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Identification of a second homolog of *N*-ethylmaleimide-sensitive fusion protein that is expressed in the nervous system and secretory tissues of *Drosophila*

(membrane transport/SEC18 homolog/docking/soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor hypothesis)

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ABSTRACT ***N*-Ethylmaleimide-sensitive fusion protein (NSF) is an ATPase known to have an essential role in intracellular membrane transport events. Recently, cDNA clones encoding a *Drosophila melanogaster* homolog of this protein, named dNSF, were characterized and found to be expressed in the nervous system. We now report the identification of a second homolog of NSF, called dNSF-2 within this species and report evidence that this ubiquitous and widely utilized fusion protein belongs to a multigene family. The predicted amino acid sequence of dNSF-2 is 84.5% identical to dNSF (hereafter named dNSF-1), 59% identical to NSF from Chinese hamster, and 38.5% identical to the yeast homolog SEC18. The highest similarity was found in a region of dNSF-2 containing one of two ATP-binding sites; this region is most similar to members of a superfamily of ATPases. dNSF-2 is localized to a region between bands 87F12 and 88A3 on chromosome 3, and *in situ* hybridization techniques revealed expression in the nervous system during embryogenesis and in several imaginal discs and secretory structures in the larvae. Developmental modulation of dNSF-2 expression suggests that quantitative changes in the secretory apparatus are important in histogenesis.**

The intracellular movement of integral membrane and secretory proteins occurs via the directional flow of transport vesicles that are formed by budding from donor membranes. Transport is completed after fusion of the transport vesicle with the appropriate target membrane (for review, see refs. 1, 2). Specificity of transport is thought to be achieved by the correct recognition of target-membrane molecules by molecules on the transport vesicles (3). Hence some of the involved molecules are predicted to be specific for individual membranes, whereas others might be common to several steps in the process.

Through the development of cell-free transport assays, Rothman and his colleagues have demonstrated that intra-Golgi transport vesicle fusion requires cytosolic factors including ATP, GTP, and several cytosolic proteins (1). Transport was blocked by the action of *N*-ethylmaleimide, a sulfhydryl alkylating agent, and a single protein was shown to be its target (4). Biochemical studies of this *N*-ethylmaleimide-sensitive fusion protein (NSF) revealed that it acted as a homotrimer, with each subunit having a molecular mass of 76 kDa (4, 5). cDNA clones encoding this protein have been isolated from Chinese hamster ovary (CHO) cell cDNA libraries (6), and their sequence analysis revealed the NSF gene product to be the mammalian counterpart of the yeast secretory mutant SEC18 (7).

Genetic studies had previously identified SEC18 as a gene product required for protein transport from the endoplasmic reticulum to Golgi complex (8). In addition, the rapid inactivation of a temperature-sensitive mutant allele of the SEC18 gene has allowed Graham and Emr (9) to show that NSF/SEC18 is required sequentially for transport from endoplasmic reticulum to Golgi complex, within the Golgi complex, and from the Golgi complex to the cell surface.

When incubated with ATP in the absence of Mg^{2+} or in the presence of nonhydrolyzable ATP analogs, NSF forms a stable 20S complex that can be solubilized from membranes with detergent (10, 11). Within this complex are cytosolic factors called soluble NSF attachment proteins (SNAPs) and the membrane SNAP receptors (SNAREs) (12). 20S complexes isolated from detergent-solubilized brain extracts contained vesicle-associated membrane protein (VAMP) (13), presumably acting as the vesicular SNARE, and the presynaptic membrane proteins syntaxin (14) and synaptosome-associated protein 25 kDa (SNAP-25) (15) as target-membrane SNAREs.

The precise role of NSF in regulating exocytosis remains unclear. NSF has been proposed to be intimately associated with the membrane fusion event, possibly promoting fusion by causing the close association of the vesicle and target-membrane SNARE proteins during ATP hydrolysis (16). Alternative hypotheses include a role for the NSF protein in initiating or maintaining the docked state between the membranes before their fusion (17). This latter possibility is supported by studies of temperature-sensitive mutations in SEC18p that show that at nonpermissive temperatures, ER-derived vesicles accumulate that are not firmly attached to the Golgi apparatus (18). To gain further insight into the mechanism of NSF action in exocytosis, additional genetic and molecular model systems are required for its study.

Recently, Ordway *et al.* (19) reported the cloning of cDNAs encoding a *Drosophila melanogaster* homolog of NSF (dNSF) that maps between bands 11D9 and 11E4 on the X chromosome. Using low-stringency Southern blot analysis, they found only a single hybridizing species, suggesting that dNSF was not a member of a family of closely related genes. In this paper we report the identification of a second homolog of NSF in *D. melanogaster* (hereafter named dNSF-2). This protein shares 84.5% sequence identity with the first dNSF (hereafter called dNSF-1) but maps to chromosome 3, between bands 87F12 and 88A3. Using *in situ* hybridization techniques, we have found that the dNSF-2-encoding gene is expressed most abundantly in the embryonic nervous system and in third-instar larval

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Abbreviations: NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; SNAP-25, synaptosome-associated protein 25 kDa; dNSF, *Drosophila melanogaster* NSF; PAS, periodic acid-Schiff; VAMP, vesicle-associated membrane protein.

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discs, salivary glands, and the ring gland. This evidence of multiple, highly related isoforms of NSF in a single species suggests that in some tissues membrane fusion and/or secretion has a specific role during development.§

MATERIALS AND METHODS

cDNA Cloning. A 50-base synthetic oligonucleotide was generated containing the sequence 5′-CAGCTGAACAACATCCTGGTGATCGGCATGACCAACCGCAAGGACCTGAT-3′, corresponding to amino acids 383–399 of sec18p and amino acids 371–387 of NSF. The oligonucleotide was end-labeled with [γ-^{32}P]ATP (Amersham) using polynucleotide kinase. Five hundred thousand plaques of a randomly primed Lambda ZAP II *Drosophila* adult head cDNA library (Stratagene) were screened with the probe in hybridization buffer containing 1 M NaCl, 20% (vol/vol) formamide, 10 mM $NaPO_4$ (pH 7.0), 0.1% SDS, 0.1% bovine serum albumin fraction V, 0.1% polyvinylpolypyrrolidone, and 0.1% Ficoll 400 at 42°C for 16 hr. Filters were washed in three changes of buffer containing 1 M NaCl and 0.1% SDS for 10 min each at 42°C and autoradiographed. Positive plaques were picked and plaque-purified in two plating cycles. cDNA inserts from purified phage clones were subcloned into Bluescript SK vectors for subsequent sequence analysis.

DNA Sequencing. Single-stranded DNA from Bluescript plasmids was rescued by superinfection with M13K107 helper phage (Stratagene). Single-stranded DNA sequencing was done by using the Sequenase version 2.0 kit (United States Biochemical). DNA sequence was compiled by sequencing both strands of three cDNAs, and sequence data were analyzed using PC/GENE software (IntelliGenetics).

Fly Stocks and Chromosomal *in Situ* Hybridization. Embryos and larval tissues were obtained from Oregon-R flies. The chromosomal location of the dNSF-2-encoding gene was mapped by *in situ* hybridization to squashed polytene salivary gland chromosomes using a full-length biotinylated cDNA probe (Detek, Enzo Biochem) essentially as described (20).

RNA Analysis. Total cellular RNA was extracted from adult female *D. melanogaster* by the cesium chloride/guanidine thiocyanate method (21). Poly(A)$^+$ mRNA was twice purified by binding to and elution from a column of oligo(dT)-cellulose, and 5 μg of poly(A)$^+$ mRNA was electrophoresed and blotted as described (13). The probe used encompassed amino acids 1–330 of dNSF-2.

***In Situ* Hybridization to RNA in Whole-Mount Embryos or Third-Instar Larval Imaginal Discs.** Embryos were prepared for hybridization as described by Bier *et al.* (22). *In situ* hybridization to whole-mount embryos was done by following the method of Tautz and Pfeifle (23), using digoxigenin-labeled probes (Boehringer Mannheim, 1093 657) with the exception that hybridization and washes were done at 48°C. The probe used in these experiments was a 1.05-kb cDNA fragment encoding amino acids 1–350; this fragment is only 78% identical to the dNSF-1 cDNA and would not cross-hybridize under the conditions used. Imaginal discs from third-instar larvae were dissected in Grace's insect medium (GIBCO) and fixed in a solution containing 0.1 M phosphate buffer (pH 7.2) and 5% (wt/vol) paraformaldehyde. No hybridization was detected using control (sense) probes or when DNA probes were omitted (data not shown).

***In Situ* Hybridization to RNA and Periodic Acid-Schiff (PAS) Staining of Third-Instar Larval Salivary Glands.** Salivary glands from third-instar larvae were dissected in Grace's insect medium (GIBCO) and fixed in a solution containing 0.1 M phosphate buffer (pH 7.2) and 5% (wt/vol) paraformaldehyde. *In situ* hybridization was done as described for embryos and imaginal discs. PAS staining was done as described (24).

RESULTS AND DISCUSSION

Approximately 500,000 clones of an adult *D. melanogaster* head cDNA library were screened with an end-labeled 50-nt probe under conditions of low stringency. From this screen three

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U28836).

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dNSF-2                             M----RAIKCPTDELSLTNKAIVNVSDFTEEVKYVDISPGPGLHYIFALEKISGPELPLGHVGFSLVQRKWATLSI   72
dNSF-1                             MAYILK.T..........R....G..P..I..A...A..Q.F......TV--.VPS.Y..........MV..   74
CHO NSF                   SEPDVSAK-MAGRSMQ.AR........S.C.V.SEK.YQSG-QH.IVRTS.NHK...T.-RTHPSAV.-.S.A...P.....GL..   82
SEC 18          MFKIPGFGKAAANHTPPDMTNMDTRTRHLKVSN..NNSYA.A.V.A.SPN..PNNI-.II.DN------L.VFTTRHSNDI.P.TI..NGN..T.GGW.L   93

dNSF-2          NQEIDVRPY---RFDASADIITLVSFETDFLQKKTTTQEPYDSDEMAKEFLMQFAGMPLTVGQTLVFQFKDKKFLGLAVKTLEAVDPRTV-GDSLPKT-R  167
dNSF-1          ...LE...---....S.V..C...........VS......Q....I.....A....S...N....L.....S...I..KSL-.EGKDTAM.  170
CHO NSF         G...E.AL.---S..KAKQC.GTMTI.I......NIDSN...T.K..A..IQ..NNQAFS...Q...S.N..-LF..L..DI..M..SILK.EPASGKRQ  178
SEC 18          ..DVQAKAFDLFKYSGKQSYLGSIDIDIS.RARGKAVSTVF.Q..L..Q.VRCYESQIFSPT.Y.IME.QGH-.FD.KIRNVQ.I.LGDIEPT.AVA.GI  192

dNSF-2          NVRFGRILGNTVVQFEKAENSVLNLQGRSKGKIVRQSIINPDWDFGKMGIGGLDKEFNAIFRRAFASRVFPPELVEQLGIKHVKGILLYGPPGTGKTLMA  267
dNSF-1          ....................S.....K...V........................S.................C.................  270
CHO NSF         KIEV.LVV..SQ.A.......S...I.KA.T.EN.........N.E..........SD..............I...M.C.............C....L.  278
SEC 18          ETK-.ILTKQ.QIN.F.GRDGLV..KSSNSLRPRSNAV.R..FK.EDL.V......TK.........I...SVI.K...S....L.........I.  291

dNSF-2          RQIGTMLNAREPKIVNGPQILDKYVGESEANIRRLFAEAEEEEKRLGPNSGLHIIIFDEIDAICKARGSVAGNSGVHDTVVNQLLAKIDGVEQLNNILVI  367
dNSF-1          .............................V...................................Q..................T.....D........  370
CHO NSF         ....K........V....E..N...........K...D....QR...A...HL.............Q...M..ST...........S.............  378
SEC 18          .K.......K.........E..S....S..E...N..KD..A.YRAK.EE.S........L.SVF.Q...RGDGT..G.N........M.-.D........  390

dNSF-2          GMTNRRDMIDEALLRPGRLEVQMEISLPNEQGRVQILNIHTKRMRDFNKIASDVDNNEIAAKTKNFSGAELEGLVRAAQSTAMNRLIK-ADSKVHVDPEA  466
dNSF-1          ...................................E...ND....K....L.....................S.......-..A..T.....  469
CHO NSF         .....P.L.............K...G..D.K..L...H...A...GHQLLSA...IK.L.VA..........................H..-.ST..E..M.K  477
SEC 18          .....K.L..S.......F...V..H..D.K..L..FD.Q..K..EN.MMSD..NLA.L..L........I...KS.S.F.I.KTVNIGKGATKLNTKD  490

dNSF-2          MEKLRVTRADFLHALDNDIKPAFGAAQEMLENLLARGIINWGPPVTELLEDGMLSVQQAKATESSGLVSVLIEGAPNSGKSALAANLAQLSDFPFVKVCS  566
dNSF-1          ....K.N.D....S.EH.......T...I.D.M....V....A..SN.......Y......P.........VA.......T....Q..KM.........  569
CHO NSF         A.S.Q...G...AS.E........TN..DYASYIMN...K..D...RV.D..E.L...T.NSDRTP.....L..P.H...T....KI.EE.N...I.I..  577
SEC 18          IA..K...E...N..N-.VT....ISE.D.KTCVEG.MMLYSER.NSI.KN.ARY.R.VRESDK.R...L..H.PAG...T....EI.LK.G...IRLI.  589

dNSF-2          PEDMVGFTESAKCLHIRKIFDDAYRSTLSCIVVDNVERLLDYGPIGPRYSNLTLQALLVLLKKQPPKGRKLLILCTSSRRDVLEEMEMLSAFTSVLHVSN  666
dNSF-1          ......Y..................M...............S.......M..........................E........T........P.  669
CHO NSF         .DK.I..S.T...QANK........K.Q...V...DI......V.....F...V..........A..Q......IG.T..K...Q.....N..STTI..PM  677
SEC 18          .NELS.MS....IAY.DNT.R...K.P.NIL.I.SL.T.V.WV.....F..NI..M.K.A..RK..QD.R...MT.T.AYS..QQ.DI..C.DNEIA.P.  689

dNSF-2          LSTPEHVLAVLDDSDLFSPEELQSIARKMAGK--RLCIGIKKLLALIDMIRQSEPHQRVIKFLSKMEEEGGLEMDRVQGH                     746
dNSF-1          ..K.D......ENT.I..KG.I.A.GK.....--.VF.......G....A..T.QS..A.............D.--.ARQ                     745
CHO NSF         IA.G.QL.EA.ELLGN.KDK.RTT..QQVK..--KVW.......M..E.SL.MD.EY..KR..ALLR...ASPL.F---D                     752
SEC 18          MTNLDELNN.MIE.NFLDDAGRVKVINELSRSCPNFNV....T.TN.ETA.HD.DP--.NELVEL.TQSA                               757
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FIG. 1. Alignment of predicted amino acid sequence of *Drosophila* NSF-2 with dNSF-1, NSF from Chinese hamster, and yeast SEC18. Single-letter amino acid code was used. Positions of amino acids identical between species are indicated with dots, whereas specific amino acids are indicated.

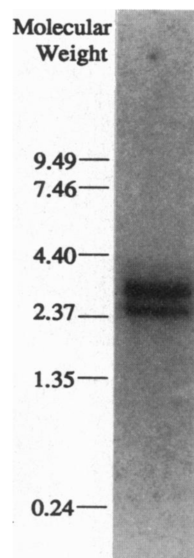

FIG. 2. Blot hybridization of *Drosophila* dNSF-2 mRNA. Five micrograms of twice-purified poly(A)$^+$ mRNA from adult female *Drosophila* was probed with a fragment corresponding to amino acids 1–330. Positions of molecular weight ($\times 10^{-3}$) markers are shown at left.

independent cDNA clones were isolated, and each contained inserts of between 2.2 and 2.4 kb. With a biotinylated probe corresponding to the entire length of the longest cDNA, the location of the dNSF-2 gene was mapped to a locus distinct from that of dNSF-1 (19) on the right arm of the third chromosome between bands 87F12 and 88A3 (data not shown). The cDNAs were subcloned for sequencing, and the nucleotide sequence was determined. The sequence of dNSF-2 differed from that of dNSF-1, confirming that they are encoded by distinct genes.

Fig. 1 compares the predicted amino acid sequences of dNSF-2, dNSF-1 (from *D. melanogaster*), CHO NSF, and yeast SEC18. The four proteins are roughly the same size; predicted lengths are 745, 746, 752, and 757 amino acids, respectively. All dNSF-2 cDNA clones terminated within 3 nt of each other, near an ATG codon predicted to be the amino terminus of the CHO NSF protein. Overall, the *Drosophila* NSF-2 protein is 84.5% identical to dNSF-1, 59% identical to CHO NSF, and 38.5% identical to yeast Sec18p.

The greatest degree of amino acid similarity occurs around and between the two domains implicated in ATP binding. Interestingly, the two ATP-binding p-loop sites, shown underlined in Fig. 1, are not identical, and the second site diverges to the greater degree. These results are consistent with recent results from Whiteheart *et al.* (5), which show that only the first ATP domain is required for ATP binding/hydrolysis. This portion of the NSF protein also shares a high degree of similarity with a variety of other proteins possessing ATP binding and ATPase activities—including the Tat-binding protein (25), the CDC48/valosin-containing protein family (26–28), and the peroxisome assembly protein family (29). As previously shown (29), there is a region that extends for ≈200 amino acids flanking the ATP-binding site, for which there is ≈45% sequence similarity between these diverse ATPases.

The molecules that regulate synaptic vesicle docking/fusion appear to be present in widely diverse eukaryotic species. To date, four molecules associated with this complex have been identified in *Drosophila*. *Drosophila* VAMP/synaptobrevin was found to share 57% sequence identity with mammalian VAMP (30, 31), as did *Drosophila* synaptotagmin/p65 (32). *Drosophila* SNAP-25 was found to share 61% sequence identity with its mammalian counterpart (33), and the *Drosophila* rop protein was found to share 65% sequence identity with its rat SEC1/UNC18 homolog (34). *Drosophila* SNAP was also found to share 62% and 61% sequence identity to bovine α-SNAP and β-SNAP (19). The similarity in the degree of sequence conservation in these proteins and NSF that is

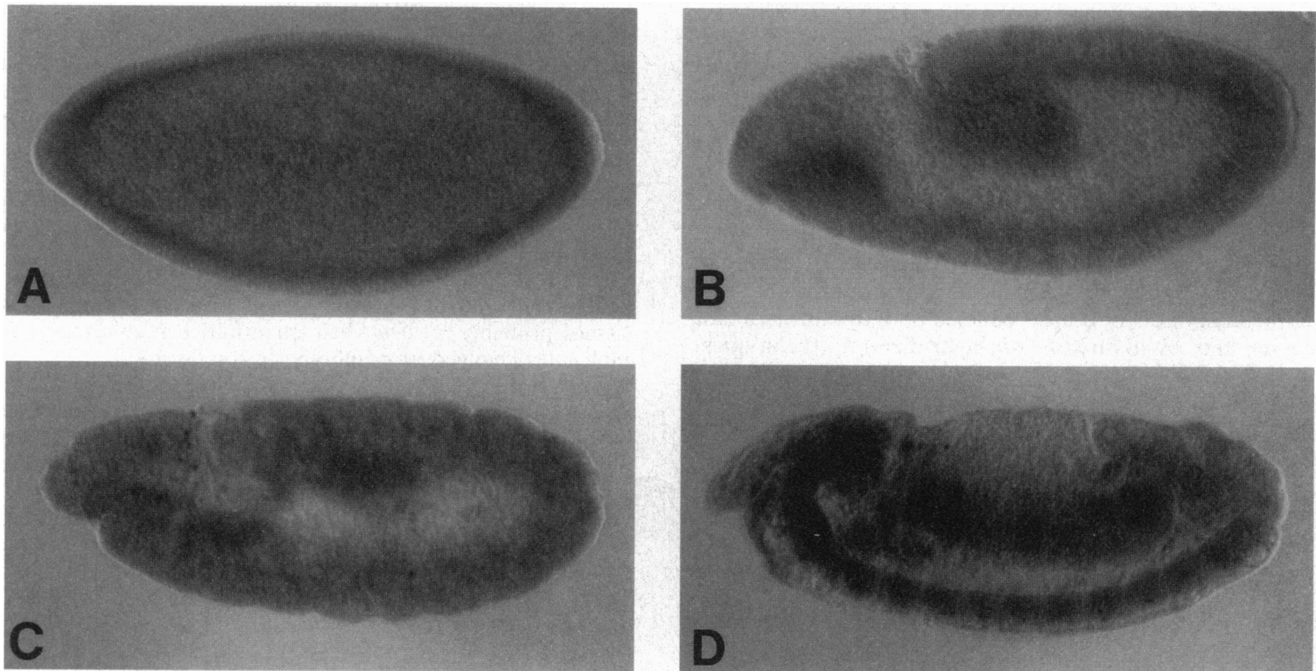

FIG. 3. *In situ* hybridization of dNSF-2 to *Drosophila* embryos. Lateral views of embryos are presented with anterior at left and posterior at right. (*A*) dNSF-2 is evenly distributed throughout cellularized embryos (stages 5 and 6). (*B*) During germ-band elongation (stages 8–10), dNSF-2 is expressed in ectoderm, mesoderm, and anterior midgut primordium. Expression is also evident in posterior midgut primordium. (*C*) In stage-11 embryos that have completely undergone germ-band elongation, dNSF-2 continues to be expressed in ectoderm and mesoderm, as described in *B*. (*D*) By stage 14 and onward, expression of dNSF-2 remains in the midgut but is also now evident in the central nervous system.

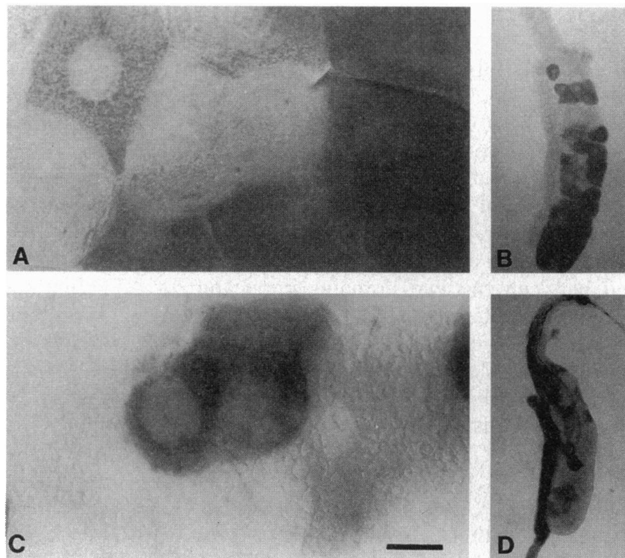

FIG. 4. *In situ* hybridization and PAS staining of third-instar larval salivary glands. (*A* and *B*) PAS staining was used to detect secretory cells within the salivary glands. (*A*) PAS stain is restricted to large vesicular structures within positive cells. (*B*) PAS-positive cells were found throughout the gland with a higher concentration at the posterior end. (*C* and *D*) *In situ* hybridization was used to detect dNSF-2-expressing cells within the salivary gland. Cells expressing dNSF-2 have a finer cytoplasm (*C*) and appear sporadically throughout the gland with the highest concentration at the anterior end (*D*). (Bar = 25 μm.)

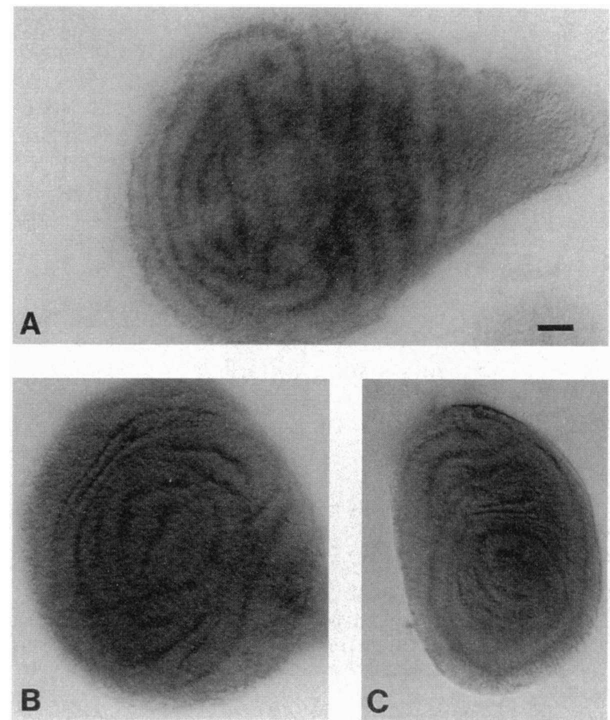

FIG. 5. *In situ* hybridization of dNSF-2 to *Drosophila* third-instar larval imaginal discs. (*A*) dNSF-2 is expressed at low levels throughout the wing imaginal disc with higher levels of expression seen in cells giving rise to distinct ring patterns. Little, if any, expression is seen in the notum region of the disc. (*B* and *C*) Expression of dNSF-2 in leg imaginal discs. As observed in wing discs, dNSF-2 is expressed throughout the leg discs with higher expression levels occurring within specific cells, giving rise to a distinct whirled pattern. (Bar = 25 μm.)

observed between *Drosophila* and mammals is of interest and may imply that their divergence occurs in parallel.

Many of these proteins are thought to have isoforms that may participate in analogous docking/fusion steps at other intracellular sites and in other tissues. The VAMP family of proteins includes a ubiquitous isoform called cellubrevin (35), which is expressed in all cell types. Similarly, syntaxin is a member of a large multigene family with some isoforms expressed in restricted patterns, while others are broadly expressed (36). In contrast, NSF has long been thought to be a single component that can interact at many intracellular sites with the different isoforms of the fusion apparatus components. Therefore, the unexpected presence of a second closely related NSF molecule means that, except for SNAP-25, all proteins in the 20S complex are members of multigene families. At present it is not known whether this plurality provides functional redundancy or is required for the formation of specific 20S complexes containing distinct components.

Expression of dNSF-2 was examined both by Northern blot analysis and by tissue *in situ*-hybridization techniques. Poly(A)$^+$ mRNA derived from adult female *Drosophila* was probed with a fragment of the dNSF-2 cDNA under stringent conditions, and several transcripts between 2.4 and 3.5 kb in size were detected (Fig. 2). The smallest of these is similar in size to the cDNAs isolated. Why multiple transcripts are observed is unclear. Under the stringent hybridization conditions used no cross-hybridization to the 3.2-kb dNSF-1 transcript should occur. Additional work will be needed to determine whether these bands are the result of alternative splicing or varied transcriptional initiation and termination sites and whether these bands reflect tissue-specific products or are present in all cells.

The widespread function of NSF in cell-membrane transport suggests that this gene is homogenously expressed in all cell types. However, *in situ*-hybridization studies of *Drosophila* embryos revealed that the level and tissue distribution of dNSF-2 expression varied during development. At present the extent to which the expression patterns of dNSF-1 and dNSF-2 overlap is unknown.

The highest level of dNSF-2 RNA was detected in embryos before and during cellularization, suggesting that much of the dNSF-2 RNA detected at early embryonic stages may derive from maternal transcripts originating in the nurse cells. At these stages (stage 5 and 6; Fig. 3*A*) [staging is according to Campos-Ortega and Hartenstein (37)], all cells appear to contain roughly equivalent dNSF-2 RNA levels. After onset of gastrulation, the highest levels of expression appear in embryonic regions that give rise to endodermal and ectodermal tissues, including the midgut and hindgut (Fig. 3*B*). Expression of dNSF-2 in germ band-retracted embryos (Fig. 3*C*) is primarily detected in the midgut, hindgut, and central nervous system (Fig. 3*D*). The higher expression levels found in these tissues probably reflects their quantitative requirements for molecules involved in membrane fusion events.

Consistent with this hypothesis is the relatively high level of dNSF-2 expression that we observed in both the ring gland (data not shown) and salivary glands of *Drosophila* larvae (Fig. 4). Both of these tissues are involved in secretion; the ring gland secretes the principal metamorphosing hormone ecdysone, whereas the salivary gland secretes glue proteins that are required for the larvae to attach to a substrate in preparation for pupariation. Interestingly, expression of dNSF-2 is not uniform within the salivary gland but rather is restricted to a few cells distributed throughout the gland (Fig. 4*D*). In addition, the cells that express dNSF-2 appear to be morphologically distinct from the cells identified by PAS. PAS staining has previously been used to identify glycoproteins that are secreted from cells within the gland (24). The PAS-positive cells appear densely packed with vesicles (Fig. 4*A*), whereas the dNSF-2-positive cells appear to have a finer, granular cytoplasm (Fig. 4*C*). The significance of this observation with respect to the

role of dNSF-2 in the regulated release of salivary gland glycoproteins remains to be determined.

In contrast to the observed expression of dNSF-2 in the embryonic nervous system, little, if any, dNSF-2 could be detected in the optic lobes and ventral ganglia of the larval nervous system. This apparent reduction in expression suggests that more secretion and fusion events are required during development of the tissue than are required for its mature functions. Alternatively, this result could reflect differential expression patterns of dNSF-1 and dNSF-2, and dNSF-1 could be primarily expressed in these mature structures.

Larval imaginal discs, which are precursors of adult structures, expressed varied levels of dNSF-2 in unusual patterns (Fig. 5). The highest expression levels were found in the antennal, wing, and leg discs. In the latter two sites, the expression was found in circular and whirled patterns that did not correlate purely with folds, invaginations, or cell-density variations in the discs. The significance of dNSF-2 expression at these sites is unclear but may reflect developmentally important patterns of secretion within the discs. A role for NSF/SEC18 proteins in development has not been previously suggested.

Availability of *Drosophila* homologs of NSF could permit a genetic study of its role in the regulation of membrane fusion and intracellular traffic in a higher eukaryote. However, the observation that at least two closely related isoforms are expressed in partially overlapping patterns will undoubtedly complicate such studies. Furthermore, we cannot rule out the existence of other isoforms. Whether these proteins are functionally redundant or not remains to be determined. The chromosomal locus of dNSF-2 coincides with the site of several mutants, most notably several embryonic recessive lethal mutations. Further studies are necessary to determine the proximity of dNSF-2 to any of these genes.

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