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Partitioning of *N*-Ethylmaleimide-Sensitive Fusion (NSF) Protein Function in *Drosophila melanogaster*: dNSF1 Is Required in the Nervous System, and dNSF2 Is Required in Mesoderm

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ABSTRACT

The *N*-ethylmaleimide-sensitive fusion protein (NSF) promotes the fusion of secretory vesicles with target membranes in both regulated and constitutive secretion. While it is thought that a single NSF may perform this function in many eukaryotes, previous work has shown that the Drosophila genome contains two distinct NSF genes, *dNSF1* and *dNSF2*, raising the possibility that each plays a specific secretory role. To explore this possibility, we generated mutations in the *dNSF2* gene and used these and novel *dNSF1* loss-of-function mutations to analyze the temporal and spatial requirements and the degree of functional redundancy between *dNSF1* and *dNSF2*. Results of this analysis indicate that dNSF1 function is required in the nervous system beginning at the adult stage of development and that dNSF2 function is required in mesoderm beginning at the first instar larval stage of development. Additional evidence suggests that dNSF1 and dNSF2 may play redundant roles during embryonic development and in the larval nervous system. Ectopic expression studies demonstrate that the *dNSF1* and *dNSF2* gene products can functionally substitute for one another. These results indicate that the Drosophila NSF proteins exhibit similar functional properties, but have evolved distinct tissue-specific roles.

VESICLE trafficking in the constitutive and regulated secretory pathways requires a set of core polypeptides that mediate interactions between the transport vesicle and the target membrane. Among these core polypeptides are vesicular membrane proteins known as v-SNAREs, target membrane proteins known as t-SNAREs, and a soluble ATPase known as the *N*-ethylmaleimide-sensitive fusion protein (NSF). Although the precise functional roles of these components in vesicle exocytosis remain unclear, a large body of work has demonstrated that v-SNAREs and t-SNAREs can assemble to form a complex and that NSF can catalyze disassembly of this complex by coupling SNARE complex disassembly to ATP hydrolysis (Jahn and Sudhof 1999; Klenchin and Martin 2000; Lin and Scheller 2000; Wickner and Haas 2000). These observations, together with other work on NSF and SNARE function, suggest several models by which these components may contribute to vesicle exocytosis. One possibility is that NSF is recruited to a SNARE complex anchoring a secretory vesicle to its target membrane and promotes membrane fusion by catalyzing complete or partial disassembly of the SNARE complex. Another model consistent with available data on NSF and SNARE function is that NSF is required after vesicle fusion to disassemble a SNARE complex formed prior to or during membrane fusion, thereby reactivating SNAREs for another round of exocytosis. Yet another possibility is that NSF plays both prefusion and postfusion roles in SNARE complex metabolism.

More recent work suggests the possibility of novel functional roles for NSF. Muller *et al.* (1999) have shown that a mutationally altered version of NSF that apparently lacks both ATPase and SNARE complex disassembly activity is capable of promoting membrane fusion in an *in vitro* Golgi reassembly assay, suggesting, among other possibilities, a role for NSF in SNARE activation prior to membrane fusion. Another recent series of studies has shown that NSF may regulate the function or subcellular distribution of the AMPA class of postsynaptic ionotropic glutamate neurotransmitter receptors in hippocampal neurons (Nishimune *et al.* 1998; Osten *et al.* 1998; Song *et al.* 1998; Luscher *et al.* 1999; Luthi *et al.* 1999; Noel *et al.* 1999). This function of NSF appears distinct from its more familiar role in vesicle trafficking, as it requires direct binding of NSF to the glutamate receptor (Nishimune *et al.* 1998; Osten *et al.* 1998; Song *et al.* 1998). Several models have been proposed to explain these results: NSF may regulate the insertion of glutamate receptors into the plasma membrane, anchor the receptors to prevent lateral diffusion away from release sites, or act as a chaperone to regulate receptor conformation or interactions with other proteins (Nishimune *et al.* 1998; Osten *et*

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al. 1998; Song *et al.* 1998; Luscher *et al.* 1999; Luthi *et al.* 1999; Noel *et al.* 1999).

To elucidate the cellular functions of NSF, we have chosen to use a genetic approach in the fruit fly, *Drosophila melanogaster*. Previous work has shown that there are two Drosophila NSF genes encoding polypeptides with 84% amino acid identity to one another, designated *dNSF1* and *dNSF2* (Boulianne and Trimble 1995; Pallanck *et al.* 1995b). *dNSF1* is expressed at high levels in adult flies and at relatively lower levels earlier in development, while *dNSF2* is expressed at similar levels during the larval and adult stages of development (Pallanck *et al.* 1995b). Temperature-sensitive alleles of *dNSF1*, termed *comatose* (here designated $dNSF1^{comt}$), were identified in a classical genetic screen for mutations causing paralysis at elevated temperature (Siddiqi and Benzer 1976). Electrophysiological and ultrastructural studies of $dNSF1^{comt}$ mutants have demonstrated that dNSF1 plays a priming role in preparing docked synaptic vesicles for fast calcium-triggered fusion (Kawasaki *et al.* 1998). Further, biochemical characterization of $dNSF1^{comt}$ mutants indicates that the dNSF1 priming role involves disassembly or rearrangement of a neural SNARE complex at the plasma membrane (Tolar and Pallanck 1998).

While studies of the $dNSF1^{comt}$ mutants have told us much about the role that dNSF1 plays in neurotransmitter release, several features of dNSF1 function remain unexplored. For example, while it is well established that dNSF1 plays an important presynaptic role in the adult nervous system, it is not known whether it plays a similar functional role prior to the adult stage of development. Further, it remains unknown whether dNSF1 functions in a postsynaptic capacity to regulate glutamate receptor function or whether dNSF1 plays secretory roles outside of the nervous system. It also remains completely unclear what function is provided by the closely related *dNSF2* gene. To investigate these issues, we generated mutations in *dNSF2* and subjected *dNSF2* mutants, along with novel loss-of-function *dNSF1* mutants, to molecular and genetic analysis. Results of these analyses show that dNSF2 function is required for viability beginning at the first instar larval stage of development, while dNSF1 function is required for viability at the adult stage of development. Transgenic rescue experiments using the *GAL4* system (Brand and Perrimon 1993) indicate that the primary function of dNSF1 resides in the nervous system, while the primary function of dNSF2 resides in mesoderm. These results, coupled with other work on NSF function, suggest the possibility that dNSF1 functions primarily in a presynaptic capacity to regulate neurotransmitter release and that dNSF2 may function in a postsynaptic capacity, perhaps in the regulation of glutamate receptor activity or synaptic maturation. Availability of *dNSF1* and *dNSF2* loss-of-function alleles will provide a foundation for directly testing these hypotheses.

MATERIALS AND METHODS

Generation of *dNSF2* mutations: Identification of chromosomes with deficiencies that remove the *dNSF2* gene was carried out using *in situ* hybridization to Drosophila polytene chromosomes as previously described (Pallanck *et al.* 1995b). To generate recessive lethal *dNSF2* mutations, male flies isogenic for a third chromosome bearing the recessive marker *scarlet* (*st*) were fed 25 mm ethylmethanesulfonate (EMS) and mated to *TM3/TM6B* females. Balanced offspring from this cross, each of which bears a unique mutagenized third chromosome, were then individually mated to *Df(3R)urd/TM6C* flies. Because the *Df(3R)urd* chromosome also bears the *st* marker, an absence of scarlet-eyed progeny from this cross indicates that a recessive lethal mutation in the *Df(3R)urd* deletion interval resides on the mutagenized chromosome. Stocks bearing recessive lethal mutations in the *Df(3R)urd* region were established by recovering these mutations *in trans* to the *TM6C* balancer chromosome. To place these mutations into complementation groups, all of the mutants recovered in this screen were mated to one another. Pairs of mutants that failed to produce scarlet-eyed progeny when mated were considered to be in the same complementation group.

Transgenic Drosophila lines: The *dNSF1* transgene consisting of a *dNSF1* cDNA under control of an *hsp70* heat-shock promoter has been described (Pallanck *et al.* 1995a). A *dNSF2* transgenic construct consisting of a full-length *dNSF2* cDNA under control of the *hsp70* promoter was generated by replacing the *dNSF1* cDNA in the *dNSF1 hsp70* transformation construct with the *dNSF2* dN2.14 cDNA (Pallanck *et al.* 1995b). *UAS-dNSF1* and *UAS-dNSF2* transgenes were created by cloning the full-length *dNSF1* dN20 cDNA (Ordway *et al.* 1994) and *dNSF2* dN2.14 cDNA into the pUAST transformation vector (Brand and Perrimon 1993). All transgenes were introduced into flies using standard germline transformation methods.

A heat-shock-inducible *dNSF2* transgenic line was used to rescue candidate *dNSF2* mutants recovered in the *Df(3R)urd* interval by crossing the candidate *dNSF2* mutants to a stock bearing the *dNSF2* transgene *in trans* to an *SM5* balancer chromosome and the *Df(3R)urd* chromosome *in trans* to the *TM3* balancer chromosome and maintaining the offspring at 25°. *dNSF2* mutants were identified as those stocks producing non-*SM5*, non-*TM3* adult flies from this cross. To rescue *dNSF2* mutants with the heat-shock *dNSF1* transgenic line, flies were subjected to heat shocks of 38° for 1 hr each day throughout development. To test for rescue of the $dNSF1^{comt}$ temperature-sensitive paralytic phenotype, adult male flies bearing a $dNSF1^{comt}$ mutation, a *UAS-dNSF1* or *UAS-dNSF2* transgenic line, and the relevant *GAL4* line were aged 3–6 days and examined in groups of 10 as described (Pallanck *et al.* 1995a). At least three groups of 10 flies each were tested for each experiment, and the standard deviation was determined for the three trials.

Sequencing of *dNSF1* and *dNSF2* mutations: To generate flies suitable for isolating the *dNSF1* mutations for sequence analysis, females bearing the $dNSF1^{dp}$ and $dNSF1^{3\text{-}13\text{-}3}$ alleles *in trans* to the *FM7* balancer chromosome were crossed to males bearing a heat-shock-inducible *dNSF1* transgene. Offspring from this cross were subjected to daily heat shocks of 1 hr at 38° to allow survival of hemizygous $dNSF1^{dp}$ and $dNSF1^{3\text{-}13\text{-}3}$ adult males. Genomic DNA was extracted from the hemizygous *dNSF1* mutants and subjected to polymerase chain reaction using primers flanking the *dNSF1* open reading frame. PCR products corresponding to the *dNSF1* mutant alleles were separated from the smaller PCR product derived from the *dNSF1* cDNA transgene using agarose gel electrophoresis. The $dNSF1^{dp}$ and $dNSF1^{3\text{-}13\text{-}3}$ PCR products were recovered from the agarose gel and subjected to dideoxy sequencing using a combination of primers that allow full coverage of the entire *dNSF1* open reading frame.

A similar strategy was used to sequence the *dNSF2* mutations. Crosses were carried out to generate flies bearing the *dNSF2* mutations *in trans* to the *Df(3R)urd* chromosome in a background of transgenic *dNSF2* expression. *dNSF2* sequences were amplified using PCR primers flanking the *dNSF2* open reading frame, and the resulting products were fractionated on agarose gels to separate the *dNSF2* mutant alleles from the smaller *dNSF2* transgenic product. The relevant PCR products were recovered from the agarose gel and subjected to dideoxy sequencing using primers that allow full coverage of the *dNSF2* open reading frame.

Germline clone analysis: To generate female germlines homozygous for *dNSF1* or *dNSF2* mutations, the *FLP/FRT, ovo^{D1}* system was used. Recombinant chromosomes bearing the *dNSF1^{dlp}* or *dNSF1$^{3\text{-}13\text{-}3}$* mutations as well as a proximal *FRT101* element were kindly provided by Rick Ordway, and a chromosome bearing the *dNSF2^{55}* mutation with a proximal *FRT82B* element was generated. *dNSF1* germline clones were generated by mating females bearing the *dNSF1^{dlp} FRT101* or *dNSF1$^{3\text{-}13\text{-}3}$ FRT101* chromosomes *in trans* to the *FM7* balancer chromosome to *FRT101 ovo^{D1}*; *hsFLP/hsFLP* males. Offspring from this cross were heat shocked at 38° for 2 hr each day for two successive days beginning at the first instar larval stage of development to induce expression of the FLP recombinase from the heat-shock promoter. Because both heterozygous and homozygous *ovo^{D1}*-bearing germline clones degenerate (Chou *et al.* 1993), the only oocytes produced in female offspring from this cross must be *dNSF1^{dlp}* or *dNSF1$^{3\text{-}13\text{-}3}$* homozygotes produced by FLP recombinase-induced mitotic recombination at the *FRT* sites (Xu and Rubin 1993). Females bearing *dNSF1^{dlp}* or *dNSF1$^{3\text{-}13\text{-}3}$* germline clones were then mated to males bearing an *FM7* balancer chromosome marked with green fluorescent protein (GFP). Non-GFP embryos corresponding to hemizygous *dNSF1^{dlp}* or *dNSF1$^{3\text{-}13\text{-}3}$* males were identified using fluorescence microscopy, transferred to fresh medium, and monitored daily until lethality was observed.

dNSF2 germline clones were generated by mating females bearing the *dNSF2^{55} FRT82B* chromosome *in trans* to a *TM3* balancer chromosome to *hsFLP*; *FRT82B ovo^{D1}* males. Offspring from this cross were heat shocked to induce expression of the FLP recombinase as described above, and female progeny were mated to males bearing the *dNSF2* deletion chromosome, *Df(3R)urd*, *in trans* to a *TM3* balancer chromosome marked with GFP. *dNSF2^{55}/Df(3R)urd* embryos were identified by their lack of GFP fluorescence and monitored daily until lethality was observed.

Preparation and analysis of SNARE complexes: Flies used for examination of SNARE complexes were aged 3–6 days. Samples for SNARE complex analysis were prepared and subjected to electrophoresis and electroblotting onto nitrocellulose membranes as described (Tolar and Pallanck 1998). Blots were incubated with an affinity-purified polyclonal antiserum that recognizes the cytoplasmic domain of n-synaptobrevin (van de Goor *et al.* 1995). After staining blots with antiserum, bands were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Quantitation of n-synaptobrevin and SNARE complexes was performed using a Digital Science Image Station 440CF (Kodak, Rochester, NY) and 1D Image Analysis software (Kodak).

Fly strains: The *GAL4* driver lines used include the following: *w- P{w+} elav^{C155}-GAL4}* (Robinow and White 1988), *w-; P{w+} 24B-GAL4}* (Brand and Perrimon 1993), *P{w+} BG57-GAL4}* (Budnik *et al.* 1996), *P{ry+} MHC-82-GAL4}* (Schuster *et al.* 1996), and *w-P{w+}Dmef2-GAL4}* (Ranganayakulu *et al.* 1996). Stocks bearing the GFP-marked *TM3* and *FM7* balancer chromosomes, as well as stocks necessary for the production of germline clones, are available from the Bloomington stock center. The specific stocks used in this work are as follows: (1) *w-; TM3 P{w+}ActGFP} Ser/Sb*; (2) *FM7i P{w+}ActGFP}/C(1)DX*; (3) *y1 f1 w-; P{ry+}neoFRT}82B P{w+}87E*, which was used to generate the *dNSF2^{55} FRT82B* recombinant chromosome; (4) *w-; P{ry+}neoFRT}82B P{w+} ovo^{D1}-18}3R1P{w+}ovo^{D1}-18}3R2/st betaTub85DD ss es/TM3*, which was used to generate *dNSF2^{55}* germline clones; (5) *y w- v P{w+}FRT101}*, which was used to generate the *FRT101 dNSF1^{dlp}* and *FRT101 dNSF1$^{3\text{-}13\text{-}3}$* recombinant chromosomes; and (6) *w-ovo^{D1} v24 P{w+} FRT101}/C(1)DX; hsFLP/hsFLP*, which was used to generate *dNSF1^{dlp}* and *dNSF1$^{3\text{-}13\text{-}3}$* germline clones.

RESULTS

Generation of *dNSF2* mutations: The Drosophila *dNSF2* gene has been previously localized to polytene region 87F14-15 on the right arm of chromosome 3 (Pallanck *et al.* 1995b). To generate mutations in this gene, chromosomes bearing deficiencies in this region were subjected to *in situ* hybridization using a *dNSF2* cDNA probe to identify deficiencies that remove the *dNSF2* gene. One of these chromosomes, *Df(3R)urd*, failed to produce a hybridization signal in this analysis, indicating that the *dNSF2* gene lies within the boundaries of the *Df(3R)urd* deficiency (data not shown). This chromosome was used in an F_2 genetic screen to identify recessive lethal mutations in the deletion interval. From a screen of ~7000 EMS-mutagenized third chromosomes, 18 mutations were recovered that were lethal *in trans* to the *Df(3R)urd* chromosome. Crosses conducted with these 18 mutants indicated that they represent six complementation groups, each containing between one and seven alleles.

To determine which of these complementation groups represent *dNSF2*, a transposable *P*-element construct containing a *dNSF2* cDNA under the control of a heat-shock-inducible promoter was introduced into the Drosophila germline and tested for its ability to rescue the lethality associated with the 18 candidate *dNSF2* mutations. The results of this analysis indicate that the *dNSF2* gene is represented by eight recessive lethal alleles. Six of the seven alleles from one complementation group (designated *dNSF2^{11}*, *dNSF2^{15}*, *dNSF2^{42}*, *dNSF2^{55}*, *dNSF2^{102}*, and *dNSF2^{103}*) were efficiently rescued from lethality by the *dNSF2* transgene, and all of the rescued flies appeared normal and were fertile in crosses. The one allele from this complementation group that could not be rescued by the *dNSF2* transgene (*dNSF2^{66}*) also failed to complement a mutation in a distinct complementation group, suggesting that the *dNSF2^{66}* mutation may affect more than one gene. The *dNSF2* transgene also rescued a mutation that complemented all of the other mutations in the *Df(3R)urd* interval (designated *dNSF2^{21}*), indicating that this mutation may represent a hypomorphic *dNSF2* allele.

Characterization of *dNSF2* mutants: In an effort to rank the different *dNSF2* alleles in terms of their severity, the *dNSF2* mutants were subjected to lethal phase analy-

TABLE 1

Lethal phase and germline clone analysis of *dNSF2* and *dNSF1* alleles

Allele	Lethal stage	Lethal stage (without maternal contribution)
dNSF2^{11}	Middle first instar larvae	—
dNSF2^{15}	Middle first instar larvae	—
dNSF2^{21}	Late first instar larvae	—
dNSF2^{42}	Pharate adults/adults	—
dNSF2^{55}	Middle first instar larvae	Middle first instar larvae
dNSF2^{102}	Late first/early second instar larvae	—
dNSF2^{103}	Middle first instar larvae	—
dNSF1^{dlp}	Pharate adults	Pharate adults
dNSF1$^{3\text{-}13\text{-}3}$	Pharate adults	Pharate adults
dNSF1$^{comt\ st17}$	Pharate adults	—
dNSF1$^{comt\ tp7}$	Pharate adults	—

The lethal phase of *dNSF2* mutants was determined by crossing flies bearing these alleles *in trans* to the *TM6C* balancer chromosome to a stock bearing the *Df(3R)urd* chromosome *in trans* to *TM6C*. Nonbalancer offspring were monitored until lethality was observed. Approximately one-half of *dNSF2*^{42} mutants die either as pharate adults or within several days of hatching when reared at low density, and lethality for this allele was nearly 100% prior to the adult stage when the mutants were reared under crowded conditions. The lethal phase of the *dNSF1* recessive lethal alleles was determined by crossing females bearing these mutations *in trans* to a GFP-marked balancer chromosome to males bearing a GFP-marked X chromosome. Non-GFP offspring were collected and monitored until lethality occurred. Lethal phase analysis of *dNSF1*$^{comt\ st17}$ and *dNSF1*$^{comt\ tp7}$ mutants was conducted by culturing flies at 29° throughout development, beginning within the first 24 hr of development. To determine the lethal phase in the absence of maternal and zygotic contribution of dNSF1 or dNSF2, the *FRT/ovo*^{D1} system was used to create mosaic females homozygous for the most severe mutant alleles of these genes in their germlines. These females were then mated to the appropriate males (see MATERIALS AND METHODS) to produce progeny lacking maternal and zygotic dNSF1 or dNSF2, and these embryos were monitored until lethality occurred. A minimum of 100 offspring were scored for each of the table entries.

sis, and the molecular nature of these alleles was determined through direct sequencing of genomic DNA. All of the *dNSF2* mutants appear to develop normally as embryos, are able to hatch from the egg case, and show normal larval locomotion as hemizygotes with the *Df(3R)urd* chromosome. However, the most severe *dNSF2* mutants ceased moving and died during the mid-first instar larval stage of development (Table 1). Two of the most severe alleles, *dNSF2*^{15} and *dNSF2*^{55}, contain premature stop codons in the *dNSF2* coding sequence and likely represent null alleles of the *dNSF2* gene (Figure 1). Another of the most severe alleles, *dNSF2*^{103}, results in the replacement of the final 314 codons of the *dNSF2* gene with 43 novel codons followed by a premature translation termination signal (Figure 1). The remaining alleles were found to be missense mutations or were devoid of mutations in the coding sequence, and most were found to cause death later in development, suggesting that many of these mutations are not null alleles of *dNSF2* (Figure 1; Table 1).

To investigate whether the lethal phase of the *dNSF2* mutants is influenced by a possible maternal deposition of dNSF2 protein, the *FLP/FRT*, *ovo*^{D1} system (CHOU and PERRIMON 1996) was used to create female flies with germlines homozygous for a putative null allele of *dNSF2*, *dNSF2*^{55}. These females were crossed to males bearing a *Df(3R)urd* chromosome *in trans* to a balancer chromosome marked with GFP, and the lethal phase of the non-GFP offspring of this cross, which are devoid of germline and zygotic expression of *dNSF2*, was monitored. Elimination of maternal and zygotic *dNSF2* expression was found to result in a first instar larval lethal phase, indicating that maternal contribution of dNSF2 protein does not affect the *dNSF2* lethal phase (Table 1).

Characterization of *dNSF1* mutants: To compare and contrast *dNSF2* and *dNSF1* function, severe loss-of-function alleles of *dNSF1* were obtained and characterized. The recessive lethal alleles of *dNSF1* chosen for this analysis, *dNSF1*^{dlp} (kindly provided by K. S. Krishnan) and *dNSF1*$^{3\text{-}13\text{-}3}$ (kindly provided by S. Titus and B. Ganetzky), were obtained from noncomplementation screens using the conditional *comatose* alleles of the *dNSF1* gene. Direct sequencing of genomic DNA corresponding to the *dNSF1*^{dlp} allele revealed a one-nucleotide deletion in the carboxy-terminal coding region of the *dNSF1* gene, which, if translated, would produce a dNSF1 polypeptide with 18 novel amino acids replacing the normal 37 carboxy-terminal amino acids (Figure 1). The *dNSF1*$^{3\text{-}13\text{-}3}$ allele contains a missense mutation that results in an E to K substitution at amino acid position 606 of the second ATPase domain (Figure 1). While the nature of the molecular lesions associated with these two *dNSF1* loss-of-function alleles leaves open the possibility for residual dNSF1 function, Western blot analysis of protein extracts obtained from *dNSF1*^{dlp} mutants indicates that this allele fails to produce detectable dNSF1 protein (data not shown). Thus, the *dNSF1*^{dlp} mutation may represent a null allele of this gene.

Lethal phase analysis conducted with males hemizygous for the *dNSF1*^{dlp} and *dNSF1*$^{3\text{-}13\text{-}3}$ alleles indicates that these mutations result in pharate adult lethality (Table 1). In many cases the flies bearing these mutations manage to protrude their heads out of the pupal case, but they are rarely able to eclose completely. When dissected from the pupal case, the *dNSF1*^{dlp} and *dNSF1*$^{3\text{-}13\text{-}3}$ mutants appear morphologically normal but are incapable of coordinated movement and die within several days. The lethal phase of these mutants cannot be explained by secondary mutations residing on the X chromosomes

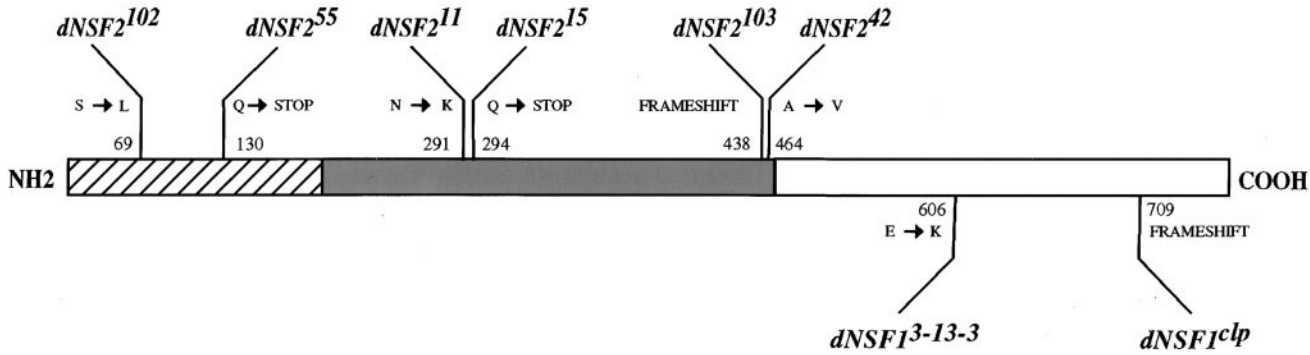

FIGURE 1.—The amino acid changes caused by six *dNSF2* and two *dNSF1* recessive lethal mutations are shown above and below the NSF domain structure, respectively (TAGAYA *et al.* 1993). Numbers refer to the amino acid position in the protein sequence. The $dNSF2^{103}$ mutation is a 334-nucleotide deletion that completely removes a 240-nucleotide intron, as well as flanking 5′ and 3′ exon sequences. This deletion fuses the first two bases of dNSF2 codon 438 to the first base of codon 470. Theoretical translation of the $dNSF2^{103}$ allele predicts a severely truncated polypeptide with 43 novel amino acids following threonine 438 before terminating at an out-of-frame stop codon. The $dNSF1^{clp}$ mutation is a one-nucleotide deletion of the first position of codon 709, which upon translation should result in replacement of the 37 carboxy-terminal dNSF1 amino acids with 18 novel amino acids. The $dNSF2^{21}$ allele was also subjected to sequence analysis, but no mutation was identified in the coding sequence, suggesting that the $dNSF2^{21}$ mutation may affect *dNSF2* transcription or splicing. Hatched box, N-domain; shaded box, ATPase domain 1; open box, ATPase domain 2.

bearing the $dNSF1^{clp}$ and $dNSF1^{3\text{-}13\text{-}3}$ mutations, as males bearing these alleles can be fully rescued by ectopic expression of dNSF1 protein from a transgene (see below).

A possible maternal contribution of dNSF1 protein does not appear to account for the survival of the most severe *dNSF1* mutants to the pharate adult stage of development. The *FLP/FRT*, ovo^{D1} system (CHOU and PERRIMON 1992) was used to create female flies bearing a germline homozygous for the $dNSF1^{clp}$ or $dNSF1^{3\text{-}13\text{-}3}$ mutations, and these females were crossed to males bearing an X chromosome balancer marked with GFP. Non-GFP male offspring from these crosses, hemizygous for one of the two *dNSF1* mutant alleles, also die as pharate adults (Table 1), indicating that wild-type dNSF1 protein is not required prior to the adult stage of development. Further support for an adult-specific requirement of dNSF1 protein for viability is provided by lethal phase analysis conducted with the temperature-sensitive *comatose* alleles of *dNSF1*. When $dNSF1^{comt\ st17}$ and $dNSF1^{comt\ tp7}$ mutants are shifted to the restrictive temperature of 29° within the first 24 hr of development, these mutants exhibit a lethal phase identical to that of the $dNSF1^{clp}$ and $dNSF1^{3\text{-}13\text{-}3}$ mutants (Table 1). Rare adult $dNSF1^{comt\ st17}$ and $dNSF1^{comt\ tp7}$ flies that manage to eclose at 29° exhibit the same uncoordinated movement as the $dNSF1^{clp}$ and $dNSF1^{3\text{-}13\text{-}3}$ mutants dissected from the pupal case and also die within several days if maintained at 29°. Control experiments carried out with wild-type flies confirmed that these phenotypes are specific to the $dNSF1^{comt}$ mutants.

Although hemizygous male larvae bearing these *dNSF1* alleles are viable, they exhibit several phenotypes. For example, when rolled onto their dorsal surface, the larvae take far longer to reorient to their ventral surface than do wild-type larvae (data not shown). In addition, $dNSF1^{clp}$ and $dNSF1^{3\text{-}13\text{-}3}$ hemizygous male larvae are often found protruding vertically from the surface of the medium for long periods of time and often burrow deeply into the medium and die, possibly as a result of suffocation. $dNSF1^{clp}$ and $dNSF1^{3\text{-}13\text{-}3}$ hemizygous male larvae also accumulate gas in their intestinal tract, which may result from a defect in secretion of digestive enzymes. These results indicate that the *dNSF1* gene provides a nonessential function during the larval stage of development.

Tissue requirements of dNSF1 and dNSF2: To identify tissues requiring dNSF1 and dNSF2 function, the *GAL4/UAS* system (BRAND and PERRIMON 1993) was used to drive ectopic expression of these proteins in specific locations in a *dNSF1* or *dNSF2* mutant background. Previous work has shown that the *dNSF1* gene product plays an important role in the regulated release of chemical neurotransmitters in the adult Drosophila nervous system (SIDDIQI and BENZER 1976; KAWASAKI *et al.* 1998; LITTLETON *et al.* 1998; TOLAR and PALLANCK 1998; KAWASAKI and ORDWAY 1999). Thus, to investigate whether dNSF1 function is required solely in neural tissue, the neuron-specific *elav-GAL4* element was used to test whether expression of dNSF1 protein in the nervous system could rescue the phenotypes associated with mutations in the *dNSF1* gene (Figure 2A). Two independently generated *UAS-dNSF1* transgenic lines (designated 21A and 73C) containing a *dNSF1* cDNA downstream of multiple *UAS* elements were tested in combination with the *dNSF1* lethal alleles, $dNSF1^{clp}$ and $dNSF1^{3\text{-}13\text{-}3}$, and the temperature-sensitive paralytic $dNSF1^{comt\ st17}$ and $dNSF1^{comt\ tp7}$ alleles. All crosses involving the *dNSF1* lethal alleles produced behaviorally and morphologically normal non-*FM7* (rescued) male progeny, indicating that expression of dNSF1 protein in the ner-

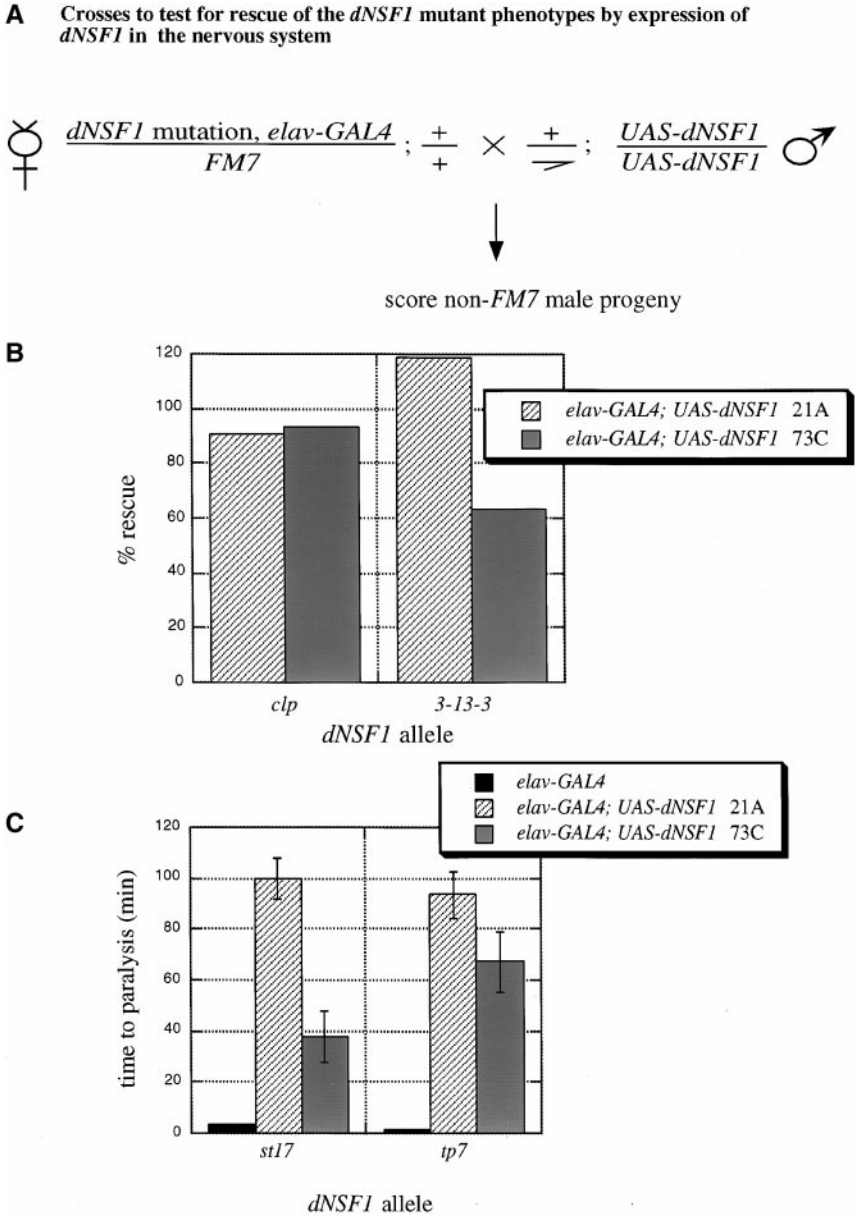

FIGURE 2.—Ectopic expression of *dNSF1* in the nervous system rescues the phenotypes of *dNSF1* mutations. (A) The crosses used for the rescue experiments. Females bearing an X chromosome with a *dNSF1* mutation (*dNSF1*^{clp}, *dNSF1*$^{3\text{-}13\text{-}3}$, *dNSF1*$^{comt\ st17}$, or *dNSF1*$^{comt\ tp7}$) and the *elav-GAL4* element *in trans* to an *FM7* balancer chromosome were crossed to transgenic males homozygous for a *UAS-dNSF1* transgene on the second chromosome. Male progeny from this cross that lack the *FM7* balancer chromosome are hemizygous for the *dNSF1* mutation. (B) Rescue of *dNSF1* lethal alleles. *dNSF1*^{clp} and *dNSF1*$^{3\text{-}13\text{-}3}$ were tested in combination with two *UAS-dNSF1* transgenic lines, designated 21A (hatched bars) and 73C (shaded bars). Percentage rescue is defined as the percentage of non-*FM7* (rescued) male progeny with respect to the total number of female progeny, divided by 0.5, the expected ratio if flies were fully rescued to Mendelian proportions. A minimum of 250 progeny were scored from each cross. (C) Rescue of *dNSF1* temperature-sensitive alleles. Time to paralysis represents the time required for one-half of the hemizygous *dNSF1* mutants to become paralyzed at 38°. Solid bars indicate the time to paralysis for flies carrying a *dNSF1* mutation and *elav-GAL4* element, but no *UAS-dNSF1* transgene. No rescue of the *dNSF1* recessive lethal or temperature-sensitive paralytic mutations was observed in flies segregating a *UAS-dNSF1* transgene in the absence of the *elav-GAL4* element.

vous system is sufficient to rescue the lethality of the *dNSF1*^{clp} and *dNSF1*$^{3\text{-}13\text{-}3}$ mutations (Figure 2B). Furthermore, neural expression of wild-type dNSF1 protein greatly alleviated the temperature-sensitive paralytic phenotype induced by the *dNSF1*$^{comt\ st17}$ or *dNSF1*$^{comt\ tp7}$ mutations (Figure 2C). In several cases, the rescued flies are indistinguishable from wild-type flies; wild-type Canton-S flies cease locomotion after roughly 80 min at 38° (data not shown). These results indicate that the *dNSF1* gene plays an essential role in the nervous system and that nervous system dysfunction underlies the temperature-sensitive paralytic phenotype of the *dNSF1*^{comt} mutants.

To examine whether similar neural expression of dNSF2 protein could rescue the lethality associated with *dNSF2* mutations, *UAS-dNSF2* transgenic lines were generated and tested for their ability to rescue *dNSF2* mutants using the *elav-GAL4* driver (Figure 3A). Three of the most severe *dNSF2* alleles (*dNSF2*^{15}, *dNSF2*^{55}, and *dNSF2*^{103}) and two independent *UAS-dNSF2* lines (designated 2A and 23A) were tested in this analysis. Crosses involving *dNSF2*^{15} and *dNSF2*^{103} alleles produced no rescued adult offspring, and those involving the *dNSF2*^{55} mutation produced only three apparently rescued adult offspring among the >750 adults scored from this cross (Figure 3C, solid bar). Further analysis of *dNSF2* mutants expressing *dNSF2* in the nervous system indicates that this manipulation results primarily in first and second instar larval lethality, with a small percentage of offspring surviving to later stages of development. These results suggest that dNSF2 plays some role in the nervous system. However, neural expression of *dNSF2* alone is insufficient for efficient rescue of the *dNSF2* mutant phenotype.

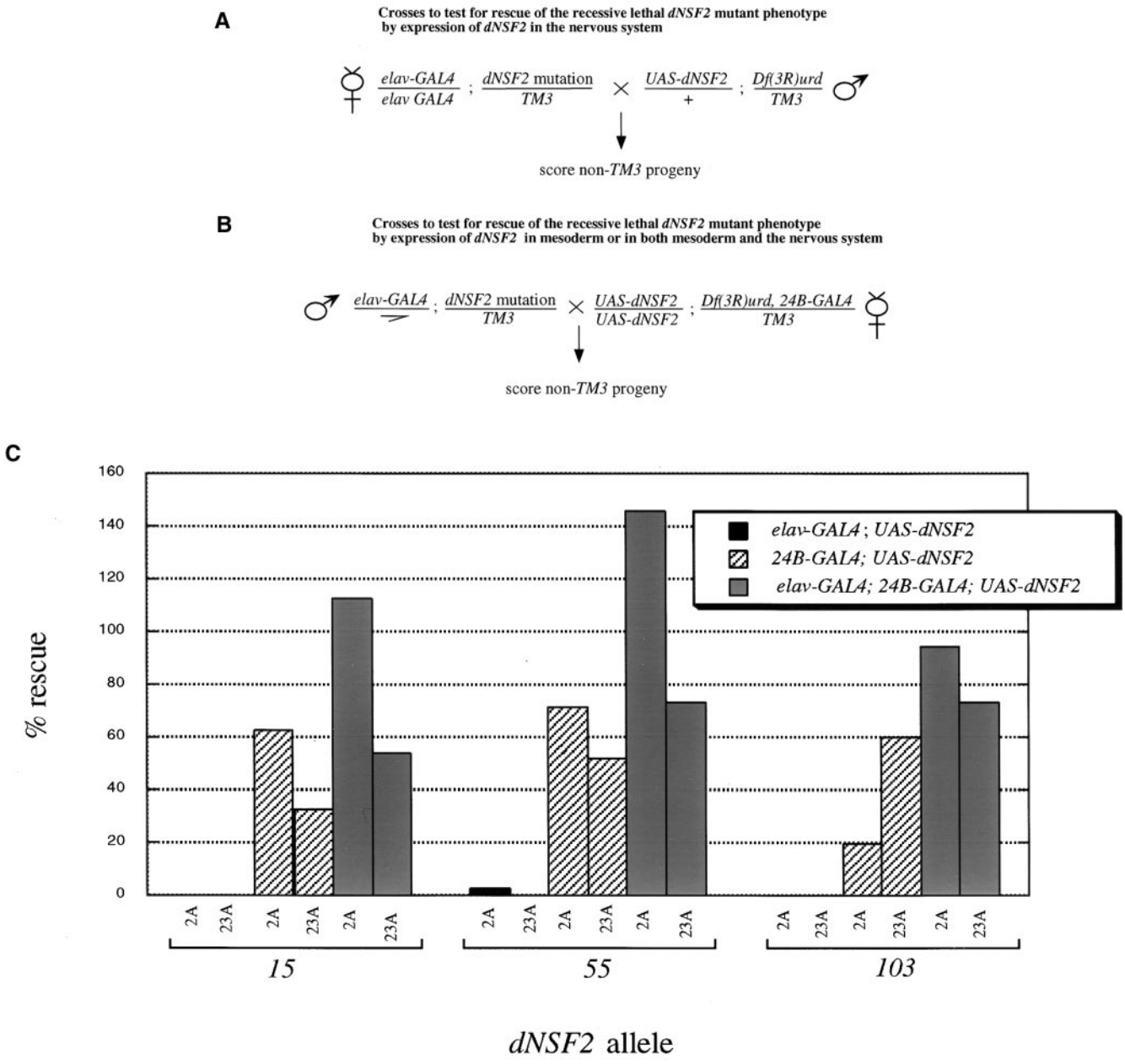

Figure 3.—Ectopic expression of *dNSF2* in mesoderm is sufficient to rescue the lethality of *dNSF2* mutations. (A) Rescue crosses involving the nerve-specific *elav-GAL4* element. Females homozygous for an *elav-GAL4* element on the X chromosome and a *dNSF2* mutation (*dNSF2*^{15}, *dNSF2*^{55}, or *dNSF2*^{103}) *in trans* to the *TM3* balancer chromosome were crossed to males bearing a *UAS-dNSF2* transgene on the second chromosome and a *Df(3R)urd* chromosome *in trans* to *TM3*. Progeny lacking the *TM3* balancer chromosome represent rescue of the *dNSF2* mutation. (B) Rescue crosses involving the mesoderm-specific *24B-GAL4* element in combination with *elav-GAL4*. Males hemizygous for an *elav-GAL4* element and bearing a *dNSF2* mutation *in trans* to *TM3* were crossed to females homozygous for a *UAS-dNSF2* element on the second chromosome and carrying a recombinant third chromosome consisting of the *Df(3R)urd* deletion and the *24B-GAL4* element *in trans* to the *TM3* balancer chromosome. Male progeny from this cross that lack the *TM3* balancer chromosome contain only the *24B-GAL4* element and thus represent *dNSF2* mutants rescued by expression of *dNSF2* in mesoderm. Female progeny from this cross that lack the *TM3* balancer chromosome contain both the *24B-GAL4* and the *elav-GAL4* elements and thus represent *dNSF2* mutants rescued by expression of *dNSF2* in both the nervous system and mesoderm. (C) Rescue of the *dNSF2* lethal alleles. Two *UAS-dNSF2* transgenic lines (2A and 23A) were tested in combination with three *dNSF2* alleles. Percentage rescue of the dNSF2 mutations mediated by nervous system expression of dNSF2 protein (solid bar), by expression of dNSF2 protein in mesoderm only (hatched bars), or by expression of dNSF2 protein in both the nervous system and mesoderm (shaded bars) is shown. Percentage rescue was calculated by dividing the percentage of non-*TM3* progeny by 0.17, the expected ratio if flies were fully rescued to expected Mendelian proportions. A minimum of 350 flies were counted for each cross. No rescue of the *dNSF2* mutants was observed in flies segregating a *UAS-dNSF2* transgene in the absence of *GAL4* elements.

Recent studies demonstrating a direct involvement of the vertebrate NSF in glutamate receptor function (Nishimune *et al.* 1998; Osten *et al.* 1998; Song *et al.* 1998; Luscher *et al.* 1999; Luthi *et al.* 1999; Noel *et al.* 1999), coupled with the fact that neuromuscular transmission in Drosophila is mediated by glutamatergic transmission (Jan and Jan 1976; Petersen *et al.* 1997; DiAntonio *et al.* 1999), led us to examine whether dNSF2 function is required in muscle. To test this hypothesis, the mesoderm-specific *24B-GAL4* line, which drives expression of GAL4 protein specifically in the tissues that give rise to somatic and visceral muscles, was used in conjunction with the *UAS-dNSF2* transgenic lines in an attempt to rescue the *dNSF2* mutant phenotype (Figure 3B). In addition, the possibility that dNSF2 function is required in both the nervous system and the musculature was tested by setting up crosses in which males segregating an *elav-GAL4* element as well as one of three *dNSF2* alleles were mated to females segregating the *Df(3R)urd* chromosome, a *24B-GAL4* element, and a *UAS-dNSF2* transgene (Figure 3B). These crosses produced behaviorally and morphologically normal rescued progeny that fell into two classes: (1) rescued males, which express dNSF2 protein only in mesoderm (Figure 3C, hatched bars); and (2) rescued females, which express dNSF2 protein simultaneously in the nervous system and mesoderm (Figure 3C, shaded bars). These results indicate that mesoderm expression of *dNSF2* is sufficient to produce viable adults. However, the extent of rescue was higher in flies expressing dNSF2 protein in both the nervous system and mesoderm than that in flies expressing dNSF2 protein only in mesoderm, indicating that *dNSF2* gene function is required primarily in mesoderm, but that dNSF2 also plays a secondary role in the nervous system.

To confirm these findings, several additional mesoderm-specific *GAL4* lines were tested for their ability to mediate rescue of the recessive lethal *dNSF2* mutant phenotype. These *GAL4* lines were also used to test whether mesoderm expression of *dNSF1* is capable of rescuing the *dNSF1* mutant phenotypes. For this analysis, only the $dNSF1^{clp}$ and $dNSF2^{15}$ alleles were used in conjunction with the *UAS-dNSF1* 21A and *UAS-dNSF2* 2A transgenes, respectively. All of the mesoderm-specific *GAL4* lines tested mediated rescue of the *dNSF2* mutation to at least 40% of expected Mendelian levels, while none of those tested produced any rescue of the *dNSF1* mutation (Table 2). These results verify that the primary function of dNSF2 resides in mesoderm and indicate that the *dNSF1* mutant phenotype can be rescued only by nervous system expression of dNSF1 protein.

Intergenic complementation analysis: To investigate the degree of functional overlap between the two Drosophila NSF proteins, the ability of *dNSF1* and *dNSF2* to substitute for one another when ectopically expressed was examined using the *GAL4/UAS* system. Because *dNSF1* gene function is required in the nervous system, we sought to test whether a *UAS-dNSF2* transgene, expressed in neural tissue under the direction of *elav-GAL4*, could rescue the phenotypes associated with mutations in the *dNSF1* gene. For these crosses, females bearing an *elav-GAL4* element and a *dNSF1* mutation were crossed to males homozygous for a *UAS-dNSF2* transgene (Figure 4A). Results of these crosses indicate that *dNSF2* expression in the nervous system is sufficient to rescue the lethality of the $dNSF1^{clp}$ and $dNSF1^{3\text{-}13\text{-}3}$ mutations (Figure 4B). Similarly, expression of *dNSF2* in the nervous system rescued the temperature-sensitive paralytic phenotype of the $dNSF1^{comt\ st17}$ and $dNSF1^{comt\ tp7}$ mutants. The extent of rescue of the *dNSF1* mutations by dNSF2 is comparable to that observed when these mutations are rescued by neural expression of dNSF1 protein.

Previous work conducted with the $dNSF1^{comt}$ alleles has demonstrated that a neural SNARE complex composed of a v-SNARE, n-synaptobrevin, and two t-SNAREs, SNAP-25 and syntaxin, accumulates at the $dNSF1^{comt}$ restrictive temperature (Tolar and Pallanck 1998). This biochemical phenotype is largely rescued by expression of wild-type dNSF1 protein from a transgene (Tolar and Pallanck 1998). To examine whether dNSF2-mediated rescue of the $dNSF1^{comt}$ temperature-sensitive paralytic phenotype is paralleled by rescue of the biochemical defect in these mutants, we compared SNARE complex levels in wild-type flies, $dNSF1^{comt}$ mutants, and $dNSF1^{comt}$ mutants expressing either *dNSF1* or *dNSF2* in the nervous system at restrictive temperature. Crosses for this analysis were performed as shown in Figures 2A and 4A. Western blot analysis of a protein lysate from wild-type fly heads using antiserum to the v-SNARE n-synaptobrevin revealed a 23-kD band representing

TABLE 2

Summary of *GAL4/UAS* rescue results

GAL4 line (expression pattern)	*dNSF1* mutations	*dNSF2* mutations
elav (nervous system)	Rescue	No rescue
24B (mesoderm)	No rescue	Rescue
Dmef2 (mesoderm)	No rescue	Rescue
MHC (muscle)	No rescue	No data
BG57 (mesoderm)	No rescue	Rescue

Transgenic lines driving expression of GAL4 protein in nerve, mesoderm, or muscle were tested for their ability to mediate rescue of the $dNSF1^{clp}$ or $dNSF2^{15}$ mutant phenotypes in combination with the *UAS-dNSF1* 21A or *UAS-dNSF2* 2A transgenes, respectively. In cases where rescue was observed, at least 40% of the expected Mendelian proportion of rescued flies was produced from the crosses. A minimum of 250 flies were scored from each cross. The *MHC-GAL4* line was not used in experiments with *dNSF2* mutants because the onset of expression of this line may not occur until after the lethal stage of the $dNSF2^{15}$ mutation (Meg Winberg, personal communication).

monomeric n-synaptobrevin and a 73-kD band representing the SNARE complex (Figure 5). As demonstrated previously, SNARE complex levels were dramatically elevated with respect to monomeric n-synaptobrevin in *dNSF1*^{comt} mutants at the restrictive temperature as compared to wild-type flies (Littleton *et al.* 1998; Tolar and Pallanck 1998). Ectopic neural expression of either *dNSF1* or *dNSF2* in the *dNSF1*^{comt} mutants dramatically alleviates the SNARE complex accumulation phenotype. All of the *UAS-dNSF1* and *UAS-dNSF2* lines reduce the ratio of SNARE complex to n-synaptobrevin monomer to near, or even below, wild-type levels. Those transgenic lines that reduce the SNARE complex accumulation phenotype most also tended to confer more efficient rescue of the paralytic phenotype of the *dNSF1*^{comt} mutants, indicating that these two phenotypes are directly related. These results show that when expressed in the nervous system, dNSF2 can catalyze disassembly of neuronal SNARE complexes and rescue the *dNSF1*^{comt} temperature-sensitive paralytic phenotype, demonstrating that dNSF2 protein can effectively substitute for dNSF1 protein.

To investigate whether ectopic expression of *dNSF1* in mesoderm can rescue the *dNSF2* mutant phenotype, we tested whether a *UAS-dNSF1* transgene expressed using *24B-GAL4* could rescue the lethality associated with the *dNSF2*^{15}, *dNSF2*^{55}, and *dNSF2*^{103} mutations. None of these crosses produced rescued progeny from among the >350 flies counted per cross, indicating that *dNSF1* expression in mesoderm is insufficient to rescue the *dNSF2* mutations. As mesoderm expression of *dNSF2* was only partially capable of rescuing lethality of the *dNSF2* mutations, we investigated whether more widespread and possibly higher levels of *dNSF1* expression could rescue the *dNSF2* mutant phenotype. To perform these experiments, we made use of a heat-shock-inducible *dNSF1* transgene. The heat-shock *dNSF1* transgene was introduced into a *dNSF2* mutant background by mating flies bearing this transgene and the *Df(3R)urd* chromosome to flies bearing one of the three *dNSF2* alleles and subjecting the progeny to daily heat shocks throughout development (Figure 6A). In contrast to the lack of rescue of *dNSF2* mutations observed when *dNSF1* expression is restricted to mesoderm, significant numbers of rescued progeny were observed using an extensive heat-shock regimen to drive ubiquitous expression of dNSF1 protein, demonstrating that dNSF1 protein is capable of rescuing mutations in *dNSF2* (Figure 6B). Together these results show that the dNSF1 and dNSF2 proteins are capable of substituting for one another under defined conditions of expression.

DISCUSSION

In an effort to explore the functions of the two NSF genes in Drosophila, we generated mutations in the *dNSF2* gene and used novel recessive lethal alleles of *dNSF1* to examine the temporal and spatial requirements and degree of functional overlap between these two genes. Our results show that both Drosophila NSF genes are required for viability. Lethal phase and germline clone analyses of these mutants show that dNSF1 function is required for viability at the adult stage of development, while the requirement for dNSF2 begins at the first instar larval stage. Ectopic expression studies demonstrate that the essential function of dNSF1 resides in the nervous system, while the essential function of dNSF2 resides in mesoderm. Despite their differing temporal and spatial requirements, ectopic expression studies show that although they do not normally do so, the dNSF1 and dNSF2 proteins are capable of substituting for one another, indicating that they have overlapping functional properties.

The finding that expression of *dNSF1* in the nervous system rescues the recessive lethal and temperature-sensitive paralytic *dNSF1* mutant phenotypes is consistent with the well-characterized functional role of dNSF1 in neurotransmission (Siddiqi and Benzer 1976; Kawasaki *et al.* 1998; Littleton *et al.* 1998; Tolar and Pallanck 1998; Kawasaki and Ordway 1999). Further experiments demonstrating that dNSF1 function is required only in adults are also consistent with the finding that this gene is expressed at highest levels at this stage of development (Pallanck *et al.* 1995b). Although these observations indicate that dNSF1 is dispensable in the embryonic and larval nervous systems, the *dNSF1* mutants exhibit a variety of larval behavioral phenotypes, including locomotion defects and temperature-sensitive paralysis (Siddiqi and Benzer 1976), suggesting that dNSF1 plays nonessential functional roles in the larval nervous system. *dNSF1* mutant larvae also display features of intestinal system dysfunction, suggesting that this gene may also provide nonneuronal functions.

In contrast to results obtained in studies of the *dNSF1* mutations, ectopic expression of dNSF2 protein in the nervous system was insufficient for significant rescue of the recessive lethal *dNSF2* mutant phenotype. Instead, rescue of the *dNSF2* mutants was produced by ectopic expression of dNSF2 protein in mesoderm. These results are supported by previous work establishing that dNSF2 is expressed in this tissue (Boulianne and Trimble 1995). Mesoderm is the precursor to several larval and adult tissues in Drosophila, principally visceral and somatic muscle (Bate 1993), suggesting that dNSF2 may play an essential functional role in muscle. The fact that *dNSF2* mutant larvae initially display normal locomotion suggests that muscle differentiation and early events in neuromuscular junction formation are not adversely affected by the *dNSF2* mutations. A model more consistent with the *dNSF2* lethal phase is that the *dNSF2* mutants are defective in the synaptic growth and maturation that occurs during larval development, a process that requires proper trafficking of muscle-specific membrane proteins and secretion of a retrograde signal that acts to regulate presynaptic activity (Pet-

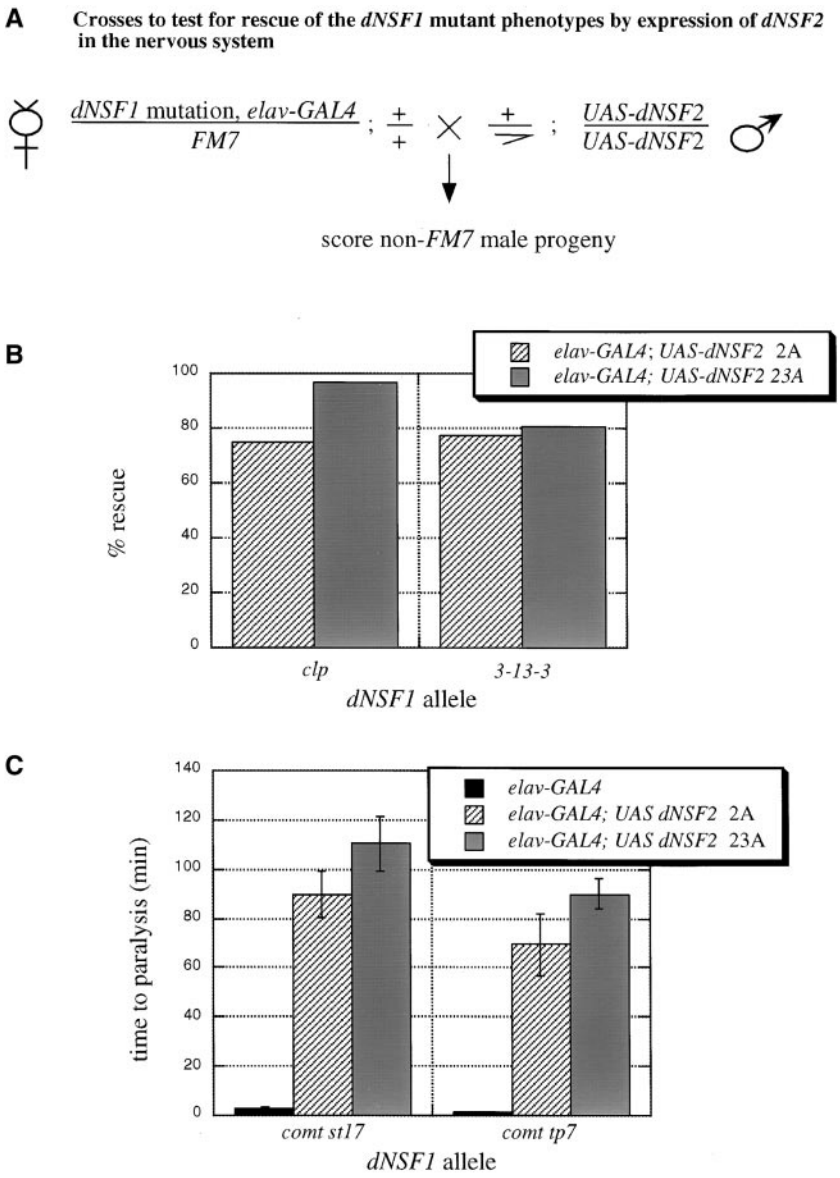

FIGURE 4.—Neural expression of *dNSF2* rescues the *dNSF1* mutant phenotypes. (A) The crosses used for the rescue experiments. Females carrying a *dNSF1* mutation and an *elav-GAL4* element were crossed to transgenic males homozygous for a *UAS-dNSF2* transgene on the third chromosome. Male progeny lacking the *FM7* balancer are hemizygous for the *dNSF1* mutation. (B) Rescue of *dNSF1* lethal alleles. $dNSF1^{clp}$ and $dNSF1^{3\text{-}13\text{-}3}$ were tested in combination with the *UAS-dNSF2* transgenic lines, 2A (hatched bars) and 23A (shaded bars). Percentage rescue was defined as the percentage of non-*FM7* (rescued) males to the total number of females, divided by 0.5, the expected ratio if flies were fully rescued to Mendelian proportions. A minimum of 350 flies were counted for each cross. (C) Rescue of *dNSF1* temperature-sensitive alleles. $dNSF1^{comt\,st17}$ and $dNSF1^{comt\,tp7}$ were tested in combination with the two *UAS-dNSF2* transgenic lines, 2A (hatched bars) and 23A (shaded bars). Time to paralysis represents the time required for one-half of the hemizygous *dNSF1* mutants to become paralyzed at 38°. Solid bars designate paralytic profiles of the $dNSF1^{comt}$ mutants in the absence of ectopic *dNSF2* expression. No rescue of the *dNSF1* recessive lethal or temperature-sensitive paralytic alleles was observed in flies segregating the *UAS-dNSF2* transgene in the absence of the *elav-GAL4* element.

ERSEN *et al.* 1997; DAVIS *et al.* 1998; DIANTONIO *et al.* 1999; GRAMATES and BUDNIK 1999).

Alternatively, another possible role for dNSF2 in muscle is suggested by recent work demonstrating that postsynaptic glutamate receptor function or subcellular localization is regulated by NSF in the vertebrate nervous system (NISHIMUNE *et al.* 1998; OSTEN *et al.* 1998; SONG *et al.* 1998; LUSCHER *et al.* 1999; LUTHI *et al.* 1999; NOEL *et al.* 1999). Glutamatergic neurotransmitter receptors reside in the Drosophila larval body wall muscles in opposition to neurotransmitter release sites and are essential for fast synaptic transmission at the larval neuromuscular junction (JAN and JAN 1976; SCHUSTER *et al.* 1991; PETERSEN *et al.* 1997; DIANTONIO *et al.* 1999). Thus, similar interactions between dNSF2 and Drosophila glutamate receptors in muscle could explain the mesoderm-specific role of dNSF2. Although the vertebrate glutamate receptor sequences that mediate interactions with NSF are poorly conserved in the Drosophila glutamate receptors (SCHUSTER *et al.* 1991; PETERSEN *et al.* 1997; NISHIMUNE *et al.* 1998; OSTEN *et al.* 1998; SONG *et al.* 1998), the dNSF2 protein may have evolved compensatory changes to allow maintenance of this interaction. Another possibility is that dNSF2 interacts with other muscle proteins involved in synapse development or function that contain sequences similar to the NSF-binding sequence present in mammalian glutamate receptors. Further experiments are currently in progress to define the precise functional role of dNSF2 in muscle.

In addition to the role of dNSF2 in mesoderm, the increased efficiency of rescue of *dNSF2* mutations mediated by simultaneous expression of dNSF2 in the nervous system and mesoderm indicates that dNSF2 also functions in the nervous system. This is also supported by the finding that expression of dNSF2 in the nervous system alone can extend the lethal phase of the *dNSF2* mutants. It is possible that dNSF2 participates in constitutive vesicle trafficking in the nervous system, and/

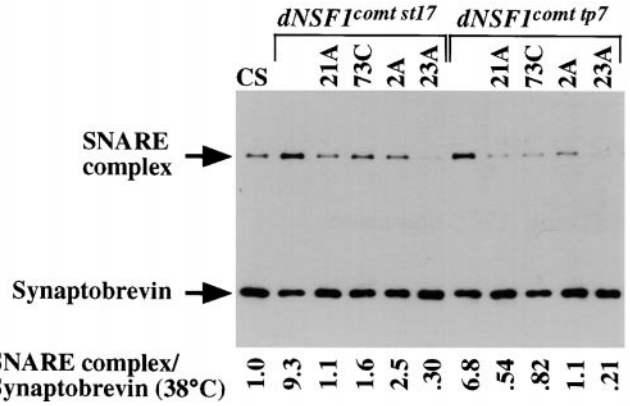

FIGURE 5.—Neural expression of *dNSF1* or *dNSF2* rescues SNARE complex accumulation in *dNSF1*^{comt} mutants. Protein extracts for Western blot analysis were prepared from wild-type (CS), *dNSF1*$^{comt\ st17}$, and *dNSF1*$^{comt\ tp7}$ flies carrying *UAS-dNSF1* (21A or 73C) or *UAS-dNSF2* (2A or 23A) transgenes driven by the *elav-GAL4* promoter, following a 20-min exposure to restrictive temperature (38°). Western blot analysis was performed using a polyclonal antibody to n-synaptobrevin (VAN DE GOOR *et al.* 1995). SNARE complex/synaptobrevin monomer ratios, normalized to wild type, are shown below each lane.

or that dNSF2 collaborates with dNSF1 in presynaptic neurotransmitter release mechanisms, as suggested by experiments showing that dNSF2 can rescue the *dNSF1* mutations and can participate in neural SNARE complex disassembly. Alternatively, dNSF2 may be involved in regulating postsynaptic membrane insertion or localization of glutamate receptors in the central nervous system. Future electrophysiological studies and glutamate receptor localization experiments involving the *dNSF2* mutants should resolve these possibilities.

A somewhat surprising result emerging from our studies is that neither dNSF1 nor dNSF2 is singly required for embryonic development and early larval locomotion. This observation could be accounted for in three ways: dNSF1 and dNSF2 may be redundant at these stages of development, a distantly related Drosophila NSF homolog may promote secretion in embryos, or there may be no requirement for NSF-like activity in developing embryos. The last possibility appears highly unlikely, since several other secretory components that interact either directly or indirectly with NSF have been shown to be required for embryonic development (HARRISON *et al.* 1994; SCHULZE *et al.* 1995; BURGESS *et al.* 1997). Although the second possibility is difficult to rule out, the finding that both dNSF1 and dNSF2 are expressed in embryos favors the idea that these two Drosophila NSF genes perform redundant functions at this stage of development (ORDWAY *et al.* 1994; BOULIANNE and TRIMBLE 1995; PALLANCK *et al.* 1995b). Lack of an essential role for either gene in the larval nervous system argues for similar redundancy of dNSF1 and dNSF2 function in this tissue.

Although previous work has shown that ectopic expression of dNSF1 protein from a heat-shock-inducible transgene can rescue the neural SNARE complex accumulation phenotype of the *dNSF1*^{comt} mutants to near wild-type levels (TOLAR and PALLANCK 1998), several of the transgenic lines used in the current study were found to reduce SNARE complex levels to below those found in wild-type flies. In particular, the *UAS-dNSF2* 23A transgenic line reduced the SNARE complex to synaptobrevin monomer ratio of the *dNSF1*$^{comt\ tp7}$ mutants by fivefold with respect to the wild-type ratio. This finding bears on the functional role of NSF. According to a recent model, the SDS-resistant neural SNARE complex is hypothesized to define a docked, readily releasable synaptic vesicle (LONART and SUDHOF 2000). If this model is correct, it predicts a sharp reduction in the readily releasable synaptic vesicle pool in flies expressing dNSF2 protein from the *UAS-dNSF2* 23A transgene, and this severe reduction in the readily releasable synaptic vesicle pool might well be expected to produce correspondingly severe behavioral manifestations. However, flies of this genotype are viable and exhibit no unusual behavioral phenotypes, suggesting that the readily releasable synaptic vesicle pool is not severely depleted. These results more readily fit a role for NSF in the disassembly of a SNARE complex formed transiently during synaptic vesicle fusion or, alternatively, a role for NSF in catalyzing partial or complete disassembly of a *trans* SNARE complex to activate synaptic vesicles for calcium-triggered fusion.

It is notable that despite their differing spatial requirements, the two Drosophila NSF proteins are capable of substituting for one another when expressed ectopically. Specifically, dNSF2 can substitute for dNSF1 when expressed in the nervous system, and dNSF1 can substitute for dNSF2 when expressed ubiquitously from a heat-shock promoter. Although it remains unclear why expression of dNSF1 in mesoderm alone fails to rescue the *dNSF2* mutants, a likely possibility is that dNSF1 does not efficiently substitute for dNSF2. Our experiments involving *dNSF2* transgenes indicate that optimal rescue of *dNSF2* mutants requires expression in both mesoderm and neural tissue. The fact that dNSF1 can rescue *dNSF2* mutants only when expressed from a heat-shock promoter may reflect the broader expression conferred by this system or possibly higher levels of expression resulting from the extensive heat-shock regimen employed (see MATERIALS AND METHODS). Additional experiments using heat-shock promoter-driven constructs, suggest that dNSF2 does not substitute efficiently for dNSF1 (R. ORDWAY, personal communication; our data not shown). These cross-rescue barriers, preventing efficient substitution of one NSF for the other, may involve subtle differences in the functional properties of the proteins, differential stabilities of the two proteins in the various tissues examined, sequence elements in the *dNSF1* and *dNSF2* mRNAs responsible for proper mRNA metabolism, or any combination of these three possibilities. Recent studies suggest that untranslated sequence elements in the *dNSF1* and *dNSF2* mRNAs are at least

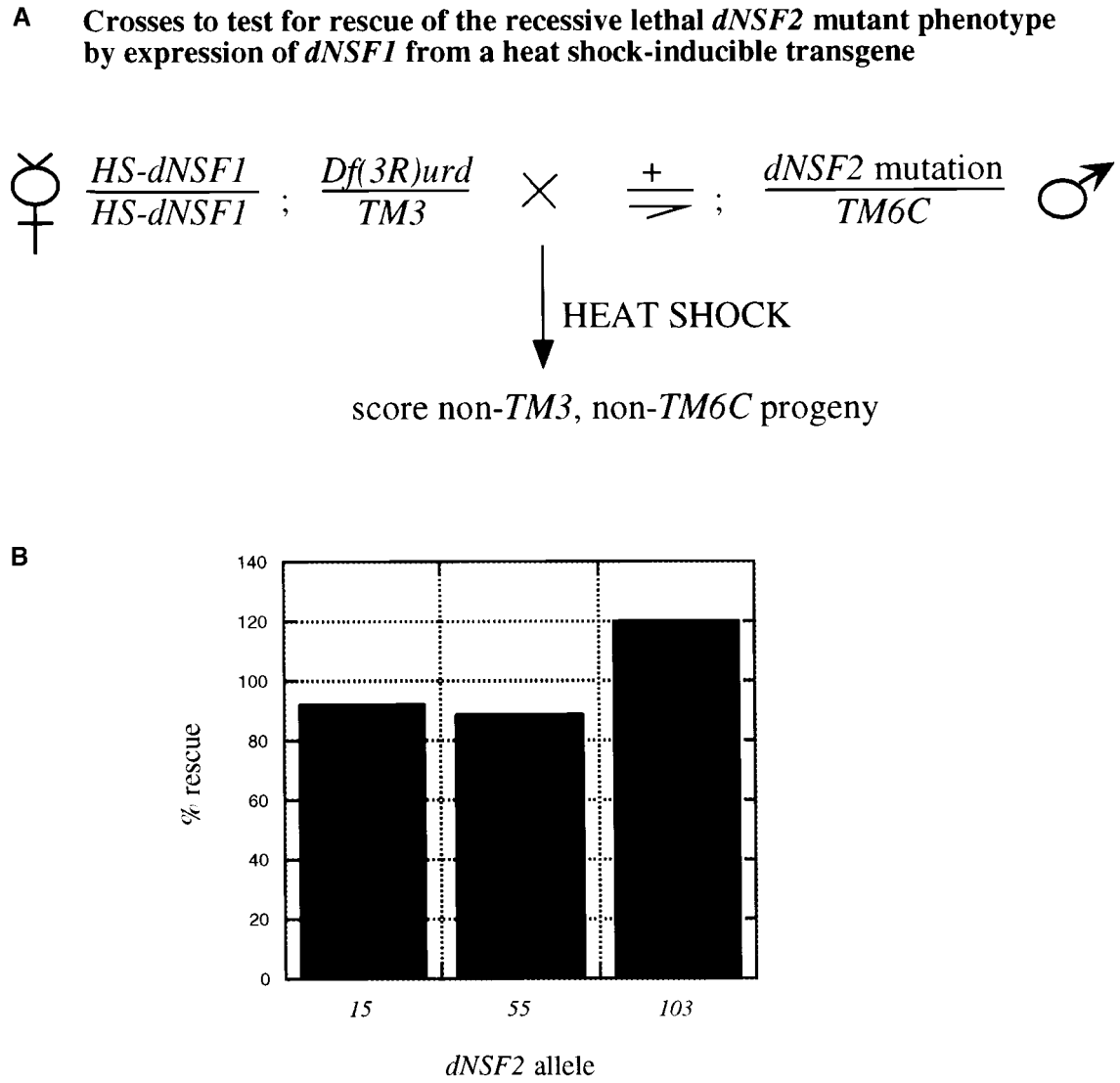

FIGURE 6.—Ectopic expression of *dNSF1* mediated by a heat-shock-inducible promoter rescues the recessive lethal *dNSF2* mutant phenotype. (A) The crosses used for the rescue experiments. Females homozygous for a heat-shock-*dNSF1* transgene (*HS-dNSF1*) on the X chromosome and carrying the *Df(3R)urd* chromosome *in trans* to the *TM3* balancer chromosome were crossed to males carrying a *dNSF2* mutation *in trans* to the *TM6C* balancer chromosome. Offspring were heat shocked at 38° for 60 min daily throughout development. Progeny lacking both the *TM3* and *TM6C* chromosomes represent rescue of the *dNSF2* mutations by *dNSF1* expression. (B) Rescue of the *dNSF2* mutations. Percentage rescue was defined as the percentage of non-*TM3*, non-*TM6C* progeny divided by 0.33, the expected ratio if flies were fully rescued to expected Mendelian proportions. No rescued progeny were observed in the absence of heat shock. A minimum of 200 flies were counted for each cross.

one important determinant preventing efficient cross-rescue (R. ORDWAY, personal communication).

The reason that Drosophila requires two closely related NSF genes remains unclear. Searches of the *Caenorhabditis elegans* and human genome databases reveal the presence of only one NSF gene in each of these two organisms, indicating that a single NSF gene is sufficient to support constitutive and regulated secretion, and possibly postsynaptic glutamate receptor regulation in these eukaryotes. However, this finding does not rule out the possible presence of multiple NSF proteins resulting from alternative splicing of the single NSF gene in these organisms. Evidence in support of this possibility is provided by Northern blot analysis of the rat NSF gene (PUSCHEL *et al.* 1994). Thus, the presence of multiple NSF genes in Drosophila may simply reflect the mechanism by which evolution has generated multiple NSF proteins with subtle functional differences in this organism. Partitioning of NSF function between two different NSF genes in Drosophila may make this organism particularly useful for teasing apart the distinct cellular roles of NSF.

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