MOLECULAR AND CELLULAR BIOLOGY, Aug. 1999, p. 5743–5758 Vol. 19, No. 8
0270-7306/99/$04.00+0

Notch and Wingless Regulate Expression of Cuticle Patterning Genes

CEDRIC SATISH WESLEY*

Laboratory of Genetics, The Rockefeller University, New York, New York 10021

Received 6 April 1999/Accepted 5 May 1999

The cell surface receptor Notch is required during *Drosophila* embryogenesis for production of epidermal precursor cells. The secreted factor Wingless is required for specifying different types of cells during differentiation of tissues from these epidermal precursor cells. The results reported here show that the full-length Notch and a form of Notch truncated in the amino terminus associate with Wingless in S2 cells and in embryos. In S2 cells, Wingless and the two different forms of Notch regulate expression of *Dfrizzled 2*, a receptor of Wg; *hairy*, a negative regulator of *achaete* expression; *shaggy*, a negative regulator of *engrailed* expression; and *patched*, a negative regulator of *wingless* expression. Analyses of expression of the same genes in mutant *N* embryos indicate that the pattern of gene regulations observed in vitro reflects regulations in vivo. These results suggest that the strong genetic interactions observed between *Notch* and *wingless* genes during development of *Drosophila* is at least partly due to regulation of expression of cuticle patterning genes by Wingless and the two forms of Notch.

The transmembrane protein Notch (N) regulates cell fates in *Drosophila melanogaster* during the development of tissues from all three germ layers (6, 11, 14, 24, 42, 83, 88). For example, embryos without zygotically contributed N produce excess neuroblasts at the expense of epidermoblasts (11, 66, 90). In this instance, N appears to function by suppressing a default fate in some members of a population of physically interacting cells. Delta (Dl), also a transmembrane protein, has been identified as the ligand for this function of N, known as lateral inhibition (1, 14, 24, 32, 46, 50, 86).

The extracellular domain of N, where extracellular ligands or factors regulating intracellular N activities are expected to bind, is made up of 36 epidermal growth factor-like (EGF-like) repeats (42, 88). In vitro analyses of deletions affecting different segments of the extracellular domain of N have shown that Dl binds N in the region of EGF-like repeats 11 and 12 (68). Serrate (Ser), the only other identified ligand of N but with functions similar to that of Dl, also binds the same two EGF-like repeats (25, 29, 68). A single-amino-acid substitution in this region can produce an embryonic lethal phenotype (18). However, these two repeats are not sufficient for wild-type N function: loss of the remaining extracellular sequence blocks formation of the embryonic cuticle (52), and single-amino-acid substitutions affecting the 2nd (nd^3), 14th (*spl*), 24th (Ax^9, Ax^{59b}, Ax^{59d}), 25th (Ax^1), 27th (Ax^{71d}), 29th (Ax^{16}, Ax^{E2}), or 32nd (N^{ts1}) EGF-like repeats or the lin12/Notch repeats [$l(1)N^B$] produce lethality or aberrant *Notch* function (41, 54, 91). Since most of these mutations alter the structure of N EGF-like repeats, it was likely that these extracellular regions mediate interactions with alternative ligands. These alternative ligands could be associated with other N functions, such as induction of cell fates observed in the development of the compound eye (2, 13, 28) or differentiation of the epidermis (15, 36). Therefore, I explored the functional significance of EGF-like repeats of N other than those involved in binding of Dl or Ser.

* Mailing address: Laboratory of Genetics, Box 288, The Rockefeller University, 1230 York Ave., New York, NY 10021. Phone: (212) 327-8233. Fax: (212) 327-7420. E-mail: wesleyc@rockvax.rockefeller.edu.

Interspecific sequence comparisons identified two possible ligand binding sites in the region containing EGF-like repeats 19 to 36. A cell surface screen of embryonic cDNA-derived peptides identified Wingless (Wg) as a possible ligand of N. In vitro analysis showed that Wg associates with N in this region. In vitro and in vivo gene expression analyses showed that Wg is associated with regulation of expression of the *Dfrizzled2*, *patched*, *shaggy*, and *hairy* genes through two distinct forms of N: the full-length form and a form of N lacking 18 or more of the amino-terminal EGF-like repeats (thereby also the Dl binding repeats). These two forms of N produce different ligand-independent and ligand-dependent gene activities in cells expressing them.

MATERIALS AND METHODS

Sequence analysis. Extracellular N sequences of *D. virilis* and of *D. pseudoobscura* were obtained by reverse transcription-PCR with *Taq* polymerase and degenerate primers. Plots were generated by using the PILEUP and PLOTSIMILARITY programs in the Genetics Computer Group sequence analysis program (27). There are only 10 single-residue, 4 two-residue, and 1 eight-residue changes in the EGF-like repeats region between N and human Notch1.

Biopanning. A 6- to 12-h *Drosophila* embryonic cDNA library was constructed in the Surfzap vector (Stratagene). Biopanning was performed as recommended by the manufacturer. Approximately 5×10^8 lambda phage (~130 times the number of primary plaques) were used for mass excision of phagemids. About 6×10^8 excised phagemids were used for filamentous phage preparation (in the presence of 1% glucose). Phage precipitate was resuspended in balanced salt solution (BSS) (89). A total of 4×10^6 to 6×10^6 heat shock-induced S2-N cells were washed twice with BSS and blocked first for 30 min at 4°C with 1 ml of BSS–5% protease-free bovine serum albumin (BSA) and then for 30 min at 4°C with 1 ml of BSS–5% BSA–100 μl of ~10^{14} M13mp8 phage per ml in BSS solution. Approximately 10^{13} to 10^{14} biopan-ready filamentous phage, mixed with 60 μl of M13mp8 phage solution, were added, and the tubes were shaken for 25 min at 4°C. The cells were washed twice with 10 ml of cold BSS–5% BSA followed by four times with 10 ml of cold BSS. The bound phages were eluted in 200 μl of 0.1 M triethylamine with protease inhibitors (1 μl each of benzamidine [0.1 mg/ml], trypsin inhibitor [10 mg/ml], pepstatin [10 mg/ml], leupeptin [10 mg/ml], and aprotinin [2.2 mg/ml] and 5 μl of phenylmethylsulfonyl fluoride [10 mg/ml]), the solution was neutralized with 200 μl of 1 M Tris-HCl (pH 7.5), and the phagemids were amplified in *Escherichia coli*. A total of ~10^{12}, ~10^{10}, and ~10^8 amplified phage were used in the second, third, and fourth rounds of biopanning, respectively. In the fifth and sixth rounds, ~10^7 and ~10^6 phagemids, respectively, were biopanned four times for 30 min consecutively on ~10^7 heat shock-induced S2 cells (each time), and the supernatant was subsequently biopanned once on heat shock-induced S2-N cells. Then 1,000 to 2,000 eluted, biopanned phagemids were screened with probes of various genes by standard procedures (73). Phages from purified *wg*-carrying phagemids were prepared as specified by the manufacturer (Stratagene).

Immunocytochemistry on cultured cells. S2, S2-N, S2-$N^{\Delta I}$, S2-$N^{\Delta EGF19-36}$, and S2-$N^{\Delta EGF1-18}$ cells were heat shock induced before use. S2-Dfz2 cells (8) were grown in the presence of copper for 12 to 14 h before use. The cells were washed twice with cold Shields and Sangs M3 medium (M3 medium; Sigma), resuspended with 400 μl of cold medium conditioned with growth of S2-Wg or S2 cells (see below), and incubated for 15 min at 4°C. The cells were washed twice with cold M3 medium, fixed in 4% paraformaldehyde–1× phosphate-buffered saline, and processed for immunofluorescence as described previously (24, 51). pv9 1.1 S2-Wg cells were used to make Wg conditioned M3 media as described previously (69). Unconcentrated medium was used for all experiments.

Immunoprecipitations. (i) From cultured cells. N and Dl proteins were induced by heat shock. Dfz2 was induced by growing S2-Dfz2 cells for 12 to 14 h in the presence of copper. For N and Dl experiments, 10^7 cells each of S2-N and S2-Dl cells, respectively, were incubated in M3 medium for 15 min for formation of aggregates (51) and used for each immunoprecipitation (i.e., each lane). The number of S2-N or S2-Dl cells was approximately the same wherever a mixture of cell lines was used, with the remainder being made up by the other cell line or S2 cells. For N and Wg experiments, 3×10^7 S2 or S2-N cells were used per immunoprecipitation. For experiments with $N^{\Delta EGF1-18}$, $N^{\Delta EGF19-36}$, and S2-Dfz2 cells, 5×10^6 cells were used per immunoprecipitation. The cells were washed twice in cold serum-free M3 medium and resuspended in 250 μl of cold M3 medium conditioned with growth of S2 cells (S2 media) or pv9 1.1 S2-Wg cells (S2-Wg media) plus protease inhibitors (20 ng each of leupeptin, pepstatin, trypsin inhibitor, and E-64 per ml, 5 ng of aprotinin per ml, and 2 mM phenylmethylsulfonyl fluoride). Where required, EGTA was added to a final concentration of 15 mM. Then 10 μl of ~1.25 mM bis(sulfosuccinimidyl suberate) (BS^3) cross-linker resuspended in 1 ml of cold phosphate-buffered BSS (pbBSS: 55 mM NaCl, 40 mM KCl, 15 mM Mg_2SO_4, 10 mM $CaCl_2$, 20 mM glucose, 50 mM sucrose, 0.74 mM KH_2PO_4, 0.35 mM Na_2HPO_4) was added to appropriate samples, and the cells were incubated for 30 min at 4°C. The cells were pelleted, resuspended in 1/10 pbBSS–10 mM Tris-HCl (pH 7.5) (to quench cross-linking)–protease inhibitors, and incubated on ice for 10 min. The membranes were pelleted, resuspended in 400 μl of cold pbBSS–60 mM Tris (pH 7.5)–0.8% Triton X-100–protease inhibitors, and incubated for 20 min on ice. A 20-μl volume of 10% deoxycholate was added, and incubation was continued for 90 min to 2 h. The extract was precleared with GammaBind Plus beads (Pharmacia) for ~2 h at 4°C and incubated overnight at 4°C with the immunoprecipitation antibody where appropriate [100 μl of the monoclonal anti-Dl antibody, 1 μl of the polyclonal anti-Wg (rb) antibody, 1 μl of the polyclonal anti-Hedgehog antibody, 4 μl of the anti-Patched antibody, or 20 μl of the anti-Dfz2 monoclonal antibody]. Immunocomplexes were captured with GammaBind Plus beads, and the beads were rinsed four times with 1 ml of cold pbBSS–protease inhibitors–10 mM Tris (pH 7.5)–0.1% Triton X-100. Bound complexes were eluted with 40 μl of 1× Laemmli buffer–protease inhibitors, boiled for 6 min, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 4% polyacrylamide gels (for anti-Dl or anti-Wg immunoprecipitations) or in 6% polyacrylamide gels (for anti-Dfz2 immunoprecipitations). Western blot analyses were performed by to standard procedures (30, 73), and signals were detected with the ECL kit (Amersham).

(ii) From embryos. Approximately 800 μl of dechorionated embryos of appropriate ages and strains, laid by circadian cycle-entrained flies (to minimize age variance in embryos), was partially crushed, with a loose-fitting pestle in a 1-ml Wheaton Dounce grinder, in the presence of 400 μl of ice-cold pbBSS–protease inhibitors, with or without ~2 mM BS^3. After 45 min of incubation on ice, 12 μl of cold 2 M Tris-HCl (pH 7.5) was added to quench the cross-linking reaction. Membrane proteins were extracted in 0.75% Triton X-100–0.5% deoxycholate. The rest of the procedure was identical to that described for immunoprecipitation from cultured cells. Anti-Dl, anti-Wg, and anti-Ser immunoprecipitates (see Fig. 4A and B) were separated by SDS-PAGE in 4% polyacrylamide gels; anti-Wg, anti-Hh, and anti-Ptc immunoprecipitates (see Fig. 4C) were separated by SDS-PAGE in 6% polyacrylamide gels.

Northern analyses. Total RNAs were extracted from cultured cells or embryos by using RNAzol B (Tel-test, Inc.). I used 0- to 20-h dechorionated embryos, collected at the indicated temperatures (with appropriate corrections for developmental times). A total of 2×10^7 cells of different cell lines (grown to confluence) were heat shocked and incubated for 2 h at room temperature before use. S2-Dfz2 cells were grown in either the presence or absence of copper for 12 to 14 h before use. The cells were washed twice with serum-free M3 medium with antibiotics, and equal volumes were aliquoted to two 1.5-ml Eppendorf tubes, pelleted, and resuspended in 600 μl of S2 medium or S2-Wg medium. After 2 h of gentle shaking at room temperature, RNAs were extracted. Then 40 μg of total RNA was loaded in each lane. Standard Northern blot procedures were used (73). For generation of $N^{\Delta EGF1-18/Ax59d}$ molecules, the fragment including *Nhe*I (nucleotide 4324) (42) and *Bgl*II (nucleotide 5160) was PCR amplified (with *Pfu* enzyme) from Ax^{59d}/*FM7* flies, cut with *Nhe*I and *Bgl*II, and used to replace the same fragment in $N^{\Delta EGF1-18}$. Samples were sequenced fully in the replaced region, and clones carrying the Ax^{59d} mutation was transfected into S2 cells. Expression of protein was confirmed by Western blotting and immunocytochemistry.

Western blot analyses. For assessment of Armadillo protein in the cytoplasm, about 3×10^6 heat shock- or metal-induced cells were processed as described by Bhanot et al. (8). The same blot was stripped and stained for ~16 h with India ink. For analyses of proteins from embryos, proteins were extracted with 0.75% Triton X-100–0.5% deoxycholate or with SDS lysis buffer (43). The amounts of proteins in different embryonic extracts were standardized by using absorbance values at 280 nm and the Bio-Rad D_C protein assay kit. The proteins were separated by SDS-PAGE in 4% polyacrylamide gels. For analysis of S2-N, S2-$N^{\Delta EGF1-18}$, S2-$N^{\Delta EGF19-36 \text{ and LN rpts}}$, and S2-$N^{\Delta EGF1-36}$ proteins, heat shock-induced cells were dissolved in 1× Laemmli buffer and separated by SDS-PAGE in 4% polyacrylamide gels. Western blotting in all cases was performed by standard procedures (30, 73) and signals were detected with the ECL kit.

In situ RNA hybridization. Batches of wild-type and mutant embryos were grown under identical conditions at 25 or 18°C and processed simultaneously under the same conditions at all steps of the procedure. For each comparative experiment, the batches of wild-type and mutant embryos were divided just before addition of probe, and different probes were added to the divided batches of embryos. A double RNA-protein hybridization procedure, described by Corbin et al. (14), was used. An anti-β-galactosidase antibody made in mouse was used to sort out FM7 or TM6 chromosome carrying embryos laid by N^{264-47}/*FM7*, *spl* Ax^{59d}/*FM7*, Ax^{9B}/*FM7*, Ax^{59d}/*FM7*, or Dl^{X}/*TM6* flies.

RESULTS

EGF-like repeats 23 to 27 and 31 to 34 are potential ligand binding sites. A chimeric *Drosophila* N protein containing EGF-like repeats 10 to 13 from *Xenopus* physically associates with the *Drosophila* Dl protein (68). Therefore, the Dl binding region in N is expected to be conserved between homologous sequences. If there are additional ligands associated with different functions of Notch and if they interact with regions other than the Dl binding region, these regions are expected to be conserved between homologous sequences also. To determine whether such conserved regions exist in the extracellular domain of N, the DNA sequences coding for the extracellular portions of N proteins in *D. virilis* and *D. pseudoobscura* were compared by plotting the running averages of conservation between sequences of these invertebrate homologs and between sequences of *D. melanogaster* N and the human homolog, hNotch 1 (Fig. 1A). Figure 1 shows that, as expected, a peak with high conservation is centered on EGF-like repeats 11, 12, and 13, which include the Dl binding region. Besides that region, there are two additional regions that are as conserved as EFG-like repeats 11 to 13 in both comparisons. In the *D. melanogaster-D. virilis* comparison, the comparison relevant to N function in *Drosophila*, one region extends from EGF-like repeats 23 to 27 and encompasses most of the domain affected by the *Ax* mutations of *N* (17, 41). A second conserved region extends from EGF-like repeats 31 to 34. The functional importance of all these conserved regions is underscored by inclusion of at least one lethal mutation in each (boldface type in Fig. 1A). As EGF-like repeats 11 and 12 bind Dl (68) and are evolutionarily conserved (Fig. 1A), the regions containing EGF-like repeats 23 to 27 and 31 to 34 were hypothesized to be conserved because they are additional ligand binding regions in the extracellular domain of N.

Cell surface biopanning screen suggests a physical affinity between N and Wg. The two identified ligands, Dl and Ser, bind N only at the region including EGF-like repeats 11 and 12 (68). This specificity suggested that if the regions including EGF-like repeats 23 to 27 and 31 to 34 bound ligands, these are likely to be novel ligands. To identify such novel N ligands, if any, phagemid biopanning (55, 74) was performed on the surfaces of live S2 cells expressing full-length N proteins (S2-N). The procedure used is schematically shown in Fig. 1B. Phagemids encoding the known N ligands, Dl and Ser, were specifically enriched by this biopanning. Enrichment was also observed for Wg, N, Big Brain, Pecanex, and Fringe phagemids but not for Scabrous and Star phagemids (Table 1). Genes encoding these proteins are known to genetically interact with *N* (5, 15, 22, 31, 40, 47, 56, 64, 67). Enrichments were not detected for *hedgehog* (*hh*), *patched* (*ptc*), and *slit* genes, whose

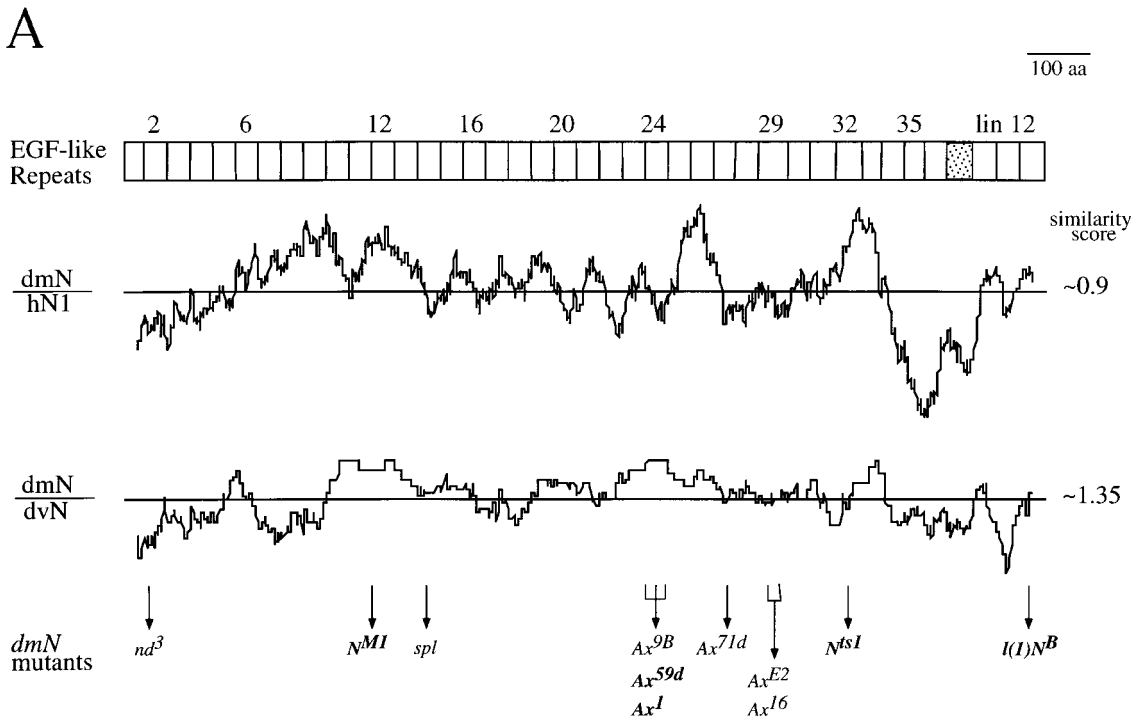
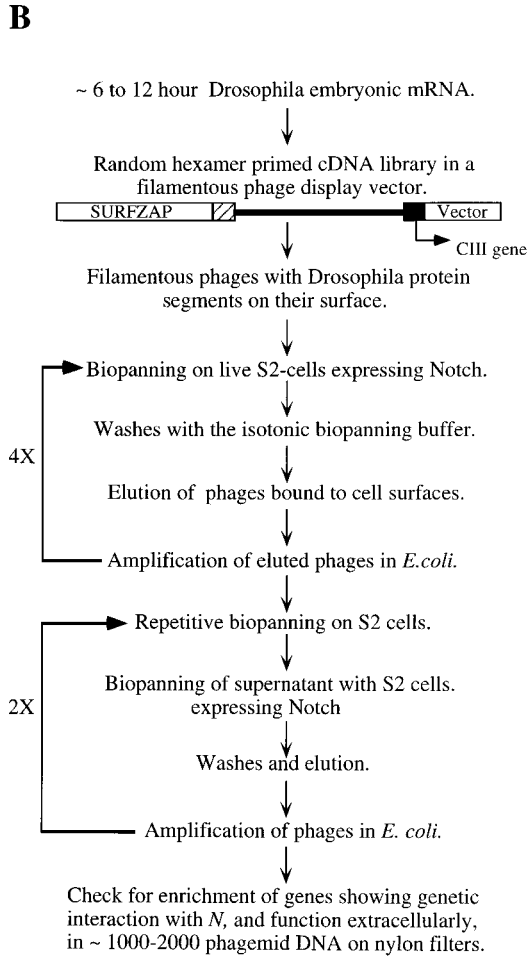


FIG. 1. Search for new N ligands. (A) Interspecific sequence comparisons reveal possible ligand binding sites in the extracellular domain of N. Each plot represents a running average of sequence conservation between two homologs, over sliding blocks of 40 amino acids (aa). At the top, the corresponding EGF-like repeats of N are graphically represented. lin12 = lin12/N repeats (42, 88). The line in the middle of each plot represents average similarity between the two sequences compared (the maximum value is 1.5). This average would include sequence conservation due to sequence elements common to all EGF-like repeats. The plot of *D. melanogaster* N and its *D. pseudoobscura* homolog is similar to that of N and the *D. virilis* homolog (differing only in the level of overall conservation). The between-lineage comparison identifies evolutionarily stable conserved regions. dmN, *D. melanogaster* N; hN1, human homolog of N (23); dvN, *D. virilis* N. Sites of mutations in *nd3* and $l(1)N^B$ are from reference 54, N^{M1} are from reference 18, *spl* and the *Ax* alleles are from reference 41, and N^{ts1} are from 91. Lethal alleles are in bold letters. (B) Schematic representation of the biopanning screen used for identification of potential N ligands (see Materials and Methods).



products function on cell surfaces (35, 45, 49, 57, 70, 81), or for several genes whose products are cytoplasmic proteins (Table 1 footnote). The high enrichment of Pecanex phagemids (from $0.3/10^5$ before biopanning to $25{,}600/10^5$ after biopanning), enrichment of phagemids of known ligands of N (Dl and Ser), enrichment of phagemids representing only a subset of genes showing interaction with the *N* gene, and lack of enrichment of EGF-like repeat sequence containing Slit phagemids indicated specificity in the enrichment process (the after-biopanning phagemid population was estimated to be composed of phagemids representing only about 15 genes).

Among the genes showing enrichment, *wg* was of particular interest. *wg* and *Ax* alleles of *N* genetically interact, and both produce similar "antineurogenic" phenotypes; N^- alleles, or the N^{M1} allele carrying a mutation in the Dl binding region, produce the other phenotypes (10, 15, 17, 18). Thus, it seemed possible that Wg physically interacts with the conserved Ax domain of N including EGF-like repeats 23 to 27 (Fig. 1). Therefore, further studies were focused on *wg*. Six of the Wg phagemids selected by biopanning were further analyzed by sequencing and Western blotting. The inserts in all six were of

TABLE 1. Change in frequency of test phagemids following biopanning of *Drosophila* embryonic cDNA carrying filamentous phages on the surfaces of S2-N cells[a]

Gene	Phagemid frequency (10^{-5}) in:	
	Biopan 0	Biopan 6
Delta	5	2,000
wingless	1	1,500
Serrate	5	1,300
Notch	0.6	4,700
big brain	6	6,200
pecanex	0.3	25,600
fringe	0.6	4,500
scabrous	0.9	<0.1
Star	4.8	<0.1
hedgehog	2	<0.1
patched	0.5	<0.1
slit	0.6	<0.1

[a] Phage enrichment was initially determined by restriction enzyme analysis of a random sample of phagemids and colony hybridization with some of the fragments. All clones in a random sample of 46 phagemids from the biopan 0 population contained inserts of different sizes, indicating a library complexity of ~1. A sample of 43 phagemid clones from the biopan 6 population was found to be composed of five sequences, indicating a maximal complexity of ~0.25. Enrichment levels of *Dl*, *wg*, *sca* phages were confirmed in several one-round biopanning experiments. Biopan 0 is the frequency before biopanning and is based on a sample of 10^5 to 10^6 phagemids; biopan 6 is the frequency after completion of the full procedure of biopanning and is based on a sample of 1,000 to 2,000 phagemids. The same membranes were also probed with gene sequences of cytoplasmic proteins *cactus*, *alcohol dehydrogenase*, and *glucose 6 phosphate dehydrogenase*. Each gene was present in biopan 0 but failed to show enrichment after biopanning.

wg coding DNA, and all six phages produced Wg protein as part of their cpIII coat protein (data not shown), suggesting that the enrichment of Wg phagemids was due to physical interactions between Wg and N.

Soluble Wg proteins form two molecular complexes with N proteins. To confirm that enrichment of *wg* phagemids on S2-N cells was due to binding of Wg protein (expressed on the surfaces of phagemids) to the surfaces of S2-N cells, immunocytochemical analyses was performed with soluble Wg and S2 cells expressing the following N molecules (see reference 52 for a complete description of these molecules): full-length N (S2-N), N lacking EGF-like repeats 19 to 36 (S2-$N^{\Delta EGF19-36}$), N lacking EGF-like repeats 1 to 18 (S2-$N^{\Delta EGF1-18}$), and N lacking the intracellular domain (S2-$N^{\Delta I}$). Wg was detected on the surfaces of S2-N cells (Fig. 2B), S2-$N^{\Delta EGF1-18}$ cells (Fig. 2C), and S2-$N^{\Delta I}$ cells (Fig. 2E) but not on surfaces of S2 cells (Fig. 2A) and S2-$N^{\Delta EGF19-36}$ cells (Fig. 2D). More than 2×10^6 cells were processed on each slide, and several such slides were examined for each cell type. Immunofluorescence signals were not detected on S2 and S2-$N^{\Delta EGF19-36}$ cell slides. Double staining with antibodies against N and Wg showed that only N-expressing cells bound Wg (Fig. 2F). Comparable frequencies of Wg-positive cells were obtained with S2-Dfz2 cells (8) treated in the same way as N-expressing cells (Fig. 2G). Dfz2 is known to bind Wg (8).

To determine whether Wg bound N or $N^{\Delta EGF1-18}$ on S2 cells expressing these proteins, immunoprecipitations were performed. In experiments with S2-N/S2-Dl cell aggregates, the anti-Dl antibody used failed to immunoprecipitate either the full-length or the extracellular domain of N. This may have been due to the particular antibody used or the disruption of N-Dl complexes during lysis and washes. Fehon et al. (24) considered disruption of the N-Dl complexes, as a consequence of the disruption of the physiological conformation of the extremely cysteine-rich extracellular domain of N, the reason for their low recovery of full-length N in Dl coimmunoprecipitations. To overcome the disruption of interaction between N and its ligands when cells are lysed for immunoprecipitation, a membrane insoluble cross-linker, BS^3, was used to covalently link proteins interacting at the cell surfaces. The activity of cross-linkers was quenched prior to lysis of cells so that cross-linking was limited to proteins interacting on the cell surfaces. BS^3 and related cross-linkers have been used successfully in studies of several cell surface protein interactions (80, 81, 87).

Immunoprecipitation of Dl from cross-linked protein extracts of S2-N/S2-Dl cell aggregates recovered a complex containing N and Dl (Fig. 3A, lane 4). The Dl-N complex was not recovered from S2-N cells in the absence of S2-Dl cells (lane 2) or from S2-Dl cells alone (lane 1). The levels of N in the supernatants of immunoprecipitates loaded in lanes 2 and 4 were comparable (lanes 3 and 5). Anti-Dl antibody failed to recover N when purified membranes of S2-N/S2-Dl cell aggregates or lysates of S2-N/S2-Dl cell aggregates were used, suggesting that the integrity of cells is indeed important for recovery of the Dl-N complex.

The capability of the cross-linking and immunoprecipitation procedure to recover proteins interacting at the cell surface was further tested with S2-Dfz2 cells treated with Wg. A Wg-Dfz2 complex, migrating at the rate of a ~100-kDa protein, was recovered (Fig. 3B, lane 2). Unlinked Dfz2 migrated at ~130 kDa (lane 4). The Wg-Dfz2 complex was not recovered in the absence of the Wg antibody (lane 3). The same Wg-Dfz2 complex was obtained when EGTA was used to chelate calcium in the medium (lane 1), indicating that calcium is not required for Wg and Dfz2 interaction.

The cross-linking and immunoprecipitation procedure that recovered the Dl-N and Wg-Dfz2 complexes was applied to S2-N and S2-$N^{\Delta EGF1-18}$ cells treated with Wg. Two N- and Wg-containing complexes, with different electrophoretic mobilities, were recovered from S2-N cells (Fig. 3C, lane 7). Only one was recovered from S2-$N^{\Delta EGF1-18}$ cells (lane 14). The same complexes were recognized by both anti-Wg and anti-N antibodies (lanes 7 and 9 and lanes 14 and 16), confirming that they contain both N and Wg. Wg- and N-containing complexes were not recovered from immunoprecipitations from S2-N or S2-$N^{\Delta EGF1-18}$ cells treated with medium that does not contain Wg (lanes 6 and 13), from S2 cells treated with Wg medium (lane 5), or from S2-N or S2-$N^{\Delta EGF1-18}$ cells treated with Wg medium without cross-linkers (lanes 8 and 15). The levels of N were similar in the protein extracts used for all these immunoprecipitations (lanes 2 to 4 and lanes 10 to 12). Note also that S2 cells do not produce N (lane 1). Since proteins or protein complexes analyzed in 4% gels migrate slower than a ~120-kDa protein, neither the ~45-kDa Wg monomers nor the Wg-Dfz2 complex migrating at ~100 kDa are expected to be retained in the gel. Wg- and N-containing complexes were not recovered from either S2-$N^{\Delta EGF19-36}$ cells treated with Wg medium (Fig. 3D, lanes 1 to 4) or S2-$N^{\Delta EGF1-18}$ cells treated with Wg medium containing EGTA (lanes 5 to 8), indicating that Wg associates with N in the region containing EGF-like repeats 19 to 36 and requires calcium for this association.

Two Wg- and N-containing complexes were recovered from Wg-treated S2-N cells (Fig. 3C, lane 7). S2-N cells are designed to produce full-length N, and most N produced by this cell line is of the size expected from full-length N (~350 kDa [lanes 2 to 4]). The Wg-N complex migrating near a ~220-kDa marker protein probably included a truncated N because S2-$N^{\Delta EGF1-18}$ cells also produce it (lane 14). $N^{\Delta EGF1-18}$ encodes a protein that lacks about half of the N

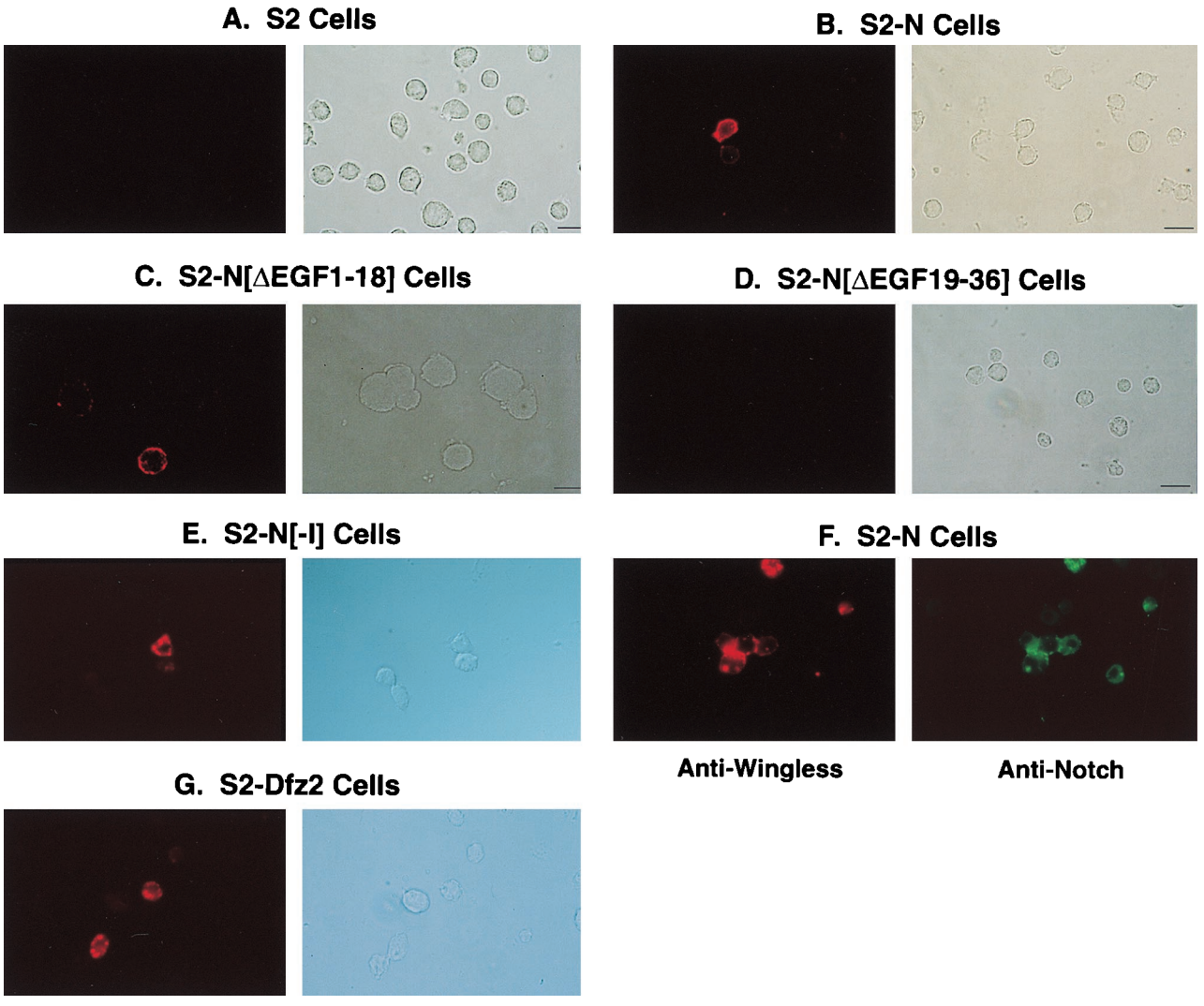


FIG. 2. Soluble Wg binds N in the region containing EGF-like repeats 19 to 36. Wg binds surfaces of S2-N (B), S2-$N^{\Delta EGF1-18}$ (C), and S2-$N^{\Delta I}$ cells (E) but not surfaces of S2 (A) and S2-$N^{\Delta EGF19-36}$ cells (D). Only N-expressing cells bind Wg (F), and the frequency of N-expressing cells binding Wg is comparable to the frequency of S2-Dfz2 cells binding Wg (G). (A to E and G) The photograph on the left shows anti-Wg immunofluorescence in a microscopic field of cells, and the photograph on the right shows Nomarski illumination of the same microscopic field of cells. (F) The photograph on the left shows immunofluorescence generated by the anti-Wg antibody, and the photograph on the right shows immunofluorescence generated in the same microscopic field of cells by an anti-N antibody. Cells were treated with unconcentrated culture medium conditioned by growth of S2-Wg cells. Wg on cell surfaces was detected immunocytochemically with anti-Wg (rb), an antibody made in rabbit (69), and a rhodamine-conjugated secondary antibody. N on cell surfaces was detected with αNI (52) and a fluorescein-conjugated secondary antibody. None of the cells incubated with culture medium conditioned by growth of S2 cells showed any detectable signals. Only cells expressing high levels of N are apparent in the photographs. An actual count of all immunofluorescent cells indicates a Wg-positive frequency of ~40% (N is expressed by only 50% of the cells stably cotransfected with the hygromycin gene). A short binding period was used because N was found to be lost from the cell surfaces within minutes of treatment with Wg.

extracellular domain EGF-like repeats (52). If N in the faster-migrating Wg-N complex is a truncated form, it was preferentially enriched or generated by Wg since a truncated form of N was not detectable in S2-N cell protein extracts (Fig. 3C, lanes 2 and 3; see also Fig. 6E, lane 4). A faster-migrating Dl-N complex was not recovered in Dl immunoprecipitations (Fig. 3A).

Wg-N complexes similar to those recovered from cultured cells are recovered from Canton S embryonic extracts. To determine whether the two Wg-N complexes produced in cultured cells treated with Wg are also produced in vivo, immunoprecipitations were performed with proteins extracted from Canton S embryos. The most frequent and predominant Wg-N complex recovered from young embryos (0 to 3 h or 0 to 6 h) was Wg complexed with a truncated N (N lacking the anti-NT epitope) (Fig. 4A, lanes 10 to 13, see Fig. 4D for epitopes of N antibodies). The electrophoretic mobility of this complex was similar to that of the faster-migrating Wg-N complex (i.e., near the ~220-kDa marker protein) recovered from cultured cells (Fig. 3C). From aliquots of the same embryonic extracts, Dl was found complexed with N recognized by all three N antibodies (Fig. 4A, lanes 7 to 9). This N in the Dl-N complex is apparently the full-length N.

Wg complexed with the truncated N was also recognized by an independently generated anti-Wg antibody (Fig. 4A, lane 14). This independent antibody (made in the rat) also immunoprecipitated the same Wg-N complex (lane 15). Probing of the blots with anti-N and anti-Wg antibodies showed that this Wg-N complex contains both Wg and N (lanes 10 and 11 and lanes 15 and 16). Similar probings showed that the Dl-N com-

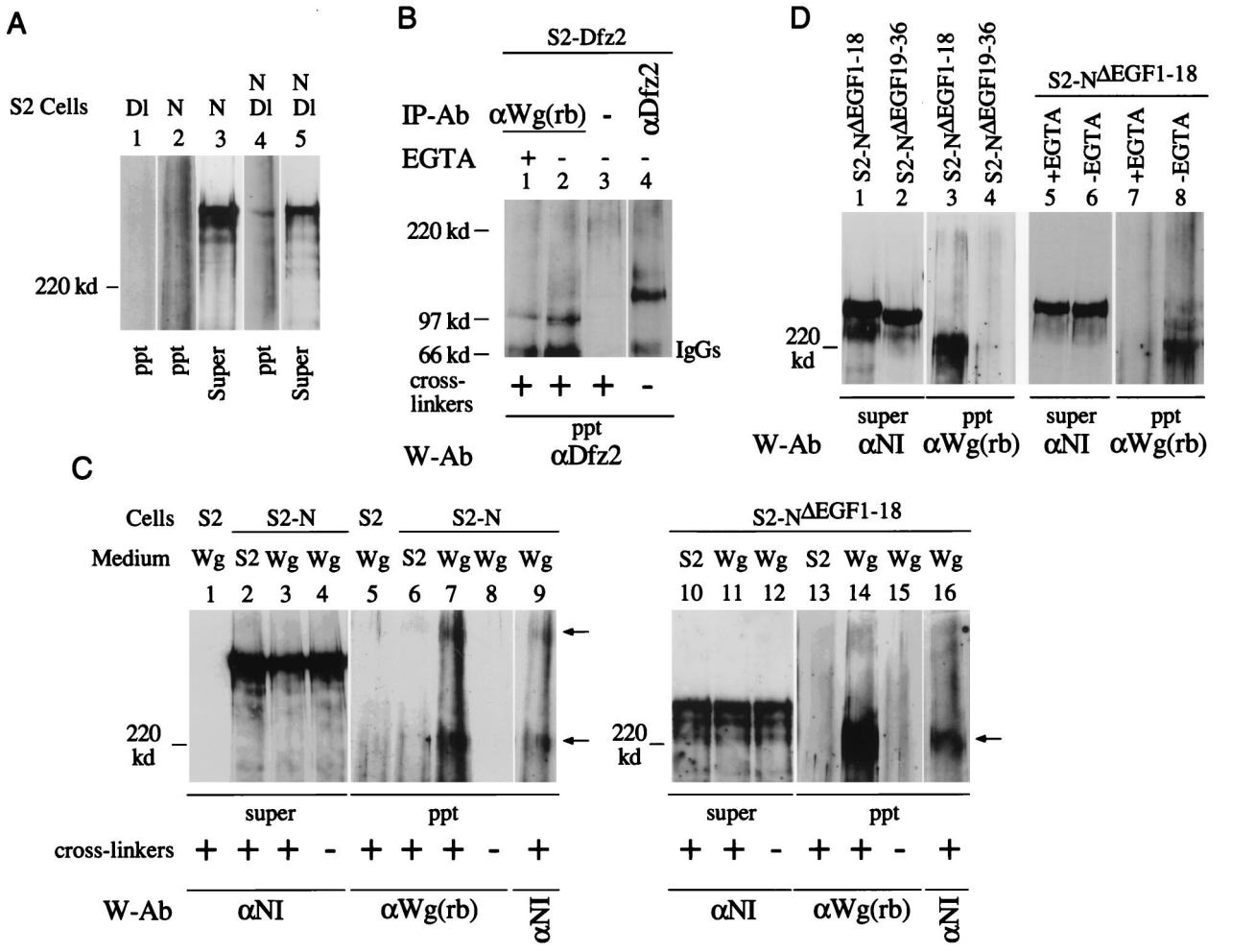


FIG. 3. Two different Wg- and N-containing complexes are recovered from N-expressing S2 cell surfaces. (A) N- and Dl-containing complexes are recovered from S2-N and S2-Dl cell aggregates in the presence of cross-linkers. Dl-containing cross-linked complexes were immunoprecipitated by the monoclonal anti-Dl antibody, MAb 202 (24) and analyzed by Western blotting with anti-NI antibody. (B) Wg- and Dfz2-containing cross-linked complexes are recovered from S2-Dfz2 cells treated with Wg medium containing cross-linkers, in the presence or absence of EGTA (lanes 1 and 2). A mouse monoclonal anti-Dfz2 antibody (kindly provided by R. Nusse) was used for immunoprecipitation (lane 4) and for detection of Wg-Dfz2 complexes by Western blotting. Wg-Dfz2 complexes were not recovered from S2-Dfz2 cells treated with medium conditioned by growth of S2 cells (not shown). (C) Two Wg- and N-containing cross-linked complexes (arrows) are immunoprecipitated from S2-N cells (lanes 7 and 9), and only one is immunoprecipitated from S2-$N^{\Delta EGF1-18}$ cells (lanes 14 and 16), treated with Wg-containing medium. N- and Wg-containing complexes were immunoprecipitated with anti-Wg(rb) and detected by Western blotting with the indicated antibodies (W-Ab). Lanes 7 and 9 and lanes 14 and 16 show reaction of the same blots with anti-Wg(rb) and anti-NI antibodies. (D) Wg and N containing cross-linked complexes are recovered from S2-$N^{\Delta EGF1-18}$ cells (lane 3) in the absence of EGTA (lane 8) but not from S2-$N^{\Delta EGF19-36}$ cells (lane 4). S2-$N^{\Delta EGF1-18}$ or S2-$N^{\Delta EGF19-36}$ cells were treated with Wg medium containing cross-linkers, immunoprecipitation was performed with anti-Wg(rb) antibody, and the Western blots were probed with the indicated antibodies. ppt, immunoprecipitated complexes eluted from GammaBind beads; Super, an aliquot of the protein extract after the last pelleting of the GammaBind beads (see Materials and Methods); IP-Ab, immunoprecipitation antibody; W-Ab, Western blotting antibody; cross-linker, BS^3. Wg, medium conditioned by growth of S2-Wg cells; S2, medium conditioned by growth of S2 cells. For panels A, C, and D, 4% polyacrylamide gels were used; for panel B, 6% polyacrylamide gels were used. Only proteins or protein complexes migrating slower than a 120-kDa marker protein are resolved in panels A, C, and D. The tops of all the blots shown coincide with the top of the resolving gel of the discontinuous SDS-PAGE gels.

plex actually contains both N and Dl (lanes 5 and 6). Anti-N antibodies do not recognize unlinked Wg, Dl, or Ser; anti-Wg antibodies do not recognize unlinked N, Dl, or Ser; anti-Dl antibodies do not recognize unlinked N, Wg, or Ser; and anti-Ser antibodies do not recognize unlinked N, Dl, or Wg (data not shown). Therefore, recognition of the same complex by two different antibodies indicates the presence of both of the proteins in the complex. Similar mobilities of the N-Dl complexes or N-Ser complexes (see below) and unlinked full-length N might be due to the resolution limitations of SDS–4% polyacrylamide gels or to anomalous mobilities of cross-linked complexes (Dfz2-Wg complexes migrate faster than unlinked Dfz2 [Fig. 3B]).

As with cultured cells, Dl was not recovered with truncated N (Fig. 4A, lanes 7 to 9) suggesting that truncated N specifically associated with Wg. The slower-migrating Wg-N complex was generally recovered at low levels in extracts prepared from 0- to 6-h embryos. Higher levels of the slower-migrating Wg-N complex were recovered from extracts prepared from 6- to 12-h embryos (lane 17), and this was the only Wg-N complex recovered from extracts prepared from 10- to 16-h embryos (lanes 18 and 19). Unlike the N-Wg complex migrating near the ~220-kDa marker protein, this slower-migrating complex reacted with all anti-N antibodies, anti-NI (lane 17), anti-NT (lane 18), and anti-NPCR (data not shown), indicating that it contains the full-length N. Neither Dl nor Wg recovered any N

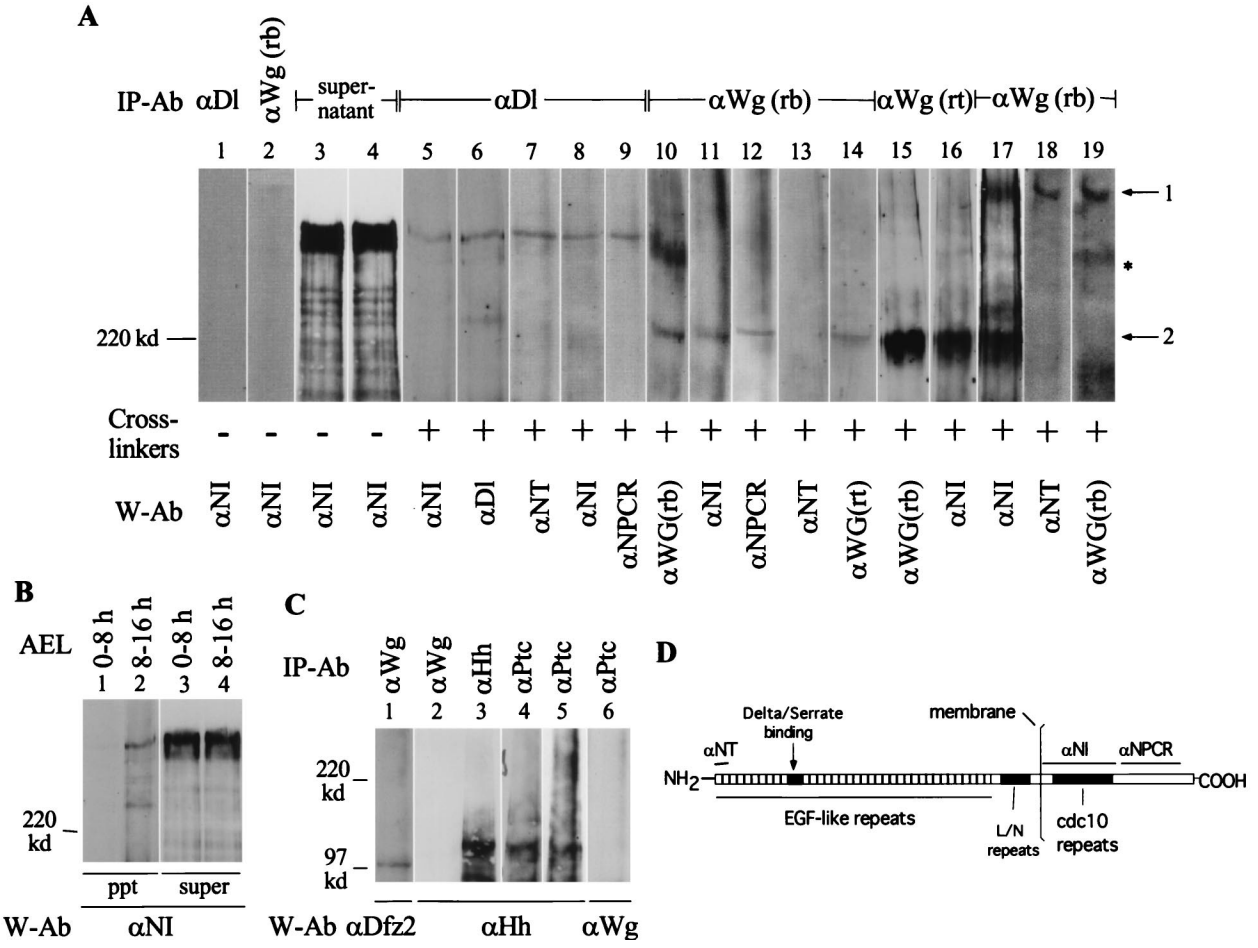


FIG. 4. Wg and N form complexes during embryogenesis. (A) Two Wg- and N-containing cross-linked complexes, similar to those recovered from cultured cells, are immunoprecipitated from Canton S embryonic extracts. Anti-Dl, is monoclonal antibody MAb 202; anti-NT is described in reference 43; anti-NPCR is described in reference 52; and anti-Wg(rt) was kindly provided by A. Martinez-Arias (See panel D for epitope regions for N antibodies). I used 0- to 3-h embryos for lanes 1 to 16, 6- to 12-h embryos for lane 17, and 10- to 16-h embryos for lanes 18 and 19. Arrow 1 shows Wg complexed with full-length N (lanes 17, 18, and 19); arrow 2 shows Wg complexed with a truncated N (lanes 10 to 17). The asterisk marks the Wg complex not containing N (lanes 10 and 19). A single blot was probed sequentially with the indicated antibodies to form lanes 10 and 11; 12, 13, and 14; 15 and 16; and 19 and 18 (numbers also indicate the sequence of probing). The same embryonic extract was used for lanes 1 to 4 and 7 to 14; lanes 5 to 6, 15, 17, and 18 are derived from different embryonic extracts. (B) Ser-N cross-linked complexes are also recovered from cross-linked embryonic extracts. Complexes were immunoprecipitated with anti-Ser antibody (kindly provided by Elizabeth Knust). Complexes migrating faster than a ~120-kDa marker protein were not analyzed in panels A and B. (C) The procedure recovering Wg-N, Dl-N, and Ser-N complexes also recovers Wg-Dfz2 (lane 1) and Hh-Ptc complexes from cross-linked embryonic extracts (lanes 3 to 6). For lanes 5 and 6, equal volumes of anti-ptc immunoprecipitate was separated in two different lanes and probed with the indicated antibodies. IP-Ab, immunoprecipitation antibody; cross-linker, BS^3; W-Ab, Western blotting antibody; AEL, after egg laying. For panels A and B, 4% polyacrylamide gels were used; for panel C, 6% polyacrylamide gels were used. The tops of all the blots shown in panels A, B, and C coincide with the top of the resolving gel of the discontinuous SDS-PAGE gels. (D) Diagram showing the N epitopes used to produce the N antibodies used in the study.

molecules in the absence of cross-linkers (lanes 1 to 4). All immunoprecipitations were repeatedly confirmed. A Wg-containing complex migrating at the rate of a 250- to 300-kDa protein was recovered at all times during embryogenesis (lanes 10 and 19). This complex appeared not to contain N, since none of the anti-N antibodies recognized it: lanes 10 and 11 and lanes 18 and 19 are the same blots probed with an anti-Wg antibody and two different anti-N antibodies (anti-NPCR also does not recognize it [lane 12]).

The immunoprecipitation procedure used to recover Wg-N and Dl-N complexes also recovered the expected Ser-N and Patched (Ptc)-Hedgehog (Hh) complexes from *Drosophila* embryonic extracts (Fig. 4B and C, lanes 3 and 4). Note that anti-Wg immunoprecipitates fail to recover Hh or Ptc (Fig. 4C, lanes 2, 5, and 6). Anti-Wg immunoprecipitates separated by SDS-PAGE in 6% polyacrylamide gels showed the Wg-Dfz2 complex migrating at ~100 kDa, similar to the complex recovered from S2-Dfz2 cells treated with Wg (Fig. 4C, lane 1). This complex will not be retained in 4% polyacrylamide gels and therefore is not seen in Fig. 4A. These results confirm that the cross-linking and immunoprecipitation procedure used to recover Wg-N complexes also recovers other complexes expected to be formed during embryogenesis. All the immunoprecipitation experiments together argue strongly in favor of the simplest proposal that Wg binds N directly.

The above-described experiments showed that (i) two forms of N associate with Wg under in vitro and in vivo conditions, (ii) one form of N lacks a portion of the amino-terminal EGF-like repeats, and (iii) the association of Wg with N is dependent on the region of N containing EGF-like repeats 19 to 36.

Wg regulates expression of *Dfrizzled2*, *hairy*, *patched*, and *shaggy* genes in S2 cells expressing N and $N^{\Delta EGF1-18}$. The cell

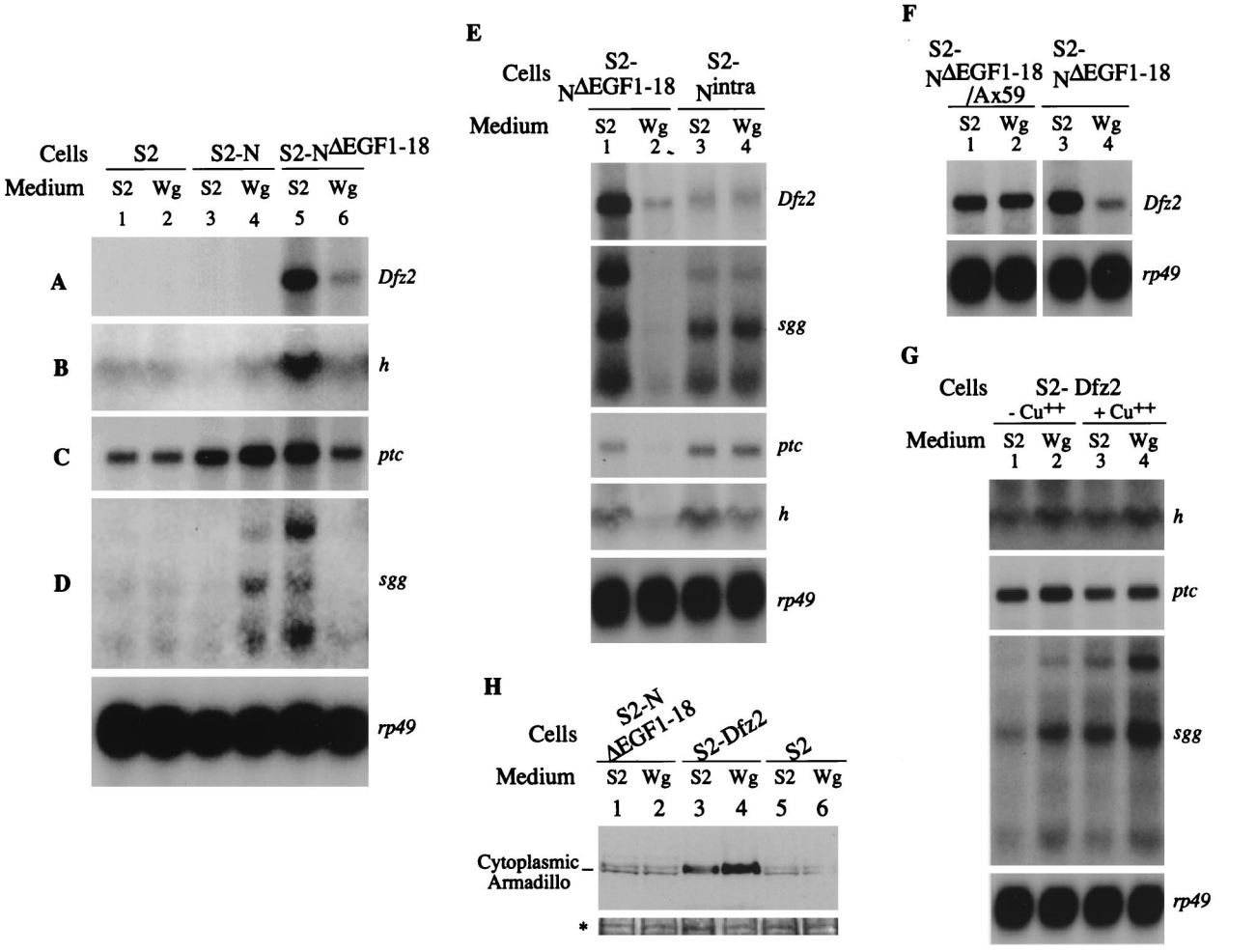


FIG. 5. N and $N^{\Delta EGF1-18}$ have different ligand-independent activities and respond differently to Wg. (A to D) Expression of *Dfz2*, *h*, *ptc*, and *sgg* in S2-N and S2-$N^{\Delta EGF1-18}$ cells are regulated by Wg. *en*, *wg*, *ac*, *hh*, and *m5* and *m8* of *E(spl)C* were not detected in any of the experiments. The sizes of transcripts of all genes were similar to published reports. *Dfz2* (8), *ptc* (35), *h* (37), *rp49* (61), and *sgg* (77). The largest *sgg* RNA corresponds in size to the embryonic transcript, while the smallest *sgg* RNA has a size expected for the ovarian transcript (77). *sgg*, *wg*, *ac*, and *m5* and *m8* of *E(spl)C* are known to genetically interact with *N* (15, 33, 71, 72, 85); *Dfz2*, *ptc*, *wg*, and *sgg*, are involved in epidermal patterning (4, 7, 8, 20, 35, 57, 65); *h* is a negative regulator of *ac* (38, 79, 84). (E) The extracellular domain of $N^{\Delta EGF1-18}$ is required for regulation of *Dfz2*, *sgg*, *ptc*, and, to a lesser extent, *h* expression. (F) Ax^{59d} mutation in $N^{\Delta EGF1-18}$ abolishes Wg-mediated down regulation of *Dfz2* expression in S2-$N^{\Delta EGF1-18}$ cells. The two autoradiographs were derived from the same blot with different exposure times. (G) S2-Dfz2 cells (S2-pMK 33 cells [8]) do not down regulate expressions of *ptc*, *sgg*, and *h* in response to Wg, with or without copper induction. (A to G) Total RNAs were extracted from the indicated cells treated with M3 medium conditioned by growth of S2 cells (S2 medium) or S2-Wg cells (Wg medium) and analyzed by Northern blotting. The same batch of S2 or Wg medium was used for all of the studies. Gene sequences used as probes are indicated on the right of each panel. *rp49* was used to indicate the relative levels of RNA in different lanes. The individual blots are exposed to film for different periods. Exposure times: *rp49* < *Dfz2* < *ptc* < *sgg* < *h*. (H) S2-$N^{\Delta EGF1-18}$ cells do not accumulate Arm in the cytoplasm in response to Wg. The bottom panel (*) shows a India ink-stained protein band visible in all lanes of the blot to indicate the amount of samples loaded. An anti-Arm antibody made in rabbits (kindly provided by Laurent Ruel) was used to detect Arm.

surface binding and immunoprecipitation experiments described above showed that N and Wg form physical complexes in vitro and in vivo. But does Wg alter the physiological state of cells through N? This question is not easily answered for the in vivo case because N is required for production of epidermal precursor cells through lateral inhibition functions associated with Dl (11, 66, 79, 90) and is also subsequently required for production of epidermis from these epidermal precursor cells (15, 36). Of these two successive developmental events, Wg is required only for production of epidermis from the epidermal precursor cells (4, 7). Any perturbation of the lateral inhibition functions of N (associated with Dl) is expected to mask epidermal functions of N associated with Wg. Therefore, I explored the response of N-expressing S2 cells to Wg in the medium and tested wild-type and mutant *N* embryos for comparable responses. Since regulation of endogenous gene activities in response to exogenous factors in the medium is a good indicator of the signaling effects of cell surface ligand-receptor interactions, the expression of a selected sample of genes that are linked to the N and Wg signaling pathways was assessed in N-expressing S2 cells treated with Wg.

The expression patterns of *m5* and *m8* genes of the *Enhancer of split Complex* [*E(spl)C*; genes associated with Dl-mediated functions of N], *wg*, *ac*, *en*, *hh*, *h*, *ptc*, *Dfz2*, *sgg*, and several housekeeping genes were tested in S2-N and S2-$N^{\Delta EGF1-18}$ cells in the presence and absence of Wg. Only *Dfz2*, *ptc*, *sgg*, and *h* expression was affected in these experiments. Interestingly, the full-length N and N truncated in the amino terminus ($N^{\Delta EGF1-18}$), the two types of N molecules found associated with Wg in cultured cells and embryos, affected the expression

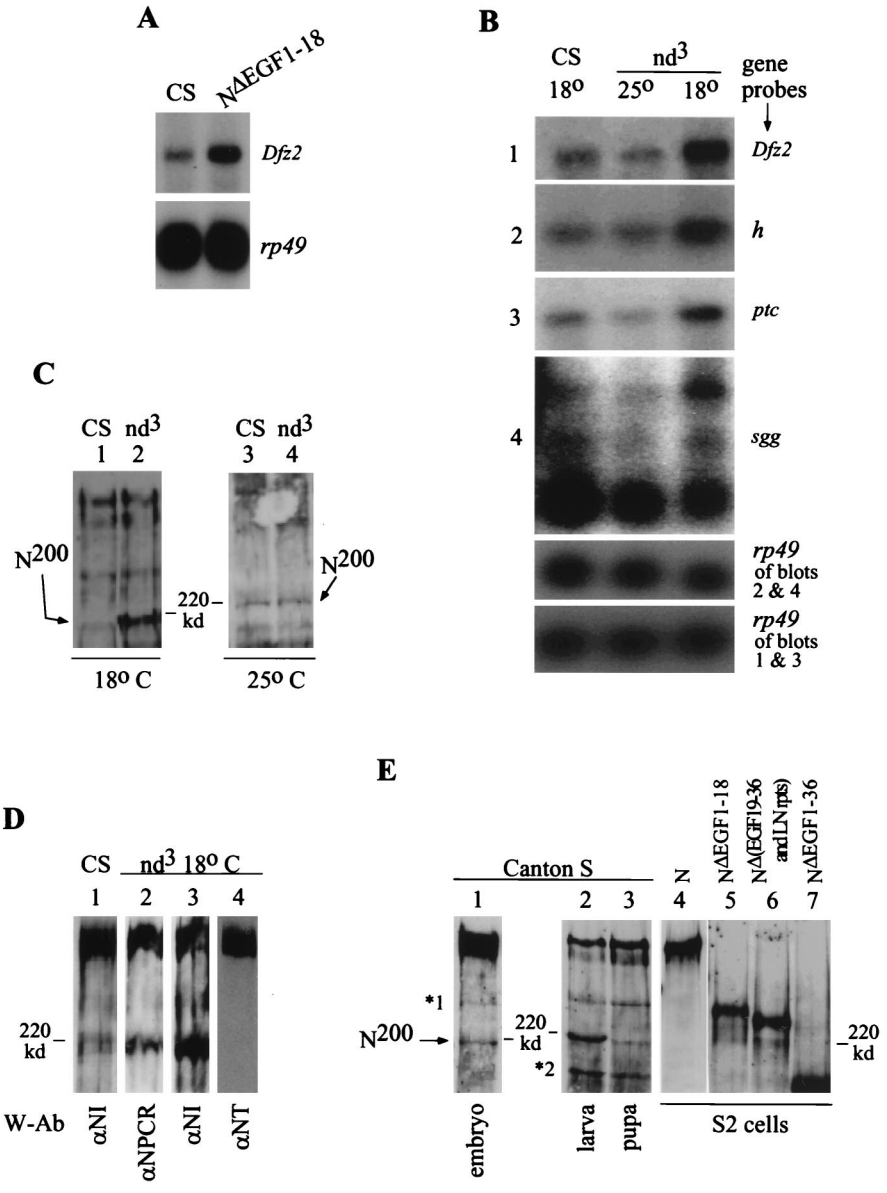
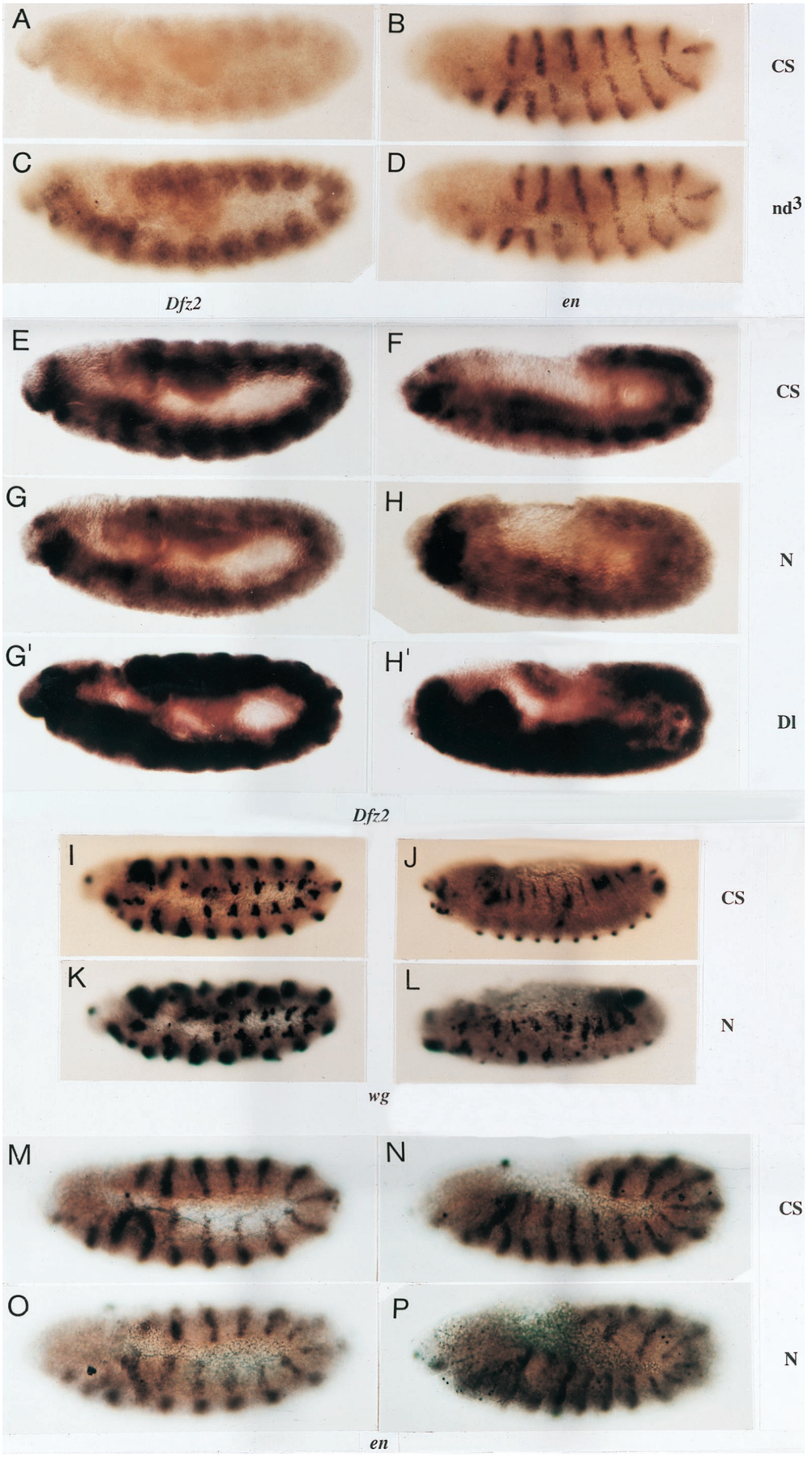


FIG. 6. $N^{\Delta EGF1-18}$ and nd^3 embryos show increased expression of genes regulated by $N^{\Delta EGF1-18}$ in vitro, and an endogenous form of N resembling $N^{\Delta EGF1-18}$ is overproduced in nd^3 embryos. (A) Embryos carrying the heat shock-inducible $N^{\Delta EGF1-18}$ transgene also overexpress *Dfz2*. We heat shocked 0- to 20-h Canton S (CS) (*yw* strain) and $N^{\Delta EGF1-18}$ (in Canton S, *yw* strain) embryos for 30 min, allowed them to recover at room temperature for 45 min, and extracted total RNAs for Northern blot analysis. *rp49* indicates relative levels of RNA in different lanes. (B) *Dfz2*, *h*, *ptc*, and *sgg* are overexpressed in nd^3 embryos at 18°C, the temperature at which the overt mutant phenotype is observed. At 25°C, expression of these genes in nd^3 does not differ from that in Canton S. Levels of expression in Canton S at 25 and 18°C are indistinguishable (expression at 18°C is shown). Total RNAs were extracted from 0- to 20-h Canton S and nd^3 embryos, reared at 25 or 18°C (with appropriate correction for developmental times), and analyzed by Northern blotting. Gene sequences used as probes are shown at the right. Exposure times: *rp*49 < *Dfz*2 < *ptc* < *sgg* < *h*. (C) nd^3 embryos at 18°C overproduce a ~200-kDa form of N, N^{200}. Because signals from high-molecular-weight forms of N interfere with assessment of levels of the less abundant N^{200}, total embryonic proteins extracted from 0- to 12-h Canton S and nd^3 embryos (at 18 or 25°C) were incubated with anti-NT and cleared prior to SDS-PAGE. Anti-NT does not react with N^{200} (see below). Extracts containing equivalent levels of ~350-kDa N were used for lanes 1 and 2. N is detected with anti-NI. (D) N^{200} is truncated in the amino terminus. N was immunoprecipitated from 0- to 12-h Canton S or nd^3 embryos (reared at 18°C) by using anti-NI and separated by SDS-PAGE (4% polyacrylamide), and the Western blots were probed with the indicated antibodies (W-Ab). See Fig. 4D for epitopes for N antibodies. nd^3 embryos were used to determine missing epitopes because they produce higher levels of N^{200} than do Canton S embryos (compare lanes 1 and 3). (E) Different developmental stages of Canton S flies express N^{200}, and N^{200} lacks more than 18 of the amino-terminal EGF-like repeats. Aliquots of total proteins extracted from Canton S embryos (0 to 3 h), one Canton S larva, one Canton S pupa, S2-N cells, S2-$N^{\Delta EGF1-18}$ cells, S2-$N^{\Delta EGF19-36}$ and LN rpts cells, and S2-$N^{\Delta EGF1-36}$ cells were separated by SDS-PAGE (4% polyacrylamide) and analyzed by Western blotting with anti-NI. *1 is recognized by all of the N antibodies studied and is therefore considered to be the partially denatured form of N (43); *2 is not recognized by anti-NT and anti-NPCR (not shown) but is recognized by anti-NI.

of these genes differently. Expression of full-length N in S2 cells did not affect expression of *Dfz2*, *ptc*, *sgg*, and *h* (Fig. 5A to D, lanes 3). However, expression of $N^{\Delta EGF1-18}$ in S2 cells strongly induced expression of these four genes, independent of any ligands (lanes 5). This difference indicated that the expression of N with a truncated extracellular domain results in ligand-independent induction of expression of *Dfz2*, *ptc*, *h*, and *sgg*. Treatment of S2-N cells with Wg induced or increased


A
B
CS
C
D
nd3
Dfz2
en
E
F
CS
G
H
N
G'
H'
Dl
Dfz2
I
J
CS
K
L
N
wg
M
N
CS
O
P
N
en

the expression of *ptc* and *sgg* (Fig. 5C and D, lanes 4). Treatment of S2-N$^{\Delta EGF1-18}$ cells, on the other hand, suppressed the expression of genes that were induced independently of ligands (Fig. 5A to D, lanes 6). Among the four genes, expression of *Dfz2* and *h* were affected solely by N$^{\Delta EGF1-18}$ (Fig. 5A and B). The level of *sgg* and *ptc* expression observed in S2-N cells treated with Wg is likely to be the net expression level due to an increase in expression promoted by the full-length N- and Wg-containing complex and to a decrease in expression promoted by the truncated N- and Wg-containing complex (also formed on S2-N$^{\Delta EGF1-18}$ cells). However, it is also possible that there is no contribution from the truncated N formed in S2-N cells, because Wg is always associated with it under the experimental conditions and prevented ligand-independent induction of gene expression.

S2-N cells do not express any other gene(s) known to bind Wg. However, S2-N$^{\Delta EGF1-18}$ cells express *Dfz2*, whose product is known to bind Wg (8). To determine whether the downregulation of genes in S2-N$^{\Delta EGF1-18}$ cells is due to N$^{\Delta EGF1-18}$ or Dfz2, *Dfz2*, *sgg*, *ptc*, and *h* expression were assessed in S2-N^{intra} cells in the presence and absence of Wg. S2-N^{intra} cells produce N without the extracellular domain (52). If association of Wg with N$^{\Delta EGF1-18}$ is required for suppression of the activities of these genes, the level of *Dfz2*, *sgg*, *ptc*, and *h* expression in S2-N^{intra} cells should not be altered by the presence of Wg in the medium. S2-N^{intra} cells were found to express these genes at lower levels than were S2-N$^{\Delta EGF1-18}$ cells. However, the levels of *Dfz2*, *sgg*, and *ptc* expression in these cells were not affected by Wg in the medium, and the level of *h* expression was only slightly reduced (Fig. 5E), indicating that the down regulation of gene expression in Fig. 5A to D, lanes 6, was due to association of Wg with N$^{\Delta EGF1-18}$. N^{intra} behaves as an activated N receptor with respect to Dl signaling (52, 82). Since N and N$^{\Delta EGF1-18}$ respond oppositely to Wg, and since N$^{\Delta EGF1-18}$ has a strong ligand-independent activity, N^{intra} activity shown in Fig. 5E is possibly a combination of N and N$^{\Delta EGF1-18}$ activities, with and without Wg.

The requirement of the extracellular domain of N$^{\Delta EGF1-18}$ for suppression of gene activities by Wg was also tested in a stable cell line transfected with N$^{\Delta EGF1-18}$ carrying the *Ax^{59d}* mutation. *Ax^{59d}* is a lethal allele of *N* because of a mutation in EGF-like repeat 24 (41). This allele manifests antineurogenic phenotypes and shows strong genetic interaction with *wg* (10, 15, 17, 18). Figure 4F shows that unlike S2-N$^{\Delta EGF1-18}$ cells, S2-N$^{\Delta EGF1-18/Ax59d}$ cells treated with Wg did not suppress expression of *Dfz2* (Fig. 5F). Thus, the region containing EGF-like repeat 24 appears to be important for Wg-mediated suppression of gene activities in S2-N$^{\Delta EGF1-18}$ cells. This region is as conserved as the Dl binding region of N (Fig. 1A).

To further clarify the roles of N$^{\Delta EGF1-18}$ and Dfz2 in suppression of gene expression in S2-N$^{\Delta EGF1-18}$ cells, the gene expression pattern was determined in S2-Dfz2 cells (pMK 33 S2-Dfz2 cells [8]). Expression of endogenous *Dfz2* cannot be assessed in this cell line because of induction of the transfected *Dfz2* gene through an ectopic promoter. Instead, the expression levels of genes that were coregulated with *Dfz2*, namely, *ptc*, *sgg*, and *h*, were determined with and without metal induction of *Dfz2* expression. S2-Dfz2 cells are responsive to Wg both with and without metal induction (8). The expression of *ptc*, *sgg*, and *h* in response to Wg was not reduced in S2-Dfz2 cells (Fig. 5G). The expression of these genes seemed to be slightly but consistently increased in response to Wg. Since Wg promotes the accumulation of Armadillo (Arm) in the cytoplasm of S2-Dfz2 cells (8), the cytoplasmic level of Arm in S2-N$^{\Delta EGF1-18}$ cells was also assessed to determine the level of Dfz2 activity in S2-N$^{\Delta EGF1-18}$ cells. The level of Arm in the cytoplasm of S2-N$^{\Delta EGF1-18}$ cells did not change in response to Wg (Fig. 5H, lanes 1 to 2). In several repetitions of the experiment, the response of S2-N$^{\Delta EGF1-18}$ cells was no different from that of S2 cells (lanes 5 and 6). On the other hand, S2-Dfz2 cells accumulated Arm in the cytoplasm (lanes 3 and 4).

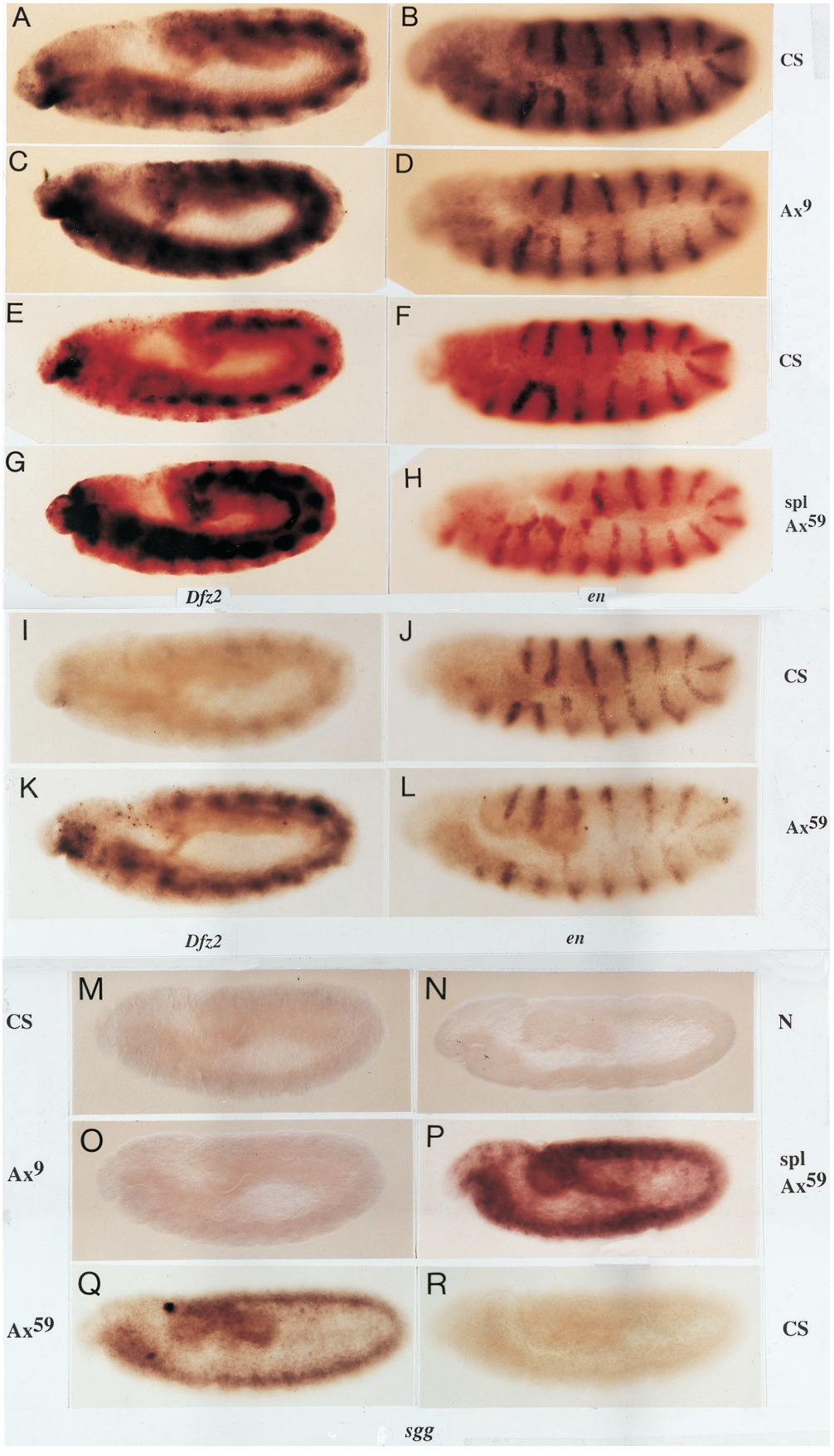
The experiments with S2-N^{intra}, S2-N$^{\Delta EGF1-18/Ax59d}$, and S2-Dfz2 cells indicated that the suppression of *Dfz2*, *ptc*, *sgg*, and *h* expression by S2-N$^{\Delta EGF1-18}$ cells in response to Wg was mediated by N$^{\Delta EGF1-18}$ rather than Dfz2. It is also likely that the increase in *ptc* and *sgg* expression in S2-N cells treated with Wg is due to N and not any other (unknown) receptor induced by N.

N lacking amino-terminal EGF-like repeats is associated with *Dfz2* expression in vivo. Full-length N and a truncated N were associated with Wg in S2 cells and embryos (Fig. 3 and 4). In S2 cells, the full-length N and the truncated N$^{\Delta EGF1-18}$ were active and responsive to Wg in different ways: N did not induce gene expression in the absence of ligands, whereas N$^{\Delta EGF1-18}$ did; N up regulated the expression of genes in response to Wg, whereas N$^{\Delta EGF1-18}$ down regulated the expression of genes in response to Wg (Fig. 5A to D). Thus, the two N-Wg complexes formed in embryos (Fig. 4) appear to have the potential to trigger different intracellular activities in vivo. To determine whether N$^{\Delta EGF1-18}$ shows the same activity in vivo that it showed in vitro, expression of *Dfz2* was assessed in embryos expressing the transgenic N$^{\Delta EGF1-18}$. *Dfz2* expression was assessed because in S2 cells it was regulated only by N$^{\Delta EGF1-18}$; S2-N cells did not induce or regulate *Dfz2* expression (Fig. 5A). Figure 6A shows that N$^{\Delta EGF1-18}$ embryos overexpress *Dfz2*, indicating that N$^{\Delta EGF1-18}$ behaves similarly in vitro and in vivo.

To find out whether endogenous N affects *Dfz2* expression in embryos, *Dfz2* RNA levels were compared between *N* mutant and Canton S embryos. Embryos of *nd^3*, a homozygous viable temperature-sensitive allele of *N* (75), were found to overexpress RNA of *Dfz2* and other genes coregulated with *Dfz2* in vitro, i.e., *ptc*, *sgg*, and *h*. These RNAs were overexpressed at 18°C (the restrictive temperature) but not at 25°C (Fig. 6B). The overexpression was specific, since the levels of RNAs of *wg*, *ac*, and the *E(spl)C* genes *m5* and *m8* were not increased in *nd^3* embryos at 18°C (data not shown; these genes were also not regulated by N or N$^{\Delta EGF1-18}$ in vitro). That a temperature-sensitive *N* allele accumulates *Dfz2* in a temperature-sensitive manner indicates that endogenous N also affects *Dfz2* expression in embryos.

Since (i) expression of *Dfz2* was associated only with N$^{\Delta EGF1-18}$ in S2 cell experiments, (ii) expression of N$^{\Delta EGF1-18}$ in embryos resulted in overexpression of *Dfz2*, and (iii) the mutation in *nd^3* is not in the region mediating suppression of *Dfz2* expression (Fig. 1A and 5), it was likely that *nd^3* embryos

FIG. 7. Expression of *Dfz2* is reduced in zygotic *N$^-$* embryos, while expression of *en* and *wg* are unaffected. (A to D) In situ hybridization of Canton S and *nd^3* embryos corroborates results from Northern blotting that *Dfz2* is overexpressed in *nd^3* embryos but *en* is not (E to H, G′, H′) *Dfz2* expression is lost in *N$^{264-47}$/Y* embryos but not in comparable stages of Canton S or *Dl^X/Dl^X* embryos. (I to L) *wg* expression is not lost in *N$^{264-47}$/Y*. (M to P) *en* expression is similar in Canton S and *N$^{264-47}$/Y* embryos. CS, Canton S; nd^3, *nd^3*; N, *N$^{264-47}$/Y*; Dl, *Dl^X/Dl^X* embryos; genes used as probes are indicated below the appropriate sets of embryos. Anterior is to the left of each embryo. *N$^{264-47}$/Y* and *Dl^X/Dl^X* embryos were identified by lack of β-galactosidase staining associated with the FM7 or TM6 balancer chromosomes (see Materials and Methods). Embryos A to D were processed simultaneously, and so were embryos E to P.


A
B
CS
C
D
Ax9
E
F
CS
G
H
spl
Ax59
Dfz2
en
I
J
CS
K
L
Ax59
Dfz2
en
CS
M
N
N
Ax9
O
P
spl
Ax59
Ax59
Q
R
CS
sgg

overproduced the endogenous form of N affecting *Dfz2* expression in vivo. Western blotting analyses revealed that nd^3 embryos indeed produced higher than Canton S levels of a ~200-kDa form of N. Just like expression of the *Dfz2*, *ptc*, *sgg*, and *h* genes, this ~200-kDa form, designated N^{200}, was overexpressed in nd^3 embryos at 18 but not 25°C (Fig. 6C). *spl*, $N^{264\text{-}47}$, *spl* Ax^{59d}, Ax^{59d}, Ax^{9B}, and other *N* alleles did not show increased level of N^{200} (data not shown). N^{200} lacks the amino-terminal EGF-like repeats, since it is not recognized by anti-NT (Fig. 6D). It is produced at low levels in embryos (relative to the full-length ~350-kDa form of N) but is expressed at much higher levels in larvae and pupae (Fig. 6E, lanes 1 to 3). N^{200} migrates between $N^{\Delta EGF1-18}$ and $N^{\Delta EGF1-36}$ (lanes 4 to 7), indicating that it is lacking more than 18 of the amino-terminal EGF-like repeats but includes a significant fraction of the carboxy-terminal half of the EGF-like repeats. The association of overexpression of *Dfz2* with overexpression of N^{200} in nd^3 embryos suggests that N^{200} is the form of N that affects *Dfz2* expression in vivo. A fine-scale developmental analysis indicated that the full-length N was also overexpressed in nd^3 embryos at certain periods of embryogenesis (data not shown). Therefore, overexpression of *Dfz2* in nd^3 embryos, and possibly that of *h* as well, is most probably due to overproduction of N^{200} (since full-length N is not associated with *Dfz2* expression in vitro) and overexpression of *ptc* and *sgg* is most probably due to overproduction of both N and N^{200} (since both N and $N^{\Delta EGF1-18}$ regulate *ptc* and *sgg* in vitro [Fig. 5A to D]). nd^3 embryos show the same level and size of *N* RNA as Canton S embryos do (data not shown). Thus, it appears that EGF-like repeat 2 (the site of mutation in the nd^3 allele [Fig. 1A]) is important for posttranslational production of N^{200} from the full-length N or for regulation of the levels of full-length N during development. However, the actual mechanism of generation of the truncated N^{200} is unknown.

If only heterodimeric N receptors are present on cell surfaces of S2 cells and embryos (9, 53, 63; see also reference 43), the cross-linkers used in immunoprecipitations must have covalently linked the two fragments composing each of the three cell surface receptors, i.e., the full length N, $N^{\Delta EGF1-18}$, and N^{200}, and their ligands (Fig. 3 and 4). The reported ~110-kDa intracellular product is not retained in the gels used in Fig. 6C to E, and the extracellular product would not be recognized by anti-NI or anti-NPCR (Fig. 4D).

N^- and Ax mutant embryos show altered expression patterns of epidermal patterning genes that are consistent with expression patterns observed in vitro. A further test of involvement of N in expression of cuticle-patterning genes in vivo would be that N^- and *Ax* embryos (which do not overproduce N^{200}) show low and high expression, respectively, of the same genes. Since N^- and *Ax* alleles are homozygous lethal, in situ hybridization rather than Northern blotting was used. In situ hybridization of nd^3 embryos with *Dfz2* and *en* probes (the latter serving as a control for levels of RNA in embryo) show that the results are qualitatively comparable to results obtained by Northern blotting (Fig. 7A to D).

Stage-specific comparisons of Canton S and zygotic N^- or *Ax* embryos showed that levels of *Dfz2* and *sgg* RNA are indeed as expected from in vitro results. $N^{264\text{-}47}/Y$ embryos expressed lower levels of *Dfz2* RNA than did Canton S embryos and Dl^X embryos (Fig. 7E to H). The levels of *wg* and *en* RNA did not differ significantly between $N^{264\text{-}47}/Y$ and Canton S embryos (Fig. 7I to L, *wg* M-P, *en*), indicating that the loss of *Dfz2* transcripts is not due to a general suppression of RNA accumulation in $N^{264\text{-}47}/Y$ embryos or loss of *wg* expression. Conversely, Ax^{59d}, Ax^{9B} (both carrying mutations in EGF-like repeat 24 [41]), and *spl* Ax^{59d} embryos overproduced *Dfz2* RNA but not *en* RNA (Fig. 8A to L; *spl* embryos do not overexpress *Dfz2* [data not shown]). A similar pattern of expression was manifest with *sgg* as well (Fig. 8M to R).

DISCUSSION

N Regions within EGF-like repeats 19 to 36 mediate interactions with Wg. Wg was identified as a putative ligand of N in a cell surface screen with phagemids carrying cDNA sequences from *Drosophila* embryos. Further analyses showed that Wg and N form molecular complexes, both in vitro and in vivo. EGF-like repeats 19 to 36 of N are required for the association of these two proteins (Fig. 2 to 4). These repeats of N include two strongly conserved regions, those containing EGF-like repeats 23 to 27 and EGF-like repeats 31 to 34 (Fig. 1A). Experiments with the Ax^{59d} allele (Fig. 5F and 8) showed that EGF-like repeat 24 is important for Wg-mediated down regulation of gene expression through $N^{\Delta EGF1-18}$. N^{200} is identified as the in vivo equivalent of $N^{\Delta EGF1-18}$. Since the nd^3 embryos (overproducing N^{200}), Ax^{59d} embryos, and S2-$N^{\Delta EGF1-18/Ax59d}$ cells overexpress the same genes down regulated by Wg and $N^{\Delta EGF1-18}$ (Fig. 5F and 8), the region containing EGF-like repeats 23 to 27 might be involved in down regulation of gene expression by Wg and N molecules in vivo. The evolutionary conservation of this region in homologous N molecules might be due to the Wg-associated functions of N. Whether Wg associates with the same region in full-length N, for induction of *sgg* and *ptc* expression, is not known. Preliminary results suggest that Wg associates at a second site within the region containing EGF-like repeats 19 to 36 of N.

Two forms of Notch regulate expression of epidermal patterning genes. Immunoprecipitations from cultured cells and embryos recovered Wg complexed with the full-length N and a form of N lacking EGF-like repeats in the amino terminus (Fig. 3 and 4). In vitro experiments showed that two forms of N regulate expression of cuticle-patterning genes, i.e., *sgg* and *ptc* by the full-length N in response to Wg, and *Dfz2*, *sgg*, *h*, and *ptc* by a form of N lacking 18 amino-terminal EGF-like repeats, $N^{\Delta EGF1-18}$, both independent of ligands and in response to Wg. Thus, Wg behaves as a ligand for two different forms of N in vitro, eliciting different responses from cells expressing these two forms of N. The significant difference between N and $N^{\Delta EGF1-18}$ is the lack of the Dl binding region in $N^{\Delta EGF1-18}$. This appears to be true of N^{200} as well. N and N^{200} might therefore regulate genes in vivo in a manner comparable to gene regulations by N and $N^{\Delta EGF1-18}$ in vitro. The in vivo relative levels of the full-length N (capable of associating with Dl and Wg) and N^{200} (capable of associating with Wg) may therefore represent a differential commitment of N to Dl or Wg signaling during embryogenesis and a differential commit-

FIG. 8. *Ax* embryos overproduce *Dfz2* and *sgg* RNA. (A to L) *Ax* embryos overproduce *Dfz2* RNA (A, C, E, G, I, and K) but not *en* RNA (B, D, F, H, J, and L). (M to R) *Ax* embryos overproduce *sgg* RNA (O to Q) but not Canton S (M and R) and $N^{264\text{-}47}/Y$ embryos (N). Due to low-level of expression in a general pattern, reduced *sgg* expression (as in panel N) and weak *sgg* overexpression (as in panel O) are more obvious in pools of embryos than in individual embryos. CS, Canton S; Ax^9, Ax^{9B}/Y; spl Ax^{59}, *spl* Ax^{59d}/Y; Ax^{59}, Ax^{59d}/Y; N, $N^{264\text{-}47}/Y$. Anterior is to the left of each embryo. Homozygous *Ax* or *N* embryos were identified by the lack of β-galactosidase staining associated with the FM7 balancer chromosomes (see Materials and Methods). Embryos A to H and M to P were processed simultaneously, and so were embryos I to L and Q to R.

ment of N to different kinds of Wg signaling. Since *N* is required not only for different developmental functions but also for sequential developmental functions (13, 76), it is quite possible that N and N^{200} constitute important components of the mechanism of *N* function at successive steps of differentiation. There is some indication that the activity of $N^{\Delta EGF1-18}$ requires the activity of N in a preceding step: expression of $N^{\Delta EGF1-18}$ in N^{264-47}/Y embryos prior to onset of the neurogenic phenotype results in overexpression of *h* and *sgg* (as expected, in the expected stage-specific pattern for *h*), but this does not occur in the neurogenic embryos (data not shown).

The intracellular pathways associated with transduction of signals by N and $N^{\Delta EGF1-18}$ do not appear to involve the expression of the *m5* and *m8* genes of *E(spl)C* that are associated with activation of N by Dl (26, 33, 39, 48, 85) or Wg-mediated stabilization of Arm in the cytoplasm (Fig. 5H) observed with Wg and Dfz2 receptor (8, 34, 60, 65). Therefore, novel pathways appear to be transducing signals to the nucleus of S2-$N^{\Delta EGF1-18}$ or S2-N cells, in both the presence and absence of Wg.

In conclusion, the strong genetic interaction between *N* and *wg* functions during *Drosophila* development (10, 15, 16, 19, 21, 44, 58, 59, 72) could be due to regulation of *wg* expression by N (19, 72), suppression of lateral inhibition signaling of N by Dsh (3), and physical associations of Wg with the two forms of N for the purpose of regulation of cuticle patterning genes. Regulation of negative regulators of *en* and *ac* expression by Wg, namely, *sgg* and *h*, respectively (38, 62, 65, 78, 79, 84), and the involvement of two different forms of N with different activities could explain previous contradictory results regarding the role of N in Wg signaling. $N^{\Delta EGF1-18}$ induces the expression of *sgg* and *h* in S2 cells (Fig. 5B and D). Loss of N and therefore loss of N^{200} (the putative in vivo equivalent of $N^{\Delta EGF1-18}$) would result in loss of *sgg* and *h* expression, leading to loss of inhibition of *en* and *ac* expression, consistent with the results of Rulifson and Blair (72) and Cadigan and Nusse (12). On the other hand, overexpression of *sgg* (Fig. 8M to R) and *h* (not shown) in *Ax* mutants could interfere with the stabilization of *en* and *ac* expression, consistent with the results of Couso and Martinez-Arias (15).

ACKNOWLEDGMENTS

I thank Michael Young for his support, advice, experimental suggestions, and review of the manuscript. I thank Lino Saez for research materials, technical advice, and critical assessment of data; Alfonso Martinez-Arias for research materials, advice, encouragement, and review of the manuscript; Toby Lieber for research materials and suggestions; Simon Kidd, Frieda Reichsman, Susan Cumberledge, Stephen Pronovost, Roel Nusse, Armen Manoukian, Vuk Stambolic, Laurent Ruel, Mark Muskavitch, Keiko Sawai, Claude Desplan, Anthony Brown, and Michael Caudy for research materials; and Amy Bejsovec for review of the manuscript.

This work was supported by NIH grant GM 25103 to Michael W. Young.

REFERENCES

1. **Alton, A. K., K. Fechtel, C. C. Kopczynski, S. B. Shepard, P. J. Kooh, and M. A. T. Muskavitch.** 1989. Molecular genetics of *Delta*, a locus required for ectodermal differentiation in *Drosophila*. Dev. Genet. **10:**261–272.
2. **Artavanis-Tsakonas, S., K. Matsuno, and M. E. Fortini.** 1995. Notch signaling. Science **268:**225–232.
3. **Axelrod, J. D., K. Matsuno, S. Artavanis-Tsakonas, and N. Perrimon.** 1996. Interaction between Wingless and Notch signaling pathways mediated by Dishevelled. Science **271:**1826–1832.
4. **Baker, N. E.** 1987. Molecular cloning of sequences from *wingless*, a segment polarity gene in *Drosophila*: the spatial distribution of a transcript in embryos. EMBO J. **6:**1765–1773.
5. **Baker, N. E., M. Mlodzik, and G. M. Rubin.** 1990. Spacing differentiation in the developing *Drosophila* eye: a fibrinogen-related lateral inhibitor encoded by *scabrous*. Science **250:**1370–1377.
6. **Bate, M., E. Rushton, and M. Frash.** 1993. A dual requirement for neurogenic genes in *Drosophila* myogenesis. Dev. Suppl. **119:**149–161.
7. **Bejsovec, A., and A. Martinez-Arias.** 1991. Roles of *wingless* in patterning the larval epidermis of *Drosophila*. Development **113:**471–485.
8. **Bhanot, P., M. Brink, C. H. Samos, J.-C. Hsieh, Y. Wang, J. P. Macke, D. Andrew, J. Nathans, and R. Nusse.** 1996. A new member of the *frizzled* family from *Drosophila* functions as a Wingless receptor. Nature **382:**225–230.
9. **Blaumueller, C. M., H. Qi, P. Zagouras, and S. Artavanis-Tsakonas.** 1997. Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell **90:**281–291.
10. **Brennan, K., R. Tateson, K. Lewis, and A. Martinez-Arias.** 1997. A functional analysis of *Notch* mutations in *Drosophila*. Genetics **147:**177–188.
11. **Cabrera, C. V.** 1990. Lateral inhibition and cell fate during neurogenesis in *Drosophila*: the interactions between *scute*, *Notch* and *Delta*. Development **109:**733–742.
12. **Cadigan, K. M., and R. Nusse.** 1996. *wingless* signaling in the *Drosophila* eye and embryonic epidermis. Development **122:**2801–2812.
13. **Cagan, R. L., and D. F. Ready.** 1989. *Notch* is required for successive cell decisions in the developing *Drosophila* retina. Genes Dev. **3:**1099–1112.
14. **Corbin, V., A. M. Michelson, S. M. Abmayr, V. Neel, E. Alcamo, T. Maniatis, and M. W. Young.** 1991. A role for the *Drosophila* neurogenic genes in mesoderm differentiation. Cell **67:**311–323.
15. **Couso, J. P., and A. Martinez-Arias.** 1994. *Notch* is required for *wingless* signaling in the epidermis of *Drosophila*. Cell **79:**259–272.
16. **Couso, J. P., E. Knust, and A. Martinez-Arias.** 1995. *Serrate* and *wingless* cooperate to induce *vestigial* gene expression and wing formation in *Drosophila*. Curr. Biol. **5:**1437–1448.
17. **de Celis, J. F., and A. Garcia-Bellido.** 1994. Modifications of the Notch function by *Abruptex* mutations in *Drosophila melanogaster*. Genetics **136:**183–194.
18. **de Celis, J. F., R. Barrio, A. del Arco, and A. Garcia-Bellido.** 1993. Genetic and molecular characterization of a Notch mutation in its Delta- and Serrate-binding domain in *Drosophila*. Proc. Natl. Acad. Sci. USA **90:**4037–4041.
19. **Diaz-Benjumea, F. J., and S. M. Cohen.** 1995. Serrate signals through *Notch* to establish a *wingless*-dependent organizer at the dorsal/ventral compartment boundary of the *Drosophila* wing. Development **121:**4215–4225.
20. **DiNardo, S., E. Sher, J. Heemskerk-Jongens, J. A. Kassis, and P. A. O'Farrell.** 1988. Two-tiered regulation of spatially patterned *engrailed* gene expression during drosophila embryogenesis. Nature **332:**604–609.
21. **Doherty, D., G. Feger, S. Younger-Sheperd, L. Y. Jan, and Y. N. Jan.** 1996. Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in *Drosophila* wing formation. Genes Dev. **10:**421–434.
22. **Doherty, D., L. Y. Jan, and Y. N. Jan.** 1997. The *Drosophila* neurogenic gene big brain, which encodes a membrane-associated protein, acts cell autonomously and can act synergistically with Notch and Delta. Development **124:**3881–3893.
23. **Ellisen, L. W., J. Bird, D. C. West, A. L. Soreng, T. C. Reynolds, S. D. Smith, and J. Sklar.** 1991. Tan-1, the human homologue of the *Drosophila* Notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell **66:**649–661.
24. **Fehon, R. G., P. J. Kooh, I. Rebay, C. L. Regan, T. Xu, M. Muskavitch, and S. Artavanis-Tsakonas.** 1990. Molecular interaction between the protein products of the neurogenic loci *Notch* and *Delta*, two EGF-homologous genes in Drosophila. Cell **61:**523–534.
25. **Fleming, R. J., T. N. Scottgale, R. J. Diedrich, and S. Artavanis-Tsakonas.** 1990. The gene *Serrate* encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in *Drosophila melanogaster*. Genes Dev. **4:**2188–2201.
26. **Fortini, M. E., and S. Artavanis-Tsakonas.** 1994. The suppressor of Hairless protein participates in Notch receptor signaling. Cell **79:**273–282.
27. **Genetics Computer Group.** 1993. Manual for the Wisconsin package, version 8. Genetic Computer Group, Madison, Wis.
28. **Greenwald, I., and G. M. Rubin.** 1992. Making a difference: The role of cell-cell interactions in establishing separate identities for equivalent cells. Cell **68:**271–281.
29. **Gu, Y., N. A. Hukriede, and R. J. Fleming.** 1995. *Serrate* expression can functionally replace *Delta* activity during neuroblast segregation in the *Drosophila* embryo. Development **121:**855–865.
30. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Heberlein, U. I. K. Hariharan, and G. M. Rubin.** 1993. *Star* is required for neuronal differentiation in the *Drosophila* retina and displays dosage-sensitive interaction with Ras 1. Dev. Biol. **160:**51–63.
32. **Heitzler, P., and P. Simpson.** 1991. The choice of cell fate in the epidermis of *Drosophila*. Cell **64:**1083–1092.
33. **Heitzler, P., M. Bourouis, L. Ruel, C. Carteret, and P. Simpson.** 1996. Genes of the *Enhancer of split* and *achaete-scute* complexes are required for a regulatory loop between *Notch* and *Delta* during lateral signalling in *Drosophila*. Development **122:**161–171.
34. **Heslip, T. R., H. Theisen, H. Walker, and J. L. Marsh.** 1997. SHAGGY and DISHEVELLED exert opposite effects on *wingless* and *decapentaplegic* ex-

pression and on positional identity in imaginal discs. Development **124:**1069–1078.
35. **Hooper, J. E., and M. P. Scott.** 1989. The Drosophila *patched* gene encodes a putative membrane protein required for segmental patterning. Cell **59:**751–765.
36. **Hoppe, P. E., and R. J. Greenspan.** 1990. The *Notch* locus of *Drosophila* is required in epidermal cells for epidermal development. Development **109:**875–885.
37. **Ish-Horowicz, D., K. R. Howard, S. M. Pinchin, and P. W. Ingham.** 1985. Molecular and genetic analysis of the hairy locus in *Drosophila*. Cold Spring Harbor Symp. Quant. Biol. **50:**135–144.
38. **Ishibashi, M., S.-L. Ang, K. Shiota, S. Nakanishi, R. Kageyama, and F. Guillemot.** 1995. Targeted disruption of mammalian *hairy* and *Enhancer of split* homolog-1 (*HES-1*) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev. **9:**3136–3148.
39. **Jennings, B., J. de Celis, C. Delidakis, A. Preiss, and S. Bray.** 1995. Role of *Notch* and *achaete-scute* complex in the expression of *Enhancer of split* bHLH proteins. Development **121:**3745–3752.
40. **Johnston, S. H., C. Rauskolb, R. Wilson, B. Prabhakaran, K. D. Irvine, and T. F. Vogt.** 1997. A family of mammalian Fringe genes implicated in boundary determination and the Notch pathway. Development **124:**2245–2254.
41. **Kelley, M. R., S. Kidd, W. A. Deutsch, and M. W. Young.** 1987. Mutations altering the structure of epidermal growth factor-like coding sequences at the *Drosophila Notch* locus. Cell **51:**539–548.
42. **Kidd, S., M. R. Kelley, and M. W. Young.** 1986. Sequence of the Notch locus of *Drosophila melanogaster*: relationship of the encoded protein to mammalian clotting and growth factors. Mol. Cell. Biol. **6:**3094–3108.
43. **Kidd, S., M. K. Baylies, G. P. Gasic, and M. W. Young.** 1989. Structure and distribution of the Notch protein in developing *Drosophila*. Genes Dev. **3:**1113–1129.
44. **Klein, T., and A. Martinez-Arias.** 1998. Different spatial and temporal interactions between *Notch*, *wingless*, and *vestigial* specify proximal and distal pattern elements of the wing in *Drosophila*. Dev. Biol. **194:**196–212.
45. **Knust, E., U. Dietrich, U. Tepass, K. A. Bremer, D. Weigel, H. Vassin, and J. A. Campos-Ortega.** 1987. EGF homologous sequences encoded in the genome of *Drosophila melanogaster* and their relation to neurogenic genes. EMBO J. **6:**761–766.
46. **Kopczynski, C. C., A. K. Alton, K. Fechtel, P. J. Kooh, and M. A. T. Muskavitch.** 1988. *Delta*, a *Drosophila* neurogenic gene, is transcriptionally complex and encodes a protein related to blood coagulation factors and epidermal growth factor of vertebrates. Genes Dev. **2:**1723–1735.
47. **LaBonne, S. G., I. Sunitha, and A. P. Mahowald.** 1989. Molecular genetics of *pecanex*, a maternal-effect neurogenic locus of *Drosophila melanogaster* that potentially encodes a large transmembrane protein. Dev. Biol. **136:**1–16.
48. **Lecourtois, M., and F. Schweisguth.** 1995. The neurogenic Suppressor of Hairless DNA-binding protein mediates the transcriptional activation of the *Enhancer of split Complex* genes triggered by Notch signaling. Genes Dev. **9:**2598–2608.
49. **Lee, J. J., D. P. von Kessler, S. Parks, and P. A. Beachy.** 1992. Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene *hedgehog*. Cell **71:**33–50.
50. **Lehmann, R., F. Jimenez, U. Dietrich, and J. A. Campos-Ortega.** 1983. On the phenotype and development of mutants of early neurogenesis in *Drosophila melanogaster*. Roux's Arch. Dev. Biol. **192:**62–74.
51. **Lieber, T., C. S. Wesley, E. Alcamo, B. Hassel, J. F. Krane, J. A. Campos-Ortega, and M. W. Young.** 1992. Single amino acid substitutions in EGF-like elements of Notch and Delta modify *Drosophila* development and affect cell adhesion in vitro. Neuron **9:**847–859.
52. **Lieber, T., S. Kidd, E. Alcamo, V. Corbin, and M. W. Young.** 1993. Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. Genes Dev. **7:**1949–1965.
53. **Logeat, F., C. Bessia, C. Brou, O. LeBail, S. Jarriault, N. G. Seidah, and A. Israel.** 1998. The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc. Natl. Acad. Sci. USA **95:**8108–8112.
54. **Lyman, D., and M. W. Young.** 1993. Further evidence for function of the *Drosophila* Notch protein as a transmembrane receptor. Proc. Natl. Acad. Sci. USA **90:**10395–10399.
55. **Marks, J. D., W. H. Ouwehand, J. M. Bye, R. Finnern, B. D. Gorick, D. Voak, S. J. Thorpe, N. C. Hughes-Jones, and G. Winter.** 1993. Human antibody fragments specific for human blood group antigens from a phage display library. Bio/Technology **11:**1145–1149.
56. **Mlodzik, M., N. E. Baker, and G. M. Rubin.** 1990. Isolation and expression of *scabrous*, a gene regulating neurogenesis in *Drosophila*. Genes Dev. **4:**1848–1861.
57. **Nakano, Y., I. Guerrero, A. Hidalgo, A. Taylor, J. R. S. Whittle, and P. W. Ingham.** 1989. A protein with several possible membrane-spanning domains encoded by the *Drosophila* segment polarity gene *patched*. Nature **341:**508–513.
58. **Neumann, C. J., and S. M. Cohen.** 1996. A hierarchy of cross-regulation involving *Notch*, *wingless*, *vestigial*, and *cut* organizes the dorsal/ventral axis of the *Drosophila* wing. Development **122:**3477–3485.
59. **Ng, M., F. J. Diaz-Benjumea, J.-P. Vincent, J. Wu, and S. M. Cohen.** 1996. Specification of the wing by localized expression of *wingless* protein. Nature **381:**316–318.
60. **Noordermeer, J., J. Kligensmith, N. Perrimon, and R. Nusse.** 1994. *dishevelled* and *armadillo* act in the wingless signaling pathway in *Drosophila*. Nature **367:**80–83.
61. **O'Connell, P., and M. Rosbash.** 1984. Sequence, structure, and codon preference of the *Drosophila* ribosomal protein 49 gene. Nucleic Acids Res. **12:**5495–5513.
62. **Ohsako, S., J. Hyer, G. Panganiban, I. Oliver, and M. Caudy.** 1994. hairy function as a DNA-binding helix-loop-helix repressor of *Drosophila* sensory organ formation. Genes Dev. **8:**2743–2755.
63. **Pan, D., and G. Rubin.** 1997. Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during *Drosophila* and vertebrate neurogenesis. Cell **90:**271–280.
64. **Panin, V. M., V. Papayannopoulos, R. Wilson, and K. D. Irvine.** 1997. Fringe modulates Notch-ligand interactions. Nature **387:**908–912.
65. **Peifer, M., D. Sweeton, M. Casey, and E. Wieschaus.** 1994. *wingless* and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo. Development **120:**369–380.
66. **Poulson, D.** 1937. Chromosomal deficiencies and embryonic development of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA **23:**133–137.
67. **Rao, Yi., L. Y. Jan, and Y. N. Jan.** 1990. Similarity of the product of the *Drosophila* neurogenic gene *big brain* to transmembrane channel proteins. Nature **345:**163–167.
68. **Rebay, I., R. J. Fleming, R. G. Fehon, L. Cherbas, P. Cherbas, and S. Artavanis-Tsakonas.** 1991. Specific repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. Cell **67:**687–699.
69. **Reichsman, F., L. Smith, and S. Cumberledge.** 1996. Glycosaminoglycans can modulate extracellular localization of the *wingless* protein and promote signal transduction. J. Cell Biol. **135:**819–827.
70. **Rothberg, J. M., D. A. Hartley, Z. Walther, and S. Artavanis-Tsakonas.** 1988. slit: an EGF-homologous locus of *Drosophila melanogaster* involved in the development of the embryonic central nervous system. Cell **55:**1047–1059.
71. **Ruel, L., M. Bourouis, P. Heitzler, V. Pantesco, and P. Simpson.** 1993. Drosophila *shaggy* kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of *Notch*. Nature **362:**557–560.
72. **Rulifson, E. J., and S. S. Blair.** 1995. Notch regulates *wingless* expression and is not required for reception of the paracrine *wingless* signal during wing margin neurogenesis in *Drosophila*. Development **121:**2813–2824.
73. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
74. **Scott, J. K., and G. P. Smith.** 1990. Searching for peptide ligands with an epitope library. Science **249:**386–390.
75. **Shellenbarger, D. L., and J. D. Mohler.** 1975. Temperature sensitive mutations of the Notch locus in *Drosophila melanogaster*. Genetics **81:**143–162.
76. **Shellenbarger, D. L., and J. D. Mohler.** 1978. Temperature-sensitive periods and autonomy of pleiotropic effects of $l(1)N^{ts1}$, a conditional Notch lethal in *Drosophila*. Dev. Biol. **62:**432–446.
77. **Siegfried, E., L. A. Perkins, T. M. Capaci, and N. Perrimon.** 1990. Putative protein kinase product of the *Drosophila* segment polarity gene *zeste-white*3. Nature **345:**825–829.
78. **Siegfried, E., T.-B. Chou, and N. Perrimon.** 1992. *wingless* signaling acts through *zeste-white* 3, the *Drosophila* homolog of *glycogen synthase kinase*-3, to regulate *engrailed* and establish cell fate. Cell **71:**1167–1179.
79. **Skeath, J. B., and S. B. Carroll.** 1991. Regulation of *achaete-scute* gene expression and sensory organ pattern formation in the *Drosophila* wing. Genes Dev. **5:**984–995.
80. **Staros, J. V., and B. P. Kakkad.** 1983. Crosslinking and chymotropic digestion of the extracytoplasmic domain of the anion exchange channel in intact human erythrocytes. J. Membr. Biol. **74:**247–254.
81. **Stone, D. M., M. Hynes, M. Armanini, T. A. Swanson, Q. Gu, R. L. Johnson, M. P. Scott, D. Pennica, A. Goddard, H. Phillips, M. Noll, J. E. Hooper, F. de Sauvage, and A. Rosenthal.** 1996. The tumor-suppressor gene *patched* encodes a candidate receptor for sonic hedgehog. Nature **384:**129–134.
82. **Struhl, G., K. Fitzgerald, and I. Greenwald.** 1993. Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. Cell **74:**331–345.
83. **Tepass, U., and V. Hartenstein.** 1995. Neurogenic and proneural genes control cell fate specification in the *Drosophila* endoderm. Development **121:**393–405.
84. **Van Doren, M., A. M. Bailey, J. Esnayra, K. Ede, and J. W. Posakony.** 1994. Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of achaete. Genes Dev. **8:**2729–2742.
85. **Vassin, H., J. Vielmetter, and J. A. Campos-Ortega.** 1985. Genetic interactions in early neurogenesis of *Drosophila melanogaster*. J. Neurogenet. **2:**291–308.
86. **Vassin, H., K. A. Bremer, E. Knust, and J. A. Campos-Ortega.** 1987. The neurogenic gene Delta of *Drosophila melanogaster* is expressed in neurogenic territories and encodes a putative transmembrane protein with EGF-like repeats. EMBO J. **6:**3431–3440.

87. **Waugh, S. M., E. E. DiBella, and P. F. Pilch.** 1989. Isolation of a proteolytically derived domain of the insulin receptor containing the major site of crosslinking/binding. Biochemistry **28:**3448–3455.
88. **Wharton, K. A., K. Johansen, T. Xu, and S. Artavanis-Tsakonas.** 1985. Nucleotide sequence from the neurogenic locus Notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell **43:**567–581.
89. **Wilcox, M.** 1986. Cell surface antigens, p. 243–276. *In* D. B. Roberts (ed.), *Drosophila*, a practical approach. IRL Press, Oxford, United Kingdom.
90. **Wright, T. R. F.** 1970. The genetics of embryogenesis in *Drosophila*. Adv. Genet. **15:**261–395.
91. **Xu, T., L. A. Caron, R. G. Fehon, and S. Artavanis-Tsakonas.** 1992. The involvement of the *Notch* locus in *Drosophila* oogenesis. Development **115:** 913–922.