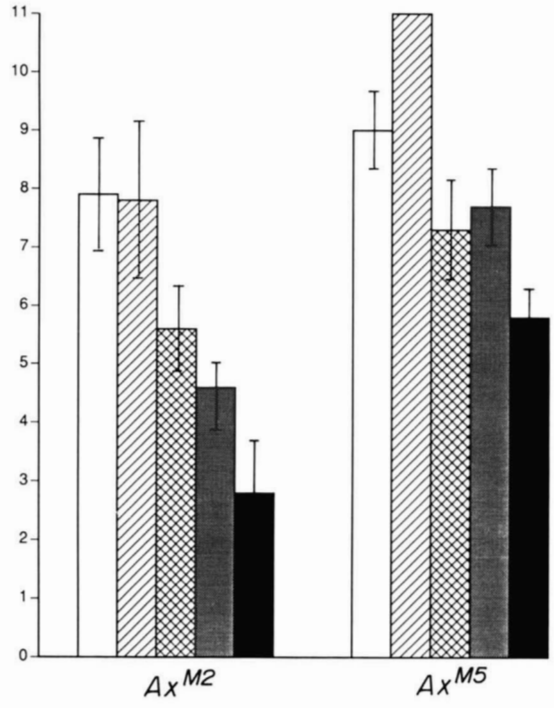


FIGURE 6.—Phenotypes mean number of present macrochaetae (in ordinates) of combinations involving the *Ax* alleles Ax^{M2} and Ax^{M5} with other viable and lethal *Ax* alleles. Columns in that order: Ax^{M2}/Y and Ax^{M5}/Y, Ax^{*}/N^{55e11}, Ax^{*}/Ax^{28}, Ax^{*}/Ax^{16172}, Ax^{*}/Ax^{M1}. Bars, standard deviations.

phenotypes are similar to that of the corresponding *Ax* viable allele at 29°. These results suggest the existence of some functional rescue of the inactivated l(1)Ax products by the presence of Ax of viable ones. The alleles Ax^{9B2}, Ax^{28} (both *Su(N)*) and Ax^{16172} (*E(N)*) have a similar macrochaetae phenotype in combinations with Ax^{M1} and with N^{55e11} at 29°, suggesting that the Ax-viable/Ax-lethal dimeric products are not functional or do not form at 29°. Ax^{E2}, Ax^{71d}, Ax^{16172} and Ax^{9B2} heterozygotes over Ax^{M1} (or Ax^{M3}) cause a strong N wing phenotype at 29°, which is similar (Ax^{E2}, Ax^{71d}) or stronger (Ax^{16172} and Ax^{9B2}) to that of the same Ax/N^{-} combinations at 29° (Figure 5H). Interestingly, all these combinations retain an Ax phenotype of absence of wing veins at 29° (not shown). In addition, Ax^{16172}/Ax^{M1} and Ax^{16172}/Ax^{M3} combinations at 29° have extra microchaetae in a pattern that resembles that of N^{ts1} flies at the restrictive temperature (SHELLENBARGER and MOHLER 1978; HARTENSTEIN and POSAKONY 1990), indicating a strong depletion of functional N products in these combinations.

The Ax^{9B2} (*Su(N)*) allele at 29° behaves, in combinations with both a *N* null allele and *l(1)Ax* alleles, as an *E(N)* allele. However, the lethality of Ax^{9B2}/Ax^{166172}, Ax^{9B2}/Ax^{E2} and Ax^{9B2}/Ax^{71d} combinations at 25° remains at 29° and the combination Ax^{9B2}/Ax^{28} is viable

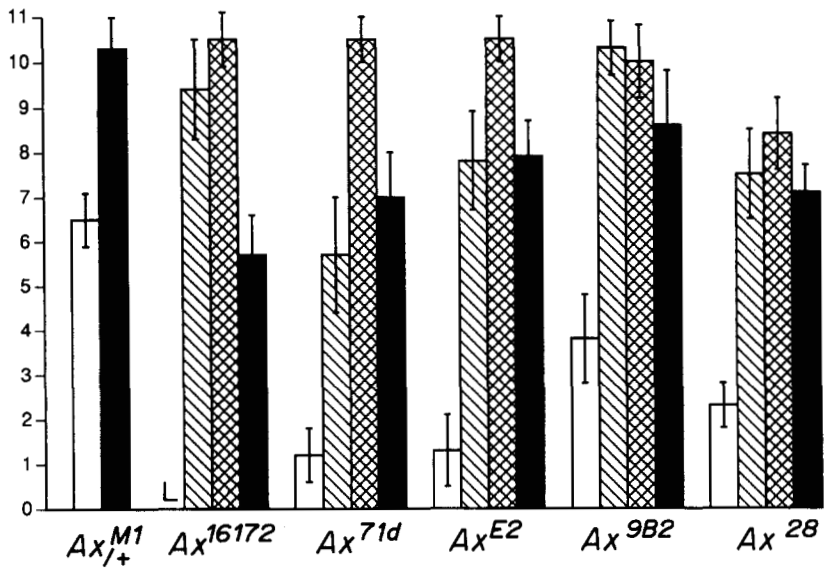


FIGURE 7.—Effects of temperature on the phenotype of absence of notum macrochaetae in different *Ax* heteroallelic combinations. First two columns are $Ax^{M1}/+$ at 25° and at 29°. Other columns in that order: Ax^*/Ax^{M1} at 25°. Ax^*/Ax^{M1} at 29°. Ax^*/N^{55e11} at 29° and Ax^*/Y at 29°. In ordinates number of macrochaetae present. Bars, standard deviations.

at both temperatures, contrary to expectation if Ax^{9B2} becomes an *E(N)* allele at 29°.

The different temperature sensitivity of Ax phenotypes, the inactivation of *l(1)Ax* alleles at 29°, and the different response of each heteroallelic combination to different temperatures suggest that *Ax* mutations cause conformational changes in the N protein. Once different Ax mutant proteins are associated in dimers these changes can be functionally corrected or exaggerated by the other partner of the dimer.

Combination of Ax alleles with the spl mutation: The phenotype of *Ax/spl* combinations depends on whether or not these mutations are in the same or in different proteins (WELSHONS 1965). In *l(1)Ax spl/+* heterozygotes the spl phenotype becomes dominant and the Ax phenotype is highly exaggerated (WELSHONS 1965).

We have carried out all the possible *Ax/spl trans*-heteroallelic combinations and found that the wing vein phenotype is not modified and that the lack of chaetae phenotypes are only weakly increased as compared with the phenotypes of *Ax/+* heterozygous flies (not shown). Combinations of *spl* with *l(1)Ax* alleles grown at 29° have Ax phenotypes of lack of macrochaetae (not shown), indicative of some functional rescue of the temperature-inactivated Ax products by the spl ones.

Combinations of *Ax* alleles with genetic variants in the loci *Dl*, *Ser*, *gro* and *H*: Antimorphic behavior can also be explained by abnormal interactions of a gene product with those of a *trans*-acting gene in heterodimers. In the case of *N* it has been shown that N product can interact with the Dl and Ser products of neighboring cells (REBAY *et al.* 1991) and with Dl in the same cell (FEHON *et al.* 1990). Furthermore, genetic tests have shown functional interactions between *N* alleles and both *Dl* and *Ser*. Thus, $N^-/+$; $Dl^-/+$ double heterozygotes have both N and Dl normalized phenotypes. The Dl wing phenotype is indistinguishable from the Confluens phenotype caused by extra doses of N^+. Interestingly, extra doses of Dl cancel the phenotype of extra doses of *N*, in $DpN^+/+/+$; $Dp\ Dl^+/+/+$ flies (not shown). These results indicate that Dl and N products titrate each other. This is in agreement with the observation that N phenotypes are exaggerated by extra Dl^+ doses and viceversa, and N^+ duplications also correct the phenotypes due to *Ser* insufficiency (VÄSSIN, VIELMETTER and CAMPOS-ORTEGA 1985; DE LA CONCHA *et al.* 1988; FLEMING *et al.* 1990; THOMAS, SPEICHER and KNUST 1991). *H* alleles correct the N insufficiency phenotype in double heterozygotes (VÄSSIN, VIELMETTER and CAMPOS-ORTEGA 1985). Interactions between these genetic variants and some *Ax* alleles have also been shown (XU *et al.* 1990; DE CELIS, MARI-BEFFA and GARCIA-BELLIDO 1991b).

We have studied the phenotypes of all the *Ax* alleles and *spl* in different combinations with *Dl*, *Ser*, *gro* and *H* alleles. Figure 8 shows the macrochaetae phenotypes of the different *Ax* alleles with 1, 2 and 3 doses of the *Dl* locus; with only one dose of the gene *Ser*, and in combinations with *H* and *gro* lethal alleles. As a rule all *Ax* alleles behave in a similar way in these combinations. Thus, Dl^+ duplication exaggerates and *Dl* lethal alleles reduce the Ax phenotype in combinations with both viable and lethal *Ax* alleles (Figures 8 and 9). These results indicate that Ax activation of N proteins is linearly dependent on interactions with Dl products. The Dl phenotypes of the *Dl/+* heterozygotes are concomitantly reduced in all *Ax/+* combinations (Figure 9). Contrary to *Dl* the heterozygosity of a *Ser* null allele does not affect the Ax phenotype of chaetae and veins in combinations with *Ax* alleles. However, doubly heterozygous $l(1)Ax/+;Ser^-/+$ flies

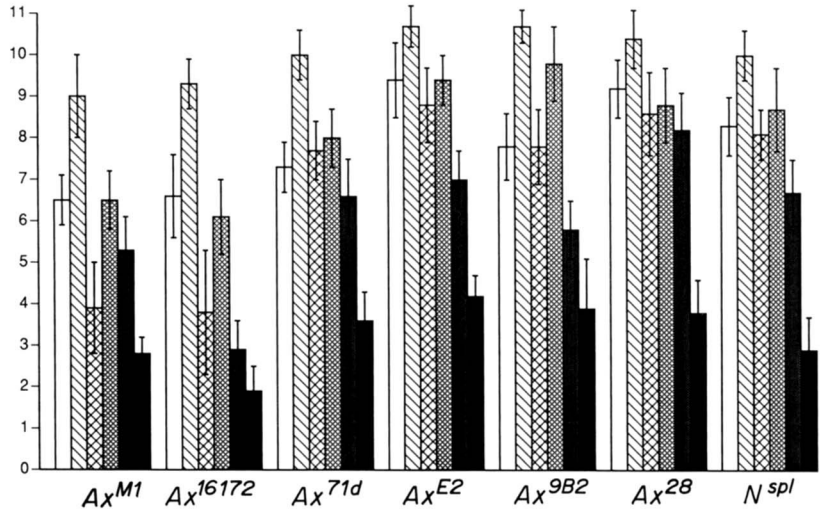


FIGURE 8.—Phenotypes (present macrochaetae) of different *Ax* alleles and *Dl*, *Ser*, *gro* and *H* genetic variants in heterozygosis. Ax^{M1} in heterozygous females, columns in that order: all other *Ax* alleles in hemizygous males, *Ax**. *Ax*;*Dl^{M1}/+. *Ax*;* *Dp*(3;3)bxd^{110},Dl^{+}/+. *Ax*;*Ser^{RX106}/+. *Ax*;l*(3)gro^{E73}/+. *Ax*;*H^{2}/+. Bars, standard deviations.

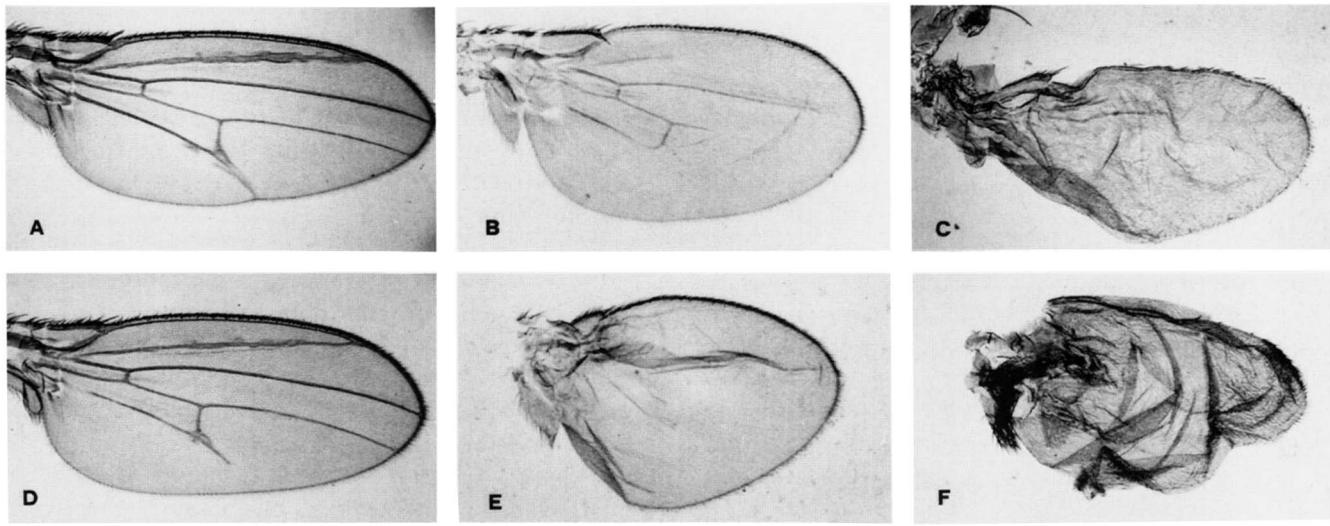


FIGURE 9.—Wing phenotypes of combinations between *Ax* alleles (males) and *Dl* and *H* genetic variants. allelic combinations: (**A**) Ax^{28};Dl^{M1}/+. (**B**) Ax^{28};*D(3;3)*bxd^{110}, $Dl^{+}Dl^{+}$/+. (**C**) Ax^{28};H^{2}/+. (**D**) Ax^{16172};Dl^{M1}/+. (**E**) Ax^{16172};*D(3;3)*bxd^{110}, $Dl^{+}Dl^{+}$/+. (**F**) Ax^{16172};H^{2}/+. All wings at the same magnification.

show a loss of chaetae in the anterior wing margin typical of *l(1)Ax* hemizygous males. Combinations of *Ax* with *H* mutations show strong Ax phenotypes (DE CELIS, MARI-BEFFA and GARCIA-BELLIDO 1991b) (Figures 8 and 9), and most of the combinations are pupal lethal. These pharate adults and scapers show phenotypes very similar to that of lethal *Ax trans*-heterozygous combinations (compare Figures 5B and 9, C and F). Combinations of *Ax* alleles with lethal alleles of *gro* also cause stronger Ax phenotypes (XU *et al.* 1990; DE CELIS, MARI-BEFFA and GARCIA-BELLIDO 1991b) (Figure 8).

The modifications of Ax phenotypes by *Dl*, *gro*, *Ser* and *H* genetic variants are indicative of a role for these gene products in the modulation of the activity of the Ax products, and by extension of that of N^{+}. Since all *Ax* alleles behave in a similar way in these *trans*-heterozygous combinations, allele-specific differences between the different *Ax* mutations in their interactions with the other gene products are not shown.

DISCUSSION

Hypermorphic nature of Ax mutations: Ax phenotypes in wing vein and chaetae differentiation appear as developmetally opposite to those caused by *N* loss of function alleles and can be interpreted as resulting from an enhanced N function. This interpretation can be extended to the phenotypes observed in *Ax* heteroallelic combinations displaying or not negative complementation, and it is compatible with the reduction of Ax phenotypes caused by a reduced amount of Ax products (homozygous *vs.* hemizygous *Ax* females). Furthermore the Ax phenotypes are serial in their effects, suggesting that mutant Ax proteins and their combinations cause different degrees of N hyperactivation. However, *l(1)Ax* flies having the strongest loss of macro- and microchaetae phenotypes

do not show the notum and wing morphological defects typical of *l(1)Ax/Ax*-viable and *Su(N)/E(N)* combinations. These results indicate that *Ax* heteroallelic combinations displaying negative complementation show different degrees of N hyperactivation in different developmental processes. The hypermorphic nature of all *Ax* alleles is contradictory with some *Ax* mutations showing antimorphic and hypomorphic behaviors (PORTIN 1975, 1977a). We discuss in the following sections the implications of the proposed hypermorphic nature of *Ax* alleles with their antimorphic and hypomorphic components in the context of a possible mechanism of N function involving N-ligand interactions and N dimerization.

Antimorphic behavior of *Ax* mutations: The reduction of Ax phenotypes by increasing the relation between N^+ and *Ax* doses defines *Ax* alleles as antimorphic. The titration of Ax mutant products by the wild-type N products could be accomplished by two non-exclusive mechanisms: the competition between N^+ and Ax products in their binding to N ligands and/or the formation of N^+/Ax dimers with wild type N function. That N products may have higher affinity than Ax ones in its binding to N ligands is consistent with the observation that mutant Ax^{E2} proteins have less ability than the N^+ ones to interact with Dl products (LIEBER *et al.* 1992).

Hypomorphic component of Ax mutations: Both lethal and *E(N) Ax* alleles cause N phenotypes in combinations with *N* null alleles. This hypomorphic component can be explained along the same line as the antimorphic one. In this case, a reduction in the amount of Ax products (in *Ax/N* females) would result in the inability of the remaining Ax products to interact with ligands and/or to form functional dimers. Following this interpretation, Su(N) products would be able to interact efficiently with Dl and/or to form functional dimers, despite a reduction in the level of their products.

The hyperactivation of N protein by *Ax* mutations and the associated anti- and hypomorphic components are closely related. Thus, *l(1)Ax* alleles have the strongest Ax phenotypes and *E(N)* alleles gives stronger phenotypes than *Su(N)* alleles. In addition, temperature sensitive *Ax* alleles (Ax^{28}, Ax^{E2} and Ax^{16172}) have stronger Ax phenotypes at 29° than at 25°, and they are almost lethal (Ax^{28}) or produce a stronger enhancement of the N phenotype (Ax^{9B2}, Ax^{E2} and Ax^{16172}) in combinations with *N* null alleles at 29°.

The products of *l(1)Ax*alleles Ax^{59d}, Ax^{59b} (PORTIN 1977a), Ax^{M1} and Ax^{M3} are inactivated at 29°. Temperature also modifies the phenotype of several viable *Ax* alleles and their hypomorphic component. These observations suggest that Ax modification of N function may depend on conformational changes in the N protein. The partial rescue of inactivated l(1)Ax products (in some *l(1)Ax/Ax-viable* combinations at the restrictive temperature), and the complementation between viable *Ax* alleles and one *N* allele with a mutation in the N ligand-binding domain (DE CELIS *et al.* 1993) suggest that different N and Ax mutant proteins can compensate each other their functional modifications. Both, functional rescue between mutant proteins and the reported relationship of N hyperactivation and associated anti- and hypomorphic components of each *Ax* allele help to understand negative complementation between some *Ax* alleles.

Heteroallelic interactions and negative complementation: *Ax* heteroallelic combinations showing negative complementation display strong Ax phenotypes. These phenotypes include loss of chaetae and wing veins and, in addition abnormalities in the shape and size of imaginal discs and in adult morphology. Again, these phenotypes are different to those resulting from extreme *N* insufficiency (SHELLENBARGER and MOHLER 1978), and might correspond to N hyperactivation caused by Ax dimers. These observations can now be considered together with the fact that negative complementation can be reverted by inactivation of one of the *Ax* allele or by mutations in some *N-trans*-acting genes (XU *et al.* 1990).

We suggest that negative complementation is the result of a stronger N hyperactivation due to the presence, in the same cell, of two different Ax proteins. By themselves, one of these Ax proteins would have stronger self-activation and lower ability to interact with ligands and/or form dimers, and the other Ax protein would have lower self-activation but stronger ability to interact with N ligands and/or to dimerize. This interpretation implies that N activation requires dimer formation, and is compatible with the observed clustering of *E(N)* and *Su(N) Ax* mutations in the *N* coding region (KELLEY *et al.* 1987). It further suggests that dimer formation depends or is facilitated by the interaction of N protein with its ligands. Similar ligand-dependent dimerization of receptors has been shown to occur in the case of the epidermal growth factor receptor (YARDEN and SCHLESSINGER 1987).

***Trans*-interactions involving *Ax* alleles:** Ax phenotypes are modified by the number of doses of different *N trans*-acting genes. Of the known N-ligand encoding genes tested (*Dl* and *Ser*) only *Dl* genetic variants modify the whole spectrum of Ax phenotypes. Thus Dl^+ duplications exaggerate while *Dl* mutations reduce the Ax phenotype. The dependence of the Ax phenotypes on the number of copies of Dl^+ suggests that Ax proteins have to interact with Dl ones to cause N hyperactivation. *Ax* mutations would then cause a non-constitutive N hyper-activation, modulated and dependent on interactions with Dl products. Interestingly *Dl* mutations also rescue the lethality of *Ax* heteroallelic combinations that show negative comple-

mentation (Xu *et al.* 1990), which is consistent with the notion that negative complementation is the result of a stronger N activation of some Ax dimers by Dl products. Surprisingly, *N* loss of function mutations behave in the same way in combinations with different doses of the gene *Dl*. Thus *Dl* mutations corrects N phenotypes and *Dl* extra copies exaggerate them. The antagonistic effects of *Dl* and *N* are reciprocal, because *N* extra doses increase Dl phenotypes and *N* mutations suppress them (Vässin, Vielmetter and Campos-Ortega 1985). In fact, the "Confluens" phenotype of extra N^+ copies is indistinguishable to that of *Dl* mutations. Thus Dl and N products seem to mutually titrate each other; more or less relative amounts of one of them causing insufficiency or excess of the other.

In wing venation whereas *N* (like *Ax*) mutations are exclusively cell autonomous, the Dl and Co phenotypes appear both in mutant cells and in the cells surrounding the mutant clone in genetic mosaics (Garcia-Bellido and de Celis 1992; our unpublished results). This directionality of the mutant effects on the receptor or ligand side of the interaction has to be taken into consideration in order to understand the mutant phenotypes. Thus, the mutual N-Dl titration could occur in the surface of the same cell (Fehon *et al.* 1990) causing a relative depletion of Dl products to act in neighboring cells. Consequently, extra doses of *N* could cause less Dl products available to bind to N receptors of neighboring cells and hence lead to a Dl (or Confluens) phenotype and to its exaggeration in *Dl* heterozygotes. Extra doses of Dl^+ cause more activation of Ax products and exaggerate Ax phenotypes, as expected. However, the same extra doses of *Dl* should activate more N receptors, both of its own and of neighboring cells but not exaggerate N insufficiency phenotypes, as observed. We then have to postulate that Dl products can bind to the N receptors of the same cell but fail to activate them, in fact binding meaning depletion of N receptors and hence N phenotypes. This interpretation applied to the Confluens phenotype as similar to that of Dl, implies that both result from an insufficiency of Dl products, *i.e.* to insufficient N activation. This is borne out by the fact that flies with three doses of N^+ and of Dl^+ have a normalized wing phenotype, as flies with only one dose of both ($N^-/+;Dl^-/+$ double heterozygotes). The observation that *Ax* mutations correct both Dl and Confluens phenotypes is consistent with that interpretation, because Ax hyperactivation would compensate for Dl insufficiency. The directionality of effects is not readily explained, but could be due to (1) conformational properties of these molecules on the cell surface vs. in intercellular interactions or (2) the requirements of a second ligand to activate the bound N-Dl heterodimers, Ax proteins being less dependent on it.

Contrary to *Dl*, *Ser* deletions, which enhance the loss of chaetae in the triple row of some *Ax* alleles, do not affect other Ax phenotypes. Interestingly, the same *Ser* deletions increase the N insufficiency phenotype of nicks in the wing margin (Fleming *et al.* 1990; Thomas, Speicher and Knust 1991). These results are compatible with the existence of competitive interactions between Dl and Ser in binding to N products in neighboring cells. Such interactions are known to occur in the same EGF repeats of N (Rebay *et al.* 1991). Thus a reduction of the amount of Ser protein may make more Ax proteins available to interact with the Dl protein. The absence of generic effects of *Ser* on Ax phenotypes indicates that N hyperactivation caused by *Ax* mutations is independent on interactions between N and Ser proteins.

The strongest interactions observed with *Ax* mutations are found in combinations between *Ax* and *H* alleles. Several of these combinations are late pupal lethal, the phenotype of the pharate adults being similar to that of *Ax* heteroallelic combinations that display negative complementation. These results indicate that the H gene product is specifically involved in the modulation of the N activation. The *H* gene codes for a putative nuclear protein with yet unknown function (Bang and Posakony 1992; Maier *et al.* 1992) and *H* alleles have Ax-like mutant phenotypes in both sensory elements and veins. H products may then be involved in reverting the ligand-dependent N activation.

Heterozygous *gro* lethal mutations also enhance Ax phenotypes (Xu *et al.* 1990; this work) but further reduction in *gro* function corrects Ax phenotypes (de Celis, Mari-Beffa and Garcia-Bellido 1991b). These results suggest that N negatively controls *gro* function, as variable amounts of gro proteins cause both neural hyper- and hypoplasia in the embryonic central nervous system (Schrons, Knust and Campos-Ortega 1992). The gene *gro* encodes a G-like protein (Preiss, Hartley and Artavanis-Tsakonas 1988) whose relation with the N pathway is unknown.

The final targets of the signal transduction pathway initiated by the activation of N are nuclear genes involved in cell differentiation as nervous elements, such as the *achaete-scute* system (de Celis, Mari-Beffa and Garcia-Bellido 1991b) and veins, such as those related to *vein* (Garcia-Bellido and de Celis 1992). N and Ax mutant phenotype on chaetae and veins can be explained by the abnormal modulation of these genes acting in two independent developmental processes.

We thank D. Gubb for constructive criticism to the manuscript. R. Hernandez, A. Hernando, A. Lopez and P. Martin contributed with skillful technical assistance. J.F.deC. is postdoctoral fellow

of the Rich Foundation. The work was supported by grants of the Spanish Dirección General de Investigación Cienfica y Técnica and the Areces Foundation.

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Communicating editor: T. W. CLINE