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Multiple Functions of a *Drosophila* Homeotic Gene, *zeste-white 3*, during Segmentation and Neurogenesis

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Lack of both maternal and zygotic gene activity at the *zeste-white 3* (*zw3*) locus causes severe developmental transformations. Embryos derived from germ cells that lack $zw3^+$ gene activity die during embryogenesis and have a phenotype that is similar to that of embryos mutant for the segment polarity gene *naked* (*nkd*). In both *nkd* and germ line clone-derived *zw3* embryos the pattern elements derived from the anterior-most part of each segment, the denticle belts, are deleted. Similar abnormal patterns of the zygotically expressed genes *engrailed* and *Ultrabithorax* are detected in both mutants, suggesting that the two genes are involved in the same developmental process. Additionally, the induction of clones of *zw3* mutant cells in imaginal discs causes homeotic transformations of noninnervated hair cells into innervated sensory bristles. The multiple roles of *zw3* during development and its possible interactions with the zygotic gene *nkd* are discussed.

INTRODUCTION

Embryonic development is directed by two separate but interacting pools of information molecules. The first pool consists of gene products expressed maternally and stored in the oocyte; the second consists of gene products derived from zygotic gene expression (see reviews by Konrad *et al.*, 1985; Akam, 1987; Perrimon and Mahowald, 1988). That these two pools of information molecules interact is made evident by the discovery that mutations in maternally and zygotically acting genes can produce identical phenotypes (Nusslein-Volhard and Wieschaus, 1980; Nusslein-Volhard *et al.*, 1984, 1987; Wieschaus *et al.*, 1984; Jurgens *et al.*, 1984; Gans *et al.*, 1975; Mohler, 1977; Perrimon *et al.*, 1986; Schupbach and Wieschaus, 1986). For example, the embryonic phenotype associated with the *tudor*-like maternal effect loci resembles the phenotype of the embryonic lethal *knirps* mutation (Nusslein-Volhard and Wieschaus, 1980, Nusslein-Volhard *et al.*, 1987). Likewise the maternal effects of *torso*-like loci are quite similar to the phenotype of the embryonic lethal mutation, *tailless* (Nusslein-Volhard *et al.*, 1982; Degelmann *et al.*, 1986; Strecker *et al.*, 1988). In only a few cases have specific genetic interactions between maternal and zygotic gene functions been demonstrated. One example is the interaction between the dorsalizing maternal effect mutation *dorsal* and the zygotic lethal mutations *snail* and *twist* (Simpson, 1983). The function of some of the maternally stored gene products may be to directly control the expression of small subsets of zygotically acting genes or to modify, receive, or transduce the information encoded by zygotically acting genes.

Genes that are involved in establishing the organization within the segment represent a late step in the regulatory hierarchy which determines pattern formation (reviewed by Ingham, 1988). According to recent models (Gergen *et al.*, 1986; O'Farrel and Scott, 1986; DiNardo *et al.*, 1988; Martinez-Arias *et al.*, 1988), there are four cell states within a segmental unit which can be defined by their requirements for segment polarity gene products. According to the simplest interpretation of this model, normal differentiation of the segment requires expression of at least one particular segment polarity gene to establish cell identity in each of four transverse rows. The anterior-most row of cells would require embryonic expression of the *naked* (*nkd*) gene, followed by rows of cells requiring *patched* (*ptc*), *wingless* (*wg*), and *engrailed* (*en*). The model does not imply that these rows of cells are the exclusive domains of expression of each gene; however, the most posterior cells of a segment have indeed been shown to express *en* (Weir and Kornberg, 1985; Ingham *et al.*, 1985a), while the cells immediately anterior to the en^+ cells express *wg* (Baker, 1987). The spatial embryonic distribution of *nkd* and *ptc* is not yet known. The correct number of segments form in embryos mutant for any one of these genes, but within those segments the organization of cuticular structures, and presumably the identity of the cells secreting them, is abnormal. In wild-type embryos, each abdominal segment secretes a belt of denticles in the anterior region and naked cuticle in the posterior

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region (Lohs-Schardin *et al.*, 1979). In *nkd* embryos the cuticle lacks denticles (Jurgens *et al.*, 1984). In *ptc* embryos the pattern of denticles suggests a substitution of the posterior half of the denticle belt by a mirror image duplication of the most anterior half (Nusslein-Volhard and Wieschaus, 1980). In *wg* embryos the naked cuticle of the posterior region is absent and is replaced by ectopic denticles, which often have a polarity opposite to that of those in the anterior region (Nusslein-Volhard and Wieschaus, 1980). In *en* embryos a rather variable pattern of denticles is observed with substantial deletions of the posterior region of even numbered segments (Nusslein-Volhard and Wieschaus, 1980). Thus, the cuticular phenotypes caused by the different segment polarities are often more complex than the above model would predict, but this complexity may be due to cell interactions and to regulation in the affected embryos.

These four segment-determining genes appear to act after genes such as the gap and pair rule genes have already established regional and segmental periodicities in the blastoderm fate map (reviewed by Ingham, 1988). Because their requirement appears rather late, it is not clear how these genes could be influenced by early-acting, maternal effect genes. More difficult to understand is how a maternal effect mutation and one of these segment polarity genes could have similar embryonic phenotypes. In a search for *X*-linked, late zygotic lethal loci with specific maternal effect phenotypes, mutations at the *zeste-white 3* (*zw3*) locus were found to exhibit a phenotype similar to the embryonic phenotype of mutations at the *nkd* locus (Perrimon *et al.*, 1989). Here we have conducted a detailed comparison of the embryonic phenotypes of mutations at these two loci. Additionally, we show that alleles of *zw3* which produce this segment polarity phenotype are also able to transform hairs into bristles as previously described by Simpson *et al.* (1988).

MATERIALS AND METHODS

Strains

The maternal effect of mutations at the *zw3* locus on embryonic segmentation was identified in a large analysis of the maternal effects of *X*-linked loci (Perrimon *et al.*, 1989). In this analysis we used five mutations at the *zw3* locus (Table 1). These mutations are maintained in *FM7c* stocks or as attached-*X* stocks: *C(1)DX, yf/Dp(1;Y)w*$^{+303}$. The duplications *Dp(1;Y)w*$^{+303}$ (2D1-2;3D3-4;*Y*) and *Dp(1;3)w*^{vco} (Dp(1;3)2B17-C1; 3C4-5;77D3-5;81) (Craymer and Roy, 1980; Perrimon *et al.*, 1984a) cover the *zw3* locus, which is located in 3B1 (Shannon *et al.*, 1972; Kaufman *et al.*, 1975). The following deficiencies were obtained from B. Judd and J. Hall: *Df(1)K95* (Df(1)3A4;B1); *Df(1)64j4* (Df(1)3A9;B1); and *Df(1)64f1* (Df(1)3B1;3B3). A larger deficiency of the region *Df(1)64c18* (Df(1)2E1-2;3C2) was also used (Craymer and Roy, 1980; Perrimon *et al.*, 1984a). The *nkd* allele used (*nkd*^{7E89}) was obtained from the Bowling Green Stock Center and was maintained in a *TM3, Sb, Ser* stock. The *X*-linked dominant female sterile mutation *Fs(1)K1237* (Busson *et al.*, 1983; Perrimon, 1984) was maintained as an attached-*X* stock: *C(1)DX, yf/Y* females crossed to *Fs(1)K1237, v*^{24}*/Y* males. The *en-lacZ* strain was obtained from C. Hama and T. Kornberg; and the *ftz-lacZ* strain from Hiromi and Gehring (1987).

TABLE 1
ORIGIN OF THE *ZESTE-WHITE 3* MUTATIONS USED IN THIS STUDY

Mutation	Origin	Reference
zw3^{k22}	El	Judd *et al.*, 1972
zw3^{b12}	X ray	Judd *et al.*, 1972
zw3^{3m1}	EMS	E. Noll and N. Perrimon
zw3^{M11-1}	Spontaneous	J. Eeken
zw3^{MA2-7}	MMS	J. Eeken

Note. Abbreviations: El, Ethylenimine; EMS, Ethyl methanesulfonate; MMS, methyl methanesulfonate.

Descriptions of balancer chromosomes and mutations, unless identified in the text, can be found in Lindsley and Grell (1968). Except where noted, stocks and matings were maintained at 25°C on standard *Drosophila* medium.

Clonal Analysis

Germline clones of *zw3* mutations were produced by using the dominant female sterile technique (Perrimon, 1984; Perrimon *et al.*, 1984b). Briefly, virgin females heterozygous for *FM7/zw3* were mated to *Fs(1)K1237 v*^{24}*/Y* males. At the end of the first larval instar stage, progeny were irradiated at a constant dose of 1000 rads (Torrex 120D X-ray machine; 100 kV, 5 mA, 3-mm aluminum filter). Mitotic recombination in the germ line of *zw3/Fs(1)K1237* females was detected by individual inspection of ovary development. The frequency of females carrying germ line clones homozygous for *zw3* was about 6%.

To induce somatic clones, egg collections were made for 24 hr from the desired genotype and larvae were irradiated between 24 and 48 hr after egg laying. Wings were dehydrated in 70% alcohol and mounted in Aquamount.

Introduction of Segmentation Fusion Genes

Females possessing homozygous germ line clones for *zw3* were crossed with males that carry either the *ftz-lacZ* or *en-lacZ* insertion. Similarly, these transfor-

mants were introduced in *nkd* mutant embryos by crossing *TM3, Sb, Ser/nkd*^{7E89}, *rucuca* flies with males carrying the *lacZ* insertion. F1 progeny which were heterozygous for both *nkd*^{7E89} and the *lacZ* insertion were intercrossed and their progeny examined.

Examination of Embryos

Scanning electron micrographs (SEM) were prepared as described by Turner and Mahowald (1976). Cuticle preparations were prepared in Hoyers mountant as described by van der Meer (1977) and the stage of lethality of mutant progeny was done as described by Perrimon *et al.* (1984b).

Immunohistochemistry

Immunohistochemistry was performed as described in Smouse *et al.* (1988) and Klingensmith *et al.* (1989). To examine the central and peripheral nervous systems of mutant embryos we used polyclonal antibody against horseradish peroxidase (anti-HRP), which labels all central and peripheral nervous system cell bodies and axons (Jan and Jan, 1982), and the *SOX2* monoclonal antibody, which recognizes the cell bodies and axons of the entire PNS and a subset of CNS neurons (Goodman *et al.*, 1984). The mouse anti-β-galactosidase primary antibody was from Promega-Biotech and anti-HRP antisera was from Cappel. The monoclonal antibody against *Ultrabithorax* (*Ubx*) was obtained from White and Wilcox (1984) and the monoclonal antibody against the engrailed homeobox domain (DiNardo *et al.*, 1988) was obtained from C. Doe. The rabbit polyclonal antibody against *even-skipped* (*eve*) was obtained from M. Frasch (Frasch *et al.*, 1987).

Silver intensification was performed with an Amersham DAB enhancement kit according to manufacturer's protocols. Subsequently, embryos were dehydrated and embedded in JB4 plastic (Polysciences). Serial 3-μm sections were cut using a Leitz 1516 microtome and stained with methylene blue. Slides were dried and mounted in Aquamount (Klingensmith *et al.*, 1989).

Pupal wings were dissected, fixed, and stained with anti-HRP as previously described (Palka *et al.*, 1983; Blair and Palka, 1985).

RESULTS

The Genetics of zw3

The *X*-linked *zw3* locus maps to position 3B1 of the salivary gland polytene chromosomes and within the region defined genetically by *zeste* and *white* (Shannon *et al.*, 1972; Kaufman *et al.*, 1975). Figure 1 shows the pattern of complementation of *zw3*, *period* (*per*), and other *zeste-white* lethal complementation groups (Shannon *et al.*, 1972; Reddy *et al.*, 1984) with three small deficiencies (*Df(1)K95*, *Df(1)64j4*, and *Df(1)64f1*, see Materials and Methods for cytology).

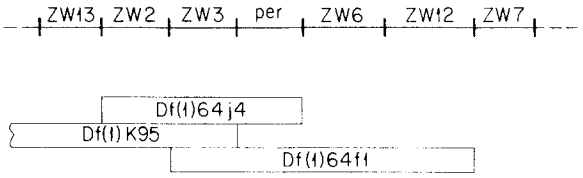


FIG. 1. Genetic map of the *zw3* region.

The lethal phases for five *zw3* alleles were analyzed (Table 2). The hemizygous progeny derived from heterozygous females for each allele die during early larval stages (Table 2, see also Shannon *et al.*, 1972). A similar lethal phase is observed when each mutation is tested *in trans* with a deficiency of the region (in Table 2 the results for only one of these complementation tests *zw3*^{k22} are shown). There is no heteroallelic complementation between the five *zw3* alleles. A similar stage of lethality and a similar phenotype were observed in transheterozygous *Df(1)K95/Df(1)64f1* (mutant for *zw3*) and *Df(1)64j4/Df(1)64f1* (mutant for both *zw3* and *per*) animals. These results suggest that the phenotype for null mutations at the *zw3* locus is early larval lethality. Mutant *zw3* larvae derived from heterozygous females do not exhibit any major cuticular defects, and their central and peripheral nervous systems develop normally as visualized with a polyclonal antibody against horseradish peroxidase and with the *SOX2* monoclonal antibody (see Materials and Methods, results not shown).

The Maternal Effect Phenotype of zw3

By using the dominant female sterile technique (see Materials and Methods), we generated germ line clones homozygous for *zw3* and examined the effect of loss of *zw3*$^{+}$ function during oogenesis. Interestingly, *zw3* mutations exhibit a fully penetrant maternal effect lethal phenotype (Table 2). All embryos derived from homozygous germ line clones die during embryonic development. A range of embryonic phenotypes is observed from eggs derived from homozygous *zw3* germ line clones crossed with wild-type males. On the basis of the level of cuticle differentiation we can define two classes of embryos: *class 1 zw3* embryos show very poor cuticle differentiation and have variable holes in their cuticle; *class 2 zw3* embryos show more cuticle differentiation and differentiate defective denticle belts (Fig. 2C). A similar range of maternal effect lethal phenotypes is exhibited by all three *zw3* alleles that were tested in

TABLE 2
LETHAL PHASES

Cross	N.	N. unh	N. unf	%E	Lethal phase
Lethal phase from heterozygous mothers					
$+/zw3^{k22} \times +/Y$	300	24	18	2	L
$+/zw3^{M11\text{-}1} \times +/Y$	236	5	1	2	L
$+/zw3^{MA2\text{-}7} \times +/Y$	170	10	2	5	L
$+/zw3^{dm1} \times +/Y$	170	12	6	4	L
$+/zw3^{b12} \times +/Y$	210	21	10	5	L
$+/zw3^{k22} \times Df(1)64c18/DpY$	300	89	60	12	L
$+/Df(1)K95 \times +/Y$	288	84	10	26.6	E
$+/Df(1)64f1 \times +/Y$	290	42	24	6.7	E-L
$+/Df(1)64j4 \times +/Y$	200	36	17	10.4	E-L
$+/Df(1)64f1 \times Df(1)64j4/DpY$	340	50	36	4.6	L
$+/Df(1)64f1 \times Df(1)K95/DpY$	280	56	40	6.6	L
Lethal phase from homozygous germ line clones					
$zw3^{M11\text{-}1} \times +/Y$	210	210	50	100	E
$zw3^{MA2\text{-}7} \times +/Y$	134	134	31	100	E
$zw3^{k22} \times +/Y$	306	306	101	100	E
$zw3^{k22} \times +/DpY$	175	175	62	100	E
$zw3^{k22} \times +/Y; Dp3/+$	164	164	51	100	E

Note. In each cross (indicated in the left-most column) the percentage of offspring dying during embryonic stages (%E) is indicated. %E = No. unh-No. unf/N.-N. unf, with N. = the total number of eggs analyzed, N. unh = the number of unhatched eggs, and N. unf = the number of unfertilized eggs. *FM7c* was used in females as the + chromosome and a *y f* chromosome was used in males. *K95*; *64j4*; *64f1*, and *64c18* refer to various deficiencies of the *zeste-white* region; *DpY* and *Dp3* to $Dp(1;Y)w^{+303}$, and $Dp(1;3)w^{vco}$, respectively (see Materials and Methods). All experiments were performed at 25°C. The stage of lethality of *Df(1)K95* animals is embryonic, *Df(1)64j4* animals die at the embryonic-larval transition and *Df(1)64f1* individuals die during larval stages. Similarly, *zw3* hemizygotes derived from heterozygotes die during larval stages (see also Shannon *et al.*, 1972). E and L indicate lethality during embryonic and larval stages, respectively, and E-L indicates lethality at the embryonic-larval transition.

germ line clone analysis (Table 2); therefore, we do not distinguish between specific alleles of *zw3* in the text.

Females with homozygous *zw3* germ line clones were crossed to wild-type males (+/*Y*) and were allowed to lay eggs. Of 306 eggs collected from these females (Table 2), 36% showed no sign of embryonic development and were most likely unfertilized eggs, 35% were *class 1 zw3* embryos, and the remaining 29% were *class 2 zw3* embryos. Although the distinction between *class 1* and *class 2 zw3* embryos is sometimes difficult to assess, two experiments were performed to test the possibility that *class 2* embryos develop from eggs that have received a wild-type copy of the *zw3* gene from the father. In the first experiment, females with homozygous *zw3* germ line clones were crossed to males that carried copies of the $zw3^+$ gene on both the *X* and *Y* chromosomes ($+/Dp(1;Y)w^{+303}$). In this cross, 175 eggs were analyzed: among the 113 that developed into embryos (Table 2), 38% clearly belonged to *class 1*, while the remaining 62% were of *class 2*. In the second experiment, females with *zw3* homozygous germ line clones were crossed to males with an autosomal duplication of the *zeste-white* region ($+/Y; Dp(1;3)w^{vco}/+$). Among the 113 eggs that developed (Table 2), 29% were of *class 1* and 71% appeared to be *class 2*. These results indicate that the zygotic expression of an extra copy of the wild-type *zw3* gene from the father improves the maternal deficiency. However, this paternal rescue is not fully penetrant because (1) less than half the embryos are of *class 2* when the females with germ line clones are crossed to +/*Y* males, and (2) not all the progeny are of *class 2* when females with germ line clones are crossed with $+/Dp(1;Y)w^{+303}$ males. Alternatively, it is possible that the introduction of other genetic factors in the males' backgrounds could account for these results. In the descriptive analysis of *zw3* embryos we do not distinguish between *class 1 zw3* and *class 2 zw3* since *class 1* embryos only represent a more extreme version of the phenotype exhibited by *class 2 zw3* embryos.

Lack of Maternal $zw3^+$ Activity Produces a Segment Polarity Phenotype Similar to That of Embryos Lacking Naked$^+$ Zygotic Activity

Class 2 zw3 embryos derived from homozygous *zw3* germ line clones are missing most ventral denticle belts and head structures (Fig. 2C1 and 2C2). Among the collection of zygotic lethal loci (Nusslein-Volhard *et al.*, 1984; Wieschaus *et al.*, 1984; Jurgens *et al.*, 1984), only embryos mutant for *naked* (*nkd*) resemble *class 2 zw3* embryos. In *nkd* embryos all segments are present but

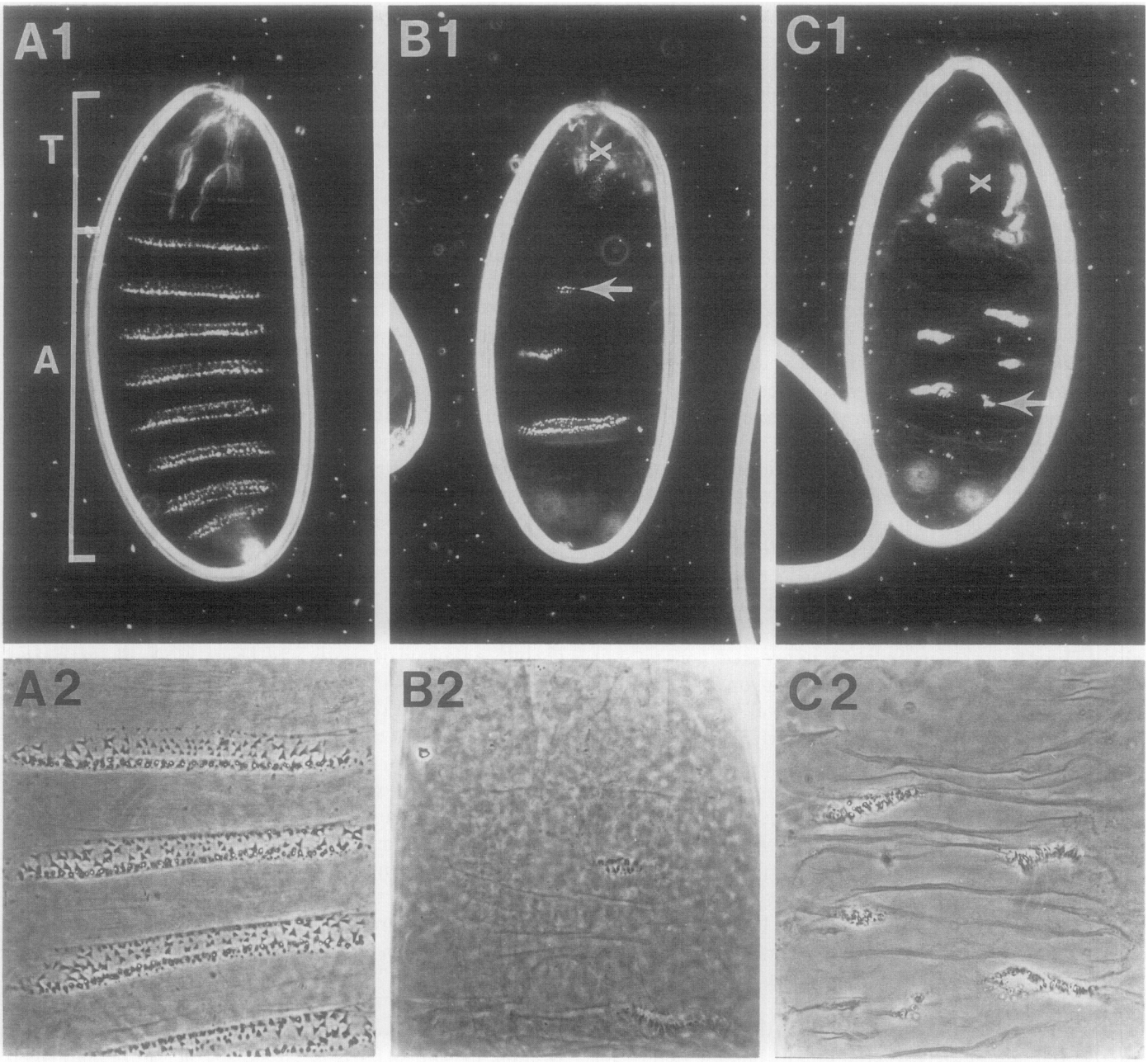


FIG. 2. The cuticle phenotypes of wild-type, *naked*, and *zw3* embryos. A1 is a dark-field micrograph of the ventral side of a wild-type embryo. Note the three thoracic (T) and eight abdominal (A) segments. A2 is a phase-contrast micrograph of the pattern of ventral denticle belts. The ventral cuticle of each wild-type abdominal segment consists of a belt of seven rows of denticles, five or six of which point posteriorly, followed by a posterior region of naked cuticle (A1, A2; Lohs-Shardin *et al.*, 1979). The ventral cuticle of *nkd* embryos is almost completely void of denticles (B1, B2). *zw3* embryos have a phenotype similar to *naked* embryos (C1, C2). Both *nkd* and *zw3* embryos have defective heads (indicated by an X) and have abnormal spiracles; the filzkorper material is very prominent. The arrows in B1 and C1 point to remnants of denticle belts in *nkd* and *zw3* embryos; the partial denticle belts are magnified in B2 and C2. Anterior is up in all figures.

their organization is abnormal; the ventral cuticle of *nkd* embryos, as in *class 2 zw3* embryos, is partially (Fig. 2B1 and 2B2) or completely devoid of denticles. Generally, *zw3* embryos have a more extreme cuticle phenotype than *nkd* embryos. We have conducted a detailed phenotypic analysis of both the *nkd* and *zw3* embryonic phenotypes to determine whether the two genes are likely to be involved in the same developmental pathway. We have examined the embryonic phenotype associated with a single allele of *nkd*, nkd^{7E89}, which most likely represents a null allele (Jurgens *et al.*, 1984; Martinez-Arias *et al.*, 1988).

To compare the phenotypes of *zw3* and *nkd* embryos, we examined the pattern of expression of three segmentation genes (*fushi tarazu* (*ftz*), *eve*, and *en*) and one homeotic gene (*Ubx*) in mutant embryos. The patterns of *ftz* and *en* expression were detected by introducing in mutant embryos chromosomes carrying *ftz-lacZ* or *en-lacZ* insertions (see Materials and Methods). During gastrulation, the *ftz-lacZ* (Hiromi and Gehring, 1987) and *en-lacZ* (C. Hama and T. Kornberg, personal communication) strains express β-galactosidase in patterns identical to the native *ftz* (Carroll and Scott, 1985) and *en* (DiNardo *et al.*, 1985) protein expression patterns, as shown by staining wild-type embryos with antibodies to β-galactosidase (Fig. 3A) (Smouse *et al.*, 1988; Klingensmith *et al.*, 1989). The patterns of *eve* and *Ubx* expression were examined using *anti-eve* and *anti-Ubx* antibodies (see Materials and Methods).

We examined the *ftz-lacZ* expression pattern in *nkd* embryos and in *zw3* embryos derived from germ line clones. In both types of embryos the early patterns of *ftz-lacZ* expression are normal (data not shown). These results are consistent with those obtained by Carroll and Scott (1986) who have shown that the seven-striped pattern of *ftz* protein is not affected by the *nkd* mutation. The *ftz-lacZ* construct is expressed correctly in wild-type embryos in a subset of neuronal precursors, the MP2's, and in their progeny as well as in the progeny of the MP1 cell (Doe *et al.*, 1988a). These precursor cells are derived from the same region of the embryo which gives rise to the anterior compartment of the epidermis. The MP2's are present in *zw3* and *nkd* embryos although they do not always divide, and their cell bodies do not migrate to their normal positions. There is no evidence of duplication or hypertrophy of the MP2's in either mutant background. However, many of the neurons which normally express *ftz-lacZ* later in neurogenesis fail to do so in *zw3* and *nkd* embryos (results not shown).

The pattern of *eve* protein expression was examined in *nkd* and *zw3* embryos using an antibody which recognizes the *eve* gene product (Frasch *et al.*, 1987). The early pattern of *eve* stripes is normal in both mutants, but in *zw3* embryos there is an abnormality associated with the late *eve* pattern. In wild-type embryos, there is a ring of cells surrounding the anal plate which express *eve* (Frasch *et al.*, 1987), but in *zw3* embryos only the cells of the ventral half of the ring are eve^+ (results not shown). It is not clear if the dorsal cells, which are also en^+ in wild-type embryos, are missing or if they are present but simply fail to express *eve*. The antibody which recognizes the *eve* gene product also stains a small subset of neuronal nuclei (Doe *et al.*, 1988b) in wild-type embryos. When *zw3* and *nkd* embryos are stained with anti-*eve*, the number of eve^+ neurons is reduced from approximately 34 per wild-type segment to roughly 12 per mutant segment. In particular, those eve^+ neurons which are born last and which make up the ventrolateral cluster are absent or fail to express *eve*. The eve^+ neurons which are present often occur in abnormal and rather disorganized patterns; it is, however, possible to identify the eve^+ "anterior and posterior corner cells" (aCC and pCC) (Doe *et al.*, 1988a,b) in mutant embryos. The aCC and pCC are siblings derived from the neuroblast 1-1, which is located in the anterior region of the segment; these two neuronal progeny then migrate into the posterior region of the next anterior segment in wild-type embryos. While it is clear that the aCC and pCC are born in mutant embryos, and express *eve*, it is not clear if they perform their anterior migration normally. Thus, the results obtained with *ftz* and *eve* probes are consistent and indicate that there are no defects in the early stripe patterns of expression of these two pair rule genes and that there are no apparent deletions of neurons derived from the anterior region of each segment, in either mutant background.

In contrast to *ftz* and *eve*, gross abnormalities in the patterns of *en-lacZ* expression are obvious after germ band extension. The domain of *en-lacZ* expression is clearly enlarged in both *nkd* (Figs. 3B1, B3) and *zw3* (Figs. 3C1, C3) embryos at 6 hr of development. The enlarged domain of *en-lacZ* expression is due to the ectopic expression of *en-lacZ* by cells which would not normally express it, since a detailed analysis of the number of cells expressing *en-lacZ* at 6 hr of development in wild-type and in both mutants indicates that *en-lacZ* is expressed in half the cells of the segmental unit in the mutants (Figs. 3B3, 3C3). No cell death is detectable at this stage (Figs. 3B3, C3). The *en-lacZ* construct is also expressed in a population of neurons derived from the posterior region of each segment. These cells include the "ventral, unpaired, medial cells," or VUMs (Goodman *et al.*, 1984), which are believed to be derived from either the posteriorly located median neuroblast or the MPs 3–5. A subset of the VUMs also expresses *Ubx* in segments T1–T3 (Fig. 7A3). When both of these probes are used in *nkd* or *zw3* embryos, we find that the VUMs are either absent or no longer expressing *en* and *Ubx* (Figs. 7B3, 7C3; data not shown).

After germ band shortening most *en* stripes in *nkd* (Fig. 3B2) and *zw3* (Fig. 3C2) embryos are irregular compared to the wild-type stripes (Fig. 3A2). To study the ontogeny of these segmentation defects, *nkd* embryos were examined by scanning electron microscopy (Fig. 4). There are no apparent defects in gastrulation of *nkd* embryos (not shown). The first defects visible in *nkd* embryos are the formation of irregular segmental borders during germ band shortening (Figs. 4A, 4B). Subsequently, partial fusion of thoracic and abdominal

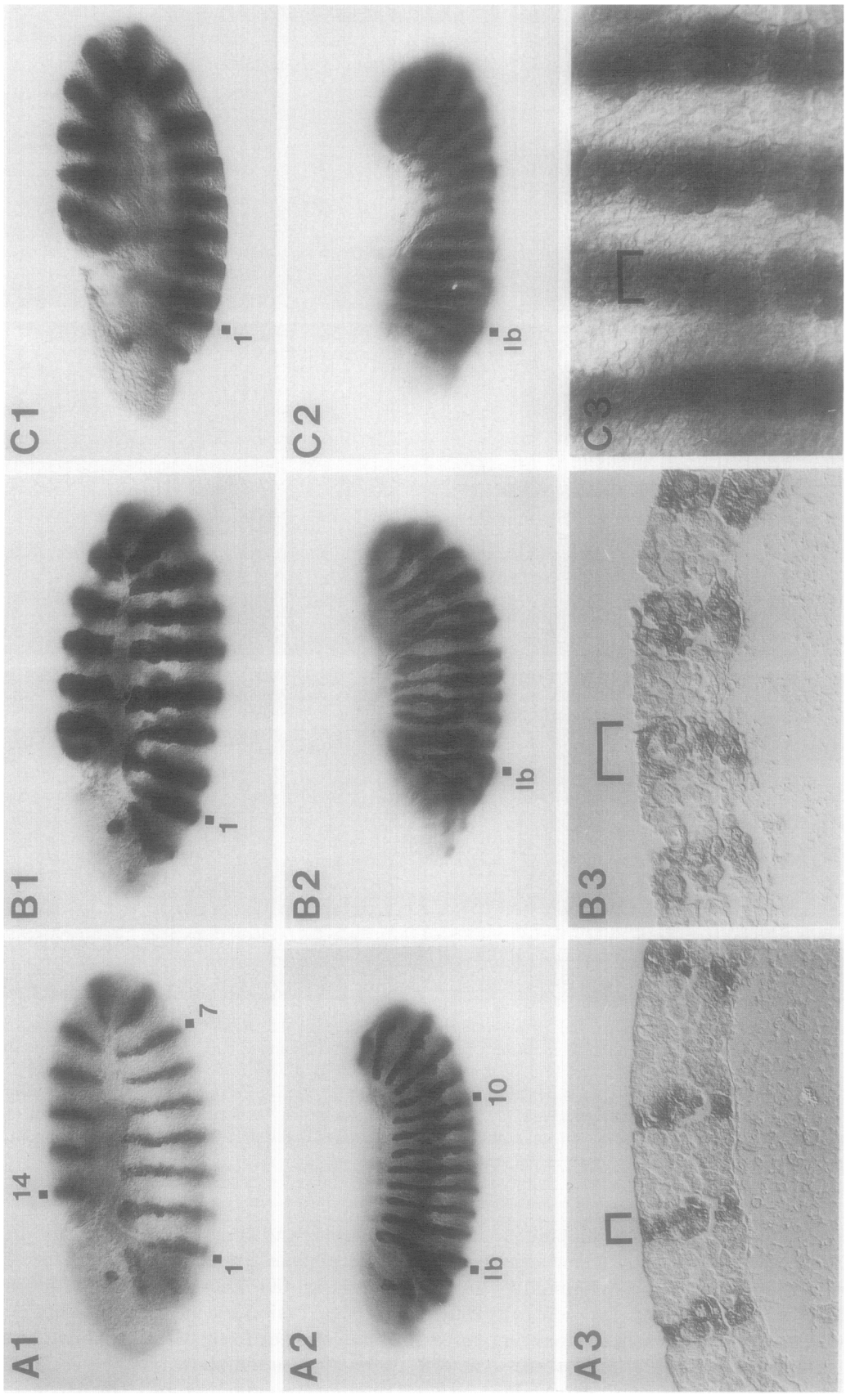


FIG. 3. Expression of *engrailed-lacZ* in wild-type, *naked*, and *zw3* embryos. A shows the pattern of *en-lacZ* in wild-type embryos at 6 (A1) and 10 (A2) hr of development, respectively. Note the 14 stripes of *en-lacZ* expression; stripes 1, 7, and 10 have been labeled as well as the labial (lb) segment. B and C show the pattern of *en-lacZ* in *nkd* (B) and *zw3* (C) embryos at similar stages. In both *nkd* and *zw3* mutant embryos the domain of *en-lacZ* expression is enlarged. At 6 hr of development *en-lacZ* in *nkd* (B1, B3) and *zw3* (C1, C3) embryos is expressed in half the cells of the segmental unit. In wild-type embryos (A3) *en-lacZ* is expressed in about a quarter of the cells of the segmental unit. Note in B3 the absence of dying cells and the location of the segmental furrows posterior to the cells expressing *en* (also visible in C1). A3 and B3 are 3-μm plastic sections; all others are whole mounts. In A1, A2, B1, B2, C1, and C2, anterior is to the left and dorsal is up. In A3 and B3, anterior is to the left and ventral is up. C3 is a ventral view with anterior to the left.

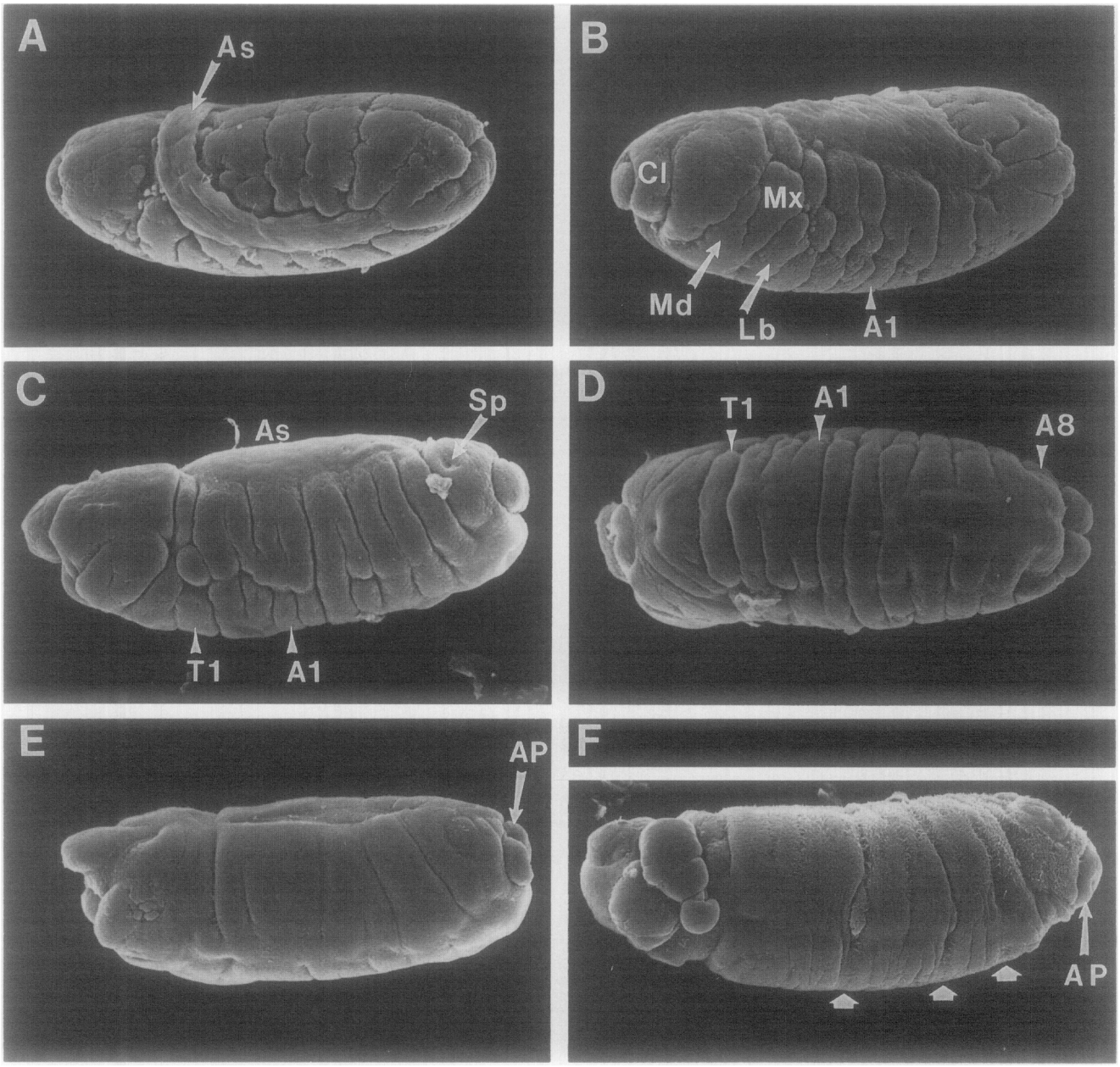


FIG. 4. Segmentation defects in *naked* embryos visualized by scanning electron micrographs. A and B are embryos of 7.5 and 9 hr, respectively. Note the irregular segmental borders. At 10 hr of embryonic development partial fusion of both thoracic and abdominal segments is observed (C is a lateral view and D is a ventral view). E is a 12-hr-old mutant embryo; note that only a few segmental grooves are present and that the head is misshapen. F is a 22-hr-old embryo; only a few denticles are present (indicated by white arrows) and some head structures are protruding. For wild-type development see Turner and Mahowald (1977). In all figures anterior is to the left and, with the exception of D, dorsal is up. Nomenclature: As, amnioserosa; Cl, clypeolabrum; Md, mandibulary; lb, labial; Mx, maxillary; T1, first thoracic segment; A1, first abdominal segment; A8, eighth abdominal segment; sp, spiracle; AP, anal pads.

segments is observed as early as 10 hr of development (Fig. 4C). At the end of dorsal closure only a few segmental grooves are present and the head is abnormally shaped. Finally, at 22 hr of development, the ventral cuticle is secreted with the variable presence of only a few denticles, while some unidentifiable head structures, which have not involuted correctly, protrude from the anterior end (Fig. 4F). A "pair rule-like" phenotype often appears in *nkd* embryos after 12 hr of development (Fig. 4E). These late segmentation defects due to segmental fusion are most likely secondary to the misexpression of *en* or to cell death that can be detected at this stage (results not shown).

Using these probes and methods, the earliest discern-

ible defects in *zw3* and *nkd* embryos occur at approximately 6 hr of development, at which time the *en-lacZ* construct is expressed in an abnormal *en* pattern. To determine if an earlier phenotype is apparent, and to confirm that the changes in *lacZ* expression reflect similar changes in native *en* expression, *zw3*-derived embryos were stained with a monoclonal antibody which recognizes the *engrailed* homeobox domain (DiNardo *et al.*, 1988). This antibody allowed detection of *en* protein, which is expressed earlier than the *en-lacZ* construct, and demonstrated that the native *en* protein is indeed expressed in wider-than-normal stripes. The abnormal *en* stripes appeared as early as 3.5 hr of development, at a time shortly after the germ band is completely extended but before overt segmentation is evident (results not shown).

A polyclonal antiserum against horseradish peroxidase (anti-HRP) (Jan and Jan, 1982) recognizes all nervous system cell bodies and axons and reveals that there is major disorganization of the CNS in *nkd* and *zw3* embryos. The very regular, ladder-like pattern of axons formed by the horizontal commissures and longitudinal connectives in wild-type embryos is replaced by a rather chaotic array of axons (Fig. 5). In each segment there is at most a single bundle of axons crossing within a segment, and there are very few axons crossing between segments. The brain often protrudes dorsally rather than being covered by epidermis during dorsal closure. The phenotype of *zw3* germ line clone-derived embryos is worse than that of comparably staged *nkd* embryos; there appear to be even fewer axons forming longitudinals and the protrusion of the brain lobes is more severe (Figs. 5C and 5F compared to 5B and 5E). Additionally, fusion of segmental ganglia is observed in *zw3* embryos consistent with the segmental fusion observed in *nkd* embryos.

The peripheral nervous system, as visualized with the *SOX2* monoclonal antibody, is also abnormal in *nkd* and *zw3* embryos (Fig. 6). In *nkd* embryos the normal numbers and types of cells (Ghysen *et al.*, 1986) appear, but their axon projections are quite aberrant (Fig. 6C, D). Many axons cross between segments, and the axons of cells in the dorsal and lateral clusters often grow horizontally rather than dorsoventrally. Occasionally lateral clusters of adjacent segments fuse and the axons of the fused clusters fasciculate together. This phenotype is more pronounced in *zw3* embryos (Fig. 6B), where the lateral clusters of several adjacent segments fuse, often resulting in giant clusters containing as many as 20 chordotonal organs. It is possible to count as few as 6–8 fused lateral clusters in the thoracic and abdominal segments of *zw3* embryos, rather than the 20 lateral clusters seen in wild-type embryos. The dorsal clusters are also more severely affected by the *zw3* mutation, since the majority of cells belonging to the dorsal clusters send axons horizontally to fasciculate with one another and rarely, if ever, send axons ventrally.

The pattern of *Ubx* gene expression was examined with a monoclonal antibody to *Ubx* (White and Wilcox, 1984). In wild-type embryos, *Ubx* is expressed most abundantly in derivatives of parasegment 6, which are the posterior compartment of T3 and the anterior compartment of A1 (White and Wilcox, 1984; Beachy *et al.*, 1985). *Ubx* is also expressed at somewhat lower levels and in fewer cells in segments A2–A7. In these segments there is a definite graded pattern of expression in that the anterior compartments express levels of *Ubx* higher than those of the posterior compartments. Thus, two patterns of *Ubx* can be discerned, one within a segment and one between segments, and these two patterns are reflected in the epidermis and the CNS (Fig. 7A). In both *nkd* (Fig. 7B) and *zw3* (Fig. 7C) embryos the pattern between segments—no expression in T1, very low expression in T2, very high expression in *T3/A1*, and high expression in A2–A7—is maintained in both the epidermis and the CNS. However, the pattern within a segment is perturbed by both mutations. The anterior-to-posterior gradient is replaced by a more uniform level of expression that is particularly evident in the ventral half of the embryos. Thus, *nkd* and *zw3* mutations do not alter the identity of segments but rather the organization of cells within a segment.

In conclusion lack of maternal $zw3^+$ activity leads to embryonic phenotypic effects that are similar to lack of zygotic nkd^+ activity: neither mutant affects the early expression of *ftz-lacZ* and *eve*, while both mutants disrupt the expression of *en-lacZ* and *Ubx* in similar ways. They have similar effects on the development of the embryonic nervous system, although the effects observed in *zw3* embryos are more severe than those of *nkd*. These effects can be summarized as a disorganization in cell body position and in axonal pathways in the CNS and the PNS and a reduction in the number of identified CNS neurons. It is not known if the apparent absence of identified neurons is the result of cell death, change of cell fate, or the direct influence on gene expression by the *nkd* and *zw3* mutations.

zw3 Is a Homeotic Locus that Transforms Hairs into Bristles

Recently, Simpson *et al.* (1988) reported that clones of mutant $zw3^{shaggy}$ cells resulting from X-ray-induced mitotic recombination, develop bristles at a density much higher than normal throughout the entire fly body; on the wing blade, these bristles are the result of transformation of hair-secreting cells into bristle-secreting cells. Interestingly, different types of bristles are

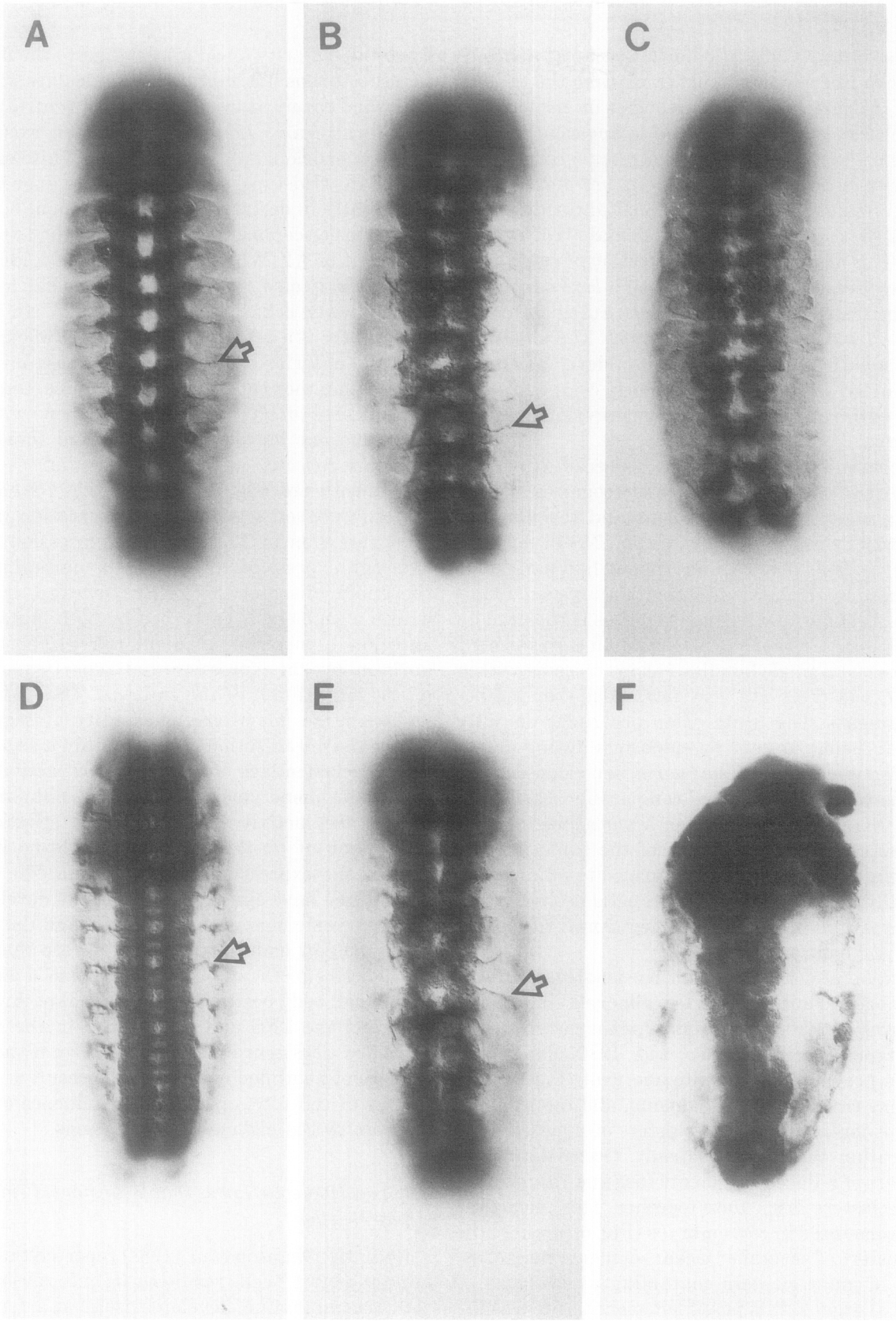


FIG. 5. CNS phenotype of *nkd* and *zw3* embryos. Ventral views of embryos stained with anti-HRP. A and D are wild-type embryos at 11 and 13 hr of development; B and E are *nkd* embryos at 11 and 12 hr; and C and F are *zw3* embryos at 11 and 13 hr. The segmental nerve (open arrow) is visible in wild-type and *nkd* embryos, but is not visible in the *zw3* embryo because of the focal plane. There is considerable disorganization of cell bodies and axons in the CNS of *nkd* and *zw3* embryos. In all panels anterior is up.

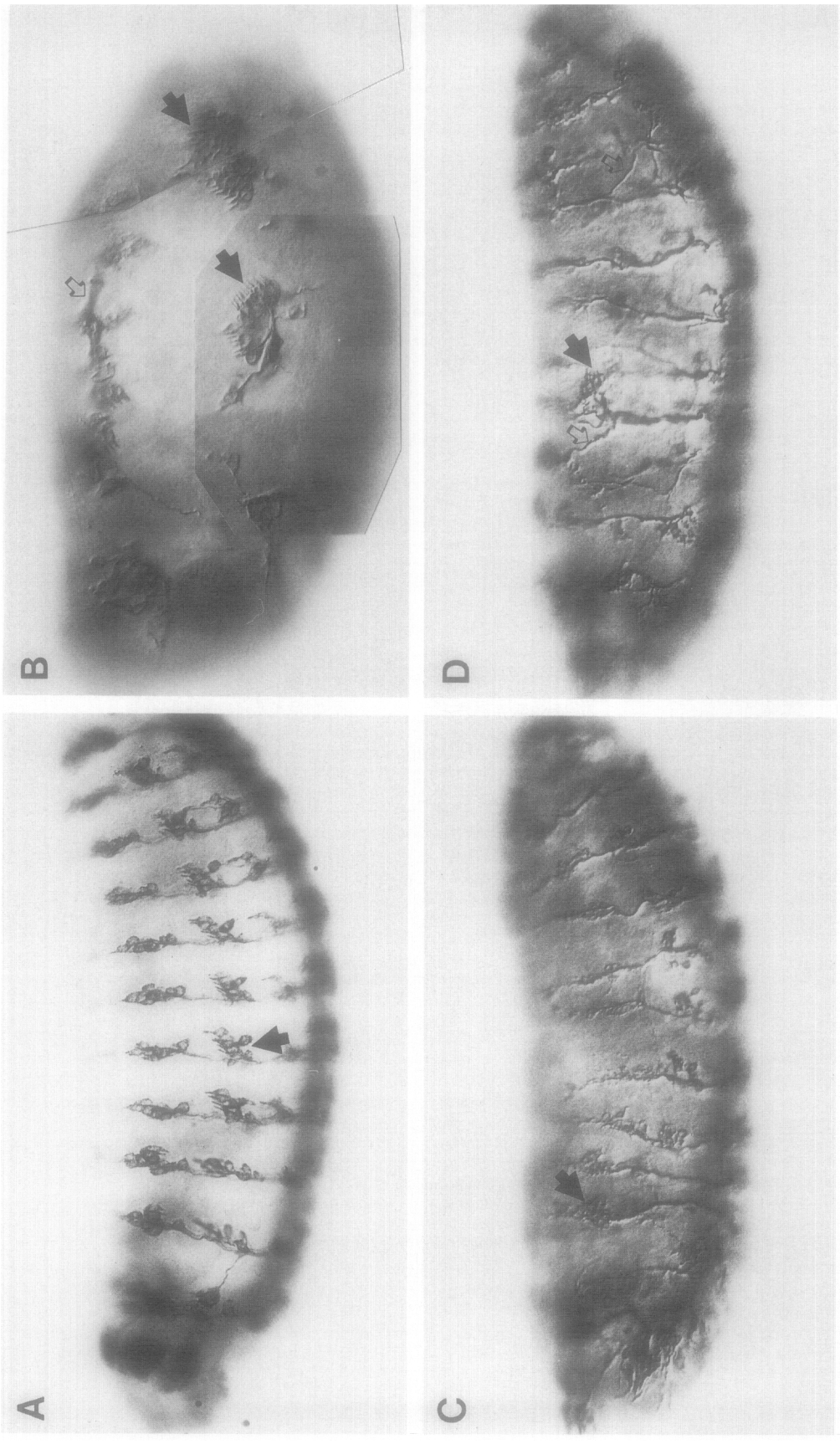
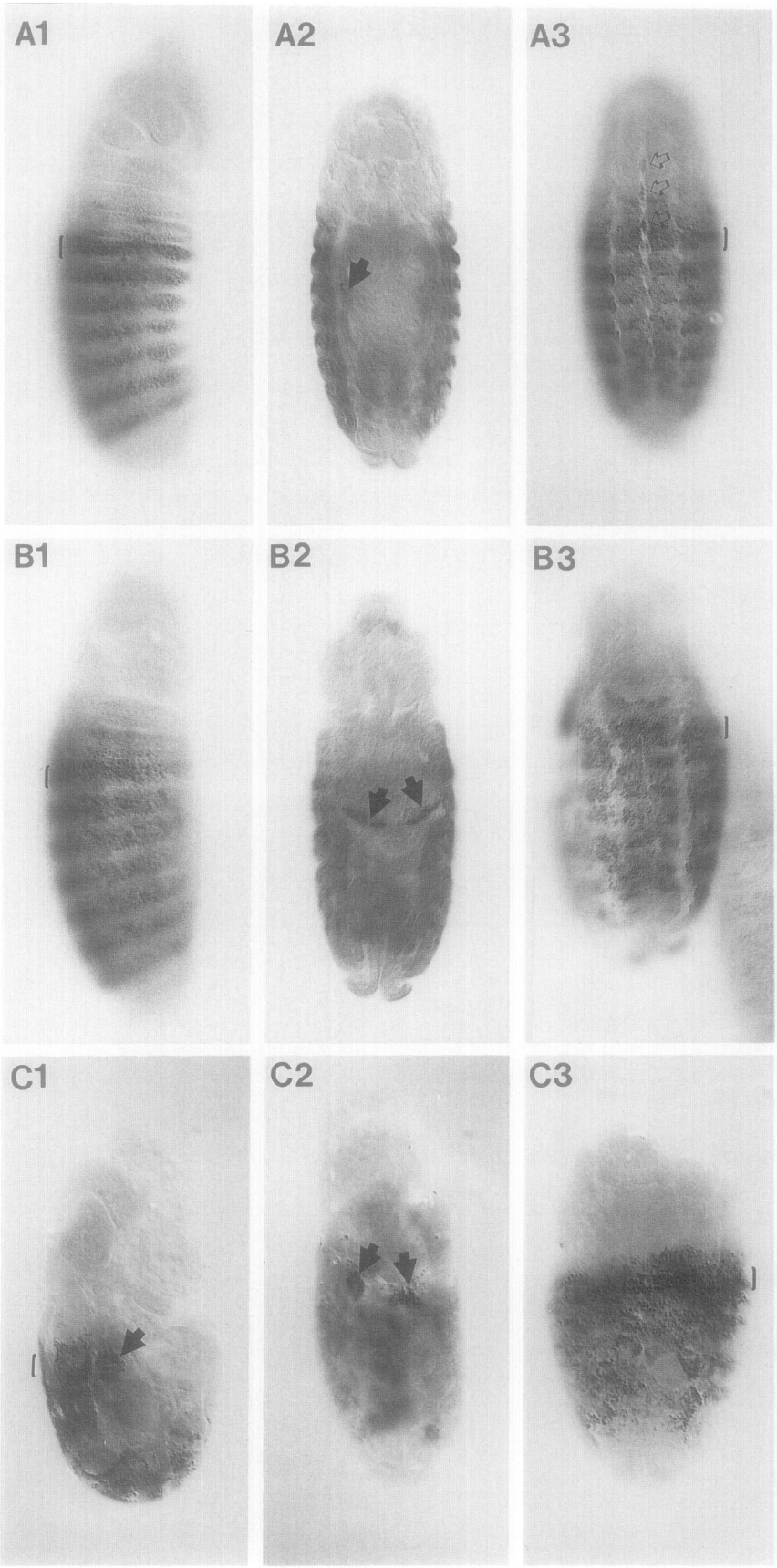


FIG. 6. PNS phenotype of *nkd* and *zw3* embryos. Embryos are stained with the SOX2 antibody; anterior is to the left and dorsal is up. The wild-type embryo shows a complex segmental pattern of sensory cells whose axons are directed ventrally (A; Campos-Ortega and Hartenstein, 1985; Ghysen *et al.*, 1986). A characteristic set of five chordotonal organs, found in A1–A7, is indicated by the arrow. A *zw3* embryo is shown in B, where the chordotonal organs of the abdominal segments are fused into three large clusters indicated by arrows. The cells of the dorsal clusters send their axons horizontally (open arrow) rather than ventrally. Embryos mutant for *nkd* are shown in C and D; fusions of adjacent segments are indicated by the dark arrows and misrouted axons which cross between segments are indicated by the open arrows.


A1
A2
A3
B1
B2
B3
C1
C2
C3

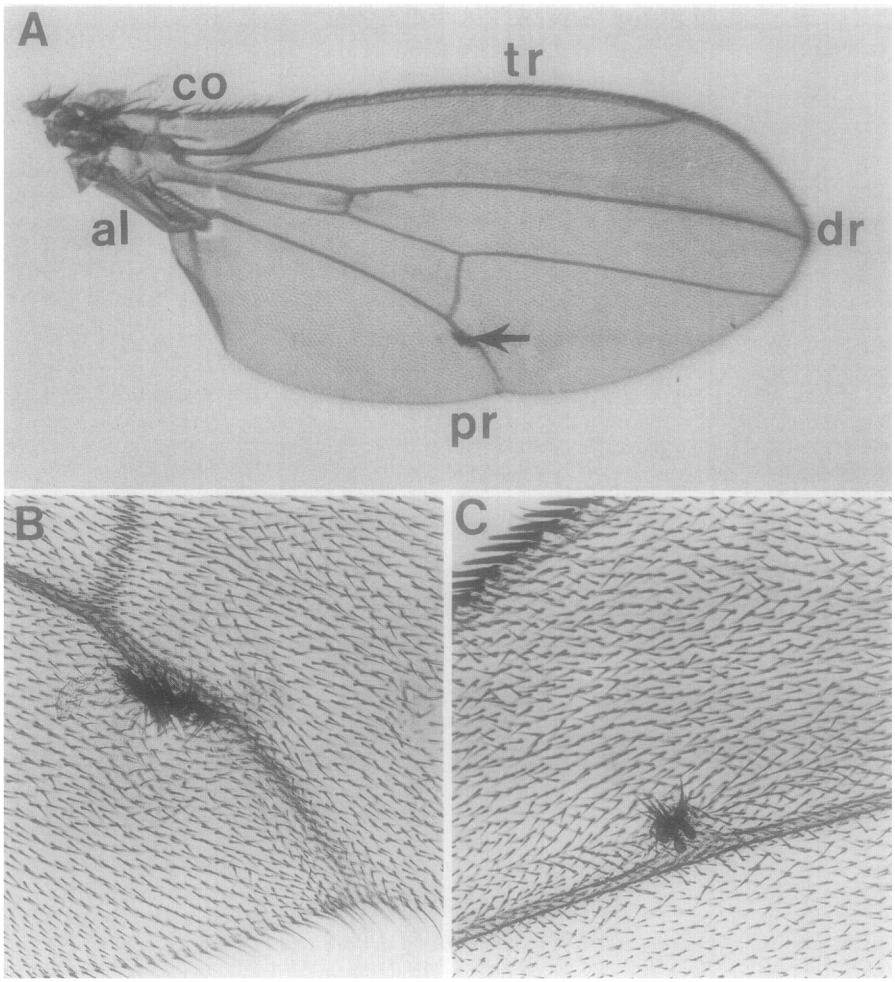
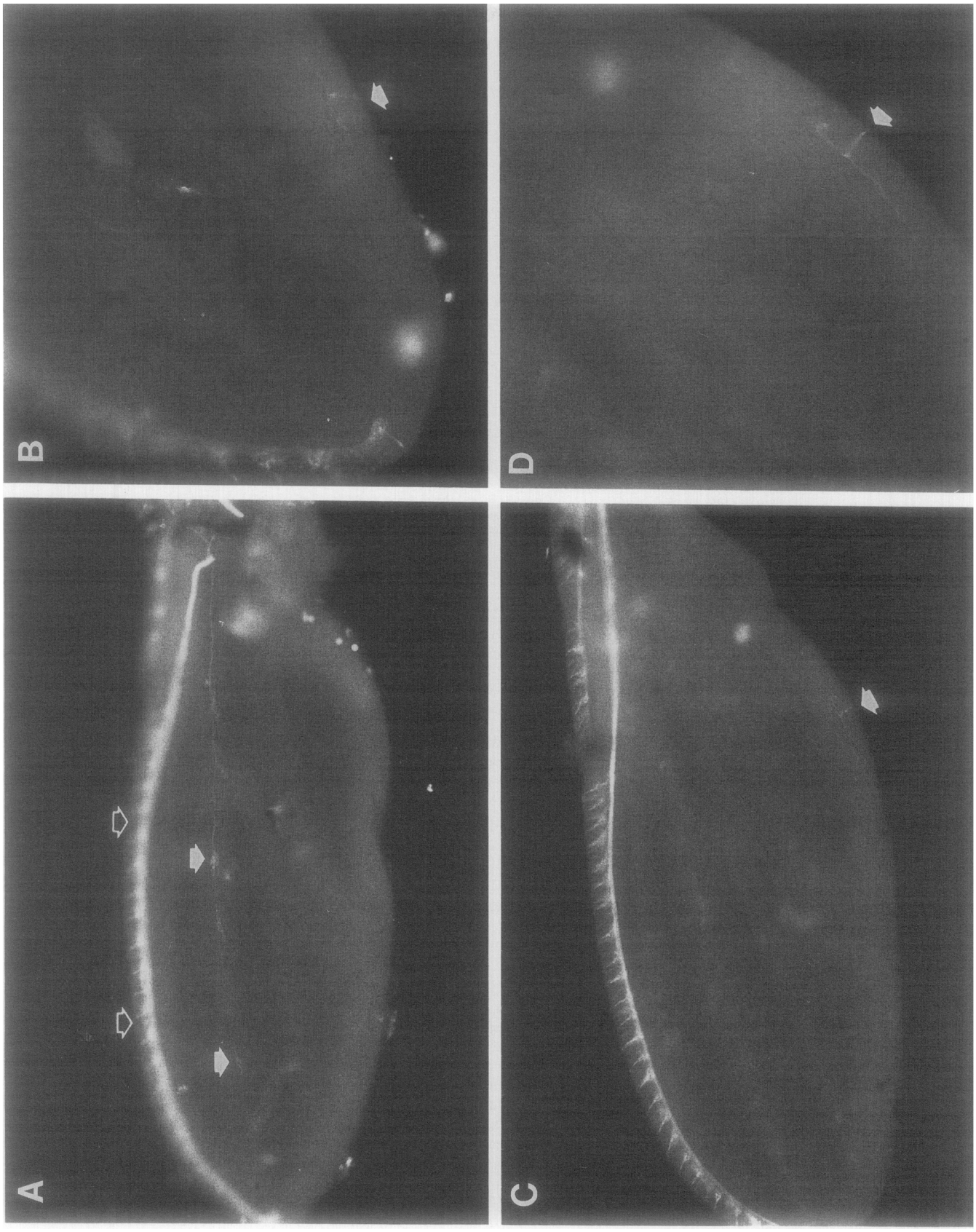


FIG. 8. *zw3* clones in the wing. The arrow in A points to a *zw3* homozygote clone induced in a *zw3*/+ adult heterozygote following irradiation during the larval stage (at 24–48 hr after egg laying). B shows an enlargement of the clone in A. Hair cells have been transformed into sensory bristles. C is an example of an anterior dorsal clone composed of dorsal triple rows bristles. Nomenclature: co, costa; tr, triple row; dr, double row; pr, posterior row; al, alula.

formed at different locations in the wing. The nature of these transformations indicates that lack of $zw3^{+}$ prevents the correct decision between an epidermal cell pathway and that of a sensory bristle. Thus, *zw3* causes homeotic transformations and these transformations reveal that *zw3* is cell autonomous, in that the genotype of a cell determines its phenotype.

To find out whether *zw3* mutations that exhibit the

FIG. 7. *Ultrabithorax* expression in wild-type, *nkd*, and *zw3* embryos. Panels labeled A are wild-type embryos, B are *nkd* embryos, and C are *zw3*. Pictures in row 1 are side views, in row 2 are optical sections through the middle of each embryo, and in row 3 focus on the ventral nerve cords. The brackets in rows 1 and 3 indicate the most prominently stained segment, A1. Note that this is still the most prominent segment in *nkd* (see also Martinez-Arias *et al.*, 1988) and *zw3* embryos, though the epidermal expression is broader and more uniform (B1) than in wild-type (A1). A common feature of *zw3* embryos is the failure to completely retract the germ band, which is evident in C1. Many steps in differentiation which normally occur after germ band retraction still take place, despite this failure. The visceral mesoderm (Bienz *et al.*, 1988) expresses *Ubx* in wild-type (A2; arrow) and mutant embryos (B2, C1, C2; arrows); however, the differentiation of these cells is abnormal in both mutants. Normally, these cells form a small cluster of muscle cells flattened and tightly apposed to the gut. In *nkd*, these cells form a large flattened sheet of cells (arrows in B2) and in *zw3*, these cells remain as large, round clusters (arrows in C1 and C2) which fail to migrate dorsally and neither flatten nor differentiate. The CNS of the wild-type (A3) has a very regular pattern of cells within each segment, and there are several nuclei, marked with open arrows, in T1–T3, which express *Ubx* as well. In *nkd* embryos (B3), the CNS appears more disorganized and the cells in T1–T3 are not present or no longer express *Ubx*. The CNS is even more disorganized in *zw3* embryos (C3), and the positive cells in T1–T3 are not evident, but the global pattern of *Ubx* expression is still visible. In all panels anterior is up.


B
D
A
C

maternal effect, *nkd*-like embryonic phenotype also produce these homeotic transformations of hairs into bristles, we generated X-ray-induced mitotic recombination on chromosomes bearing one of the *zw3* alleles. Clones in the wing blades were obtained and homeotic transformations of hairs into bristles were observed (Fig. 8) for all five *zw3* mutations (Table 1), indicating that both the segment polarity maternal effect phenotype and the adult transformation of hairs into bristles are not allele specific.

The sensory bristles of the wing are normally innervated by neurons which project axons proximally through the developing wing. Normally a common precursor cell gives rise to both the cuticular projection of a sensory bristle and the neurons associated with it (Bodenstein, 1950). To test whether the ectopic bristles produced by somatic clones of *zw3* cells are in fact innervated, pupae were dissected 30 hr after the initiation of pupation and everted wings were removed and stained with anti-HRP (see Materials and Methods). As shown in Fig. 9, clones of ectopic neurons can be found in wings of *zw3* heterozygotes following irradiation. This indicates that the transformation caused by the *zw3* mutation is complete and must change the fate of presumptive epidermal cell precursors to that of sensory cell precursors.

DISCUSSION

Multiple Functions of zeste-white 3

In this paper we describe *zw3*, a new homeotic gene. Lack of either maternal or zygotic activity at the *zw3* locus causes distinct developmental transformations.

Lack of maternal $zw3^+$ activity leads to a maternal effect lethal phenotype that is similar to the zygotic lethal phenotype of mutations at the *naked* locus (Jurgens *et al.*, 1984). This similarity is seen both in the altered expression of segmentation and homeotic genes and in the cuticle defects in mutant embryos (Figs. 2, 3, 7). It is important to note that *zw3* embryos have in general a phenotype more severe than that of *nkd* embryos. This may be due to a maternal component of *nkd* gene expression which would ameliorate its zygotic effects. Alternatively, it is possible that *zw3* has additional functions during embryogenesis which might contribute to the embryonic phenotype.

There are two zygotic requirements of *zw3* that can be distinguished. The first one is during larval development since mutant larvae derived from heterozygous mothers die. The second one is observed when clones of homozygous cells are generated by mitotic recombination. Lack of zygotic function of *zw3* during pupation causes homeotic transformations of hair cells into sensory bristles. This pupal phenotype of *zw3* is an imaginal equivalent to the classic neurogenic phenotype, that is, the conversion of an epidermal precursor cell to a neuronal precursor cell (Campos-Ortega, 1988). However, we found no evidence for a similar neurogenic phenotype in embryos derived from germ line clones homozygous for *zw3*. Similarly, there are no obvious nervous system or cuticular defects in *zw3* larvae derived from heterozygous females that would implicate a relationship to the maternal or pupal requirements.

The Segment Polarity Phenotypes of zw3 and nkd Are Similar

Mutations in both *nkd* and *zw3* are associated with a segment polarity phenotype in which the anterior part of every segment is deleted and replaced by naked cuticle reminiscent of the normal posterior region.

The most likely explanation for this phenotype is that cells which require expression of $zw3^+$ and nkd^+ for their normal fate are transformed into cells expressing en^+ (see also Martinez-Arias *et al.*, 1988). This transformation affects the nature of the cuticle that they secrete leading to the naked appearance of the cuticle. Possibly because of the ectopic expression of *engrailed*, segmentation is abnormal in *zw3* and *nkd* embryos. This can be clearly seen in the SEMs of *nkd* embryos (Fig. 4). These segmentation defects may directly cause the global defects observed in the nervous system (Fig. 5) and the misrouting of PNS axons (Fig. 6). The embryonic phenotype is characterized by a reduction in identified neurons rather than an increase. Neurons which arise from the anterior region of the segment (aCC, pCC, MP2's, most of the Ubx^+ cells) are present. However, at least some of the neurons derived from the posterior region, such as the VUMs, are absent (or undetectable using the probes described here), indicating that the deletions and duplications of pattern elements seen in the cuticle are not directly reflected by deletions and duplications of similar pattern elements in the

FIG. 9. *zw3* clones in developing wings are innervated. Everted wing discs were dissected from pupae 30 hr after pupation and stained with anti-HRP. In the wild-type wing (A) there are sensory cells on the longitudinal veins L1 (open arrows) and L3 (closed arrows). B and C show small clones of one or two neurons in wings of *zw3* heterozygotes following irradiation. Note that in each case the ectopic neurons have axons which bifurcate and grow both proximally and distally. D is a higher magnification of the clone shown in C.

CNS. It is possible that the nervous system phenotype of *nkd* and *zw3* mutations is due to independent requirements for the genes during segmentation and neurogenesis, as has been found for a number of other segmentation genes (Doe *et al.*, 1988a,b). The large clusters of chordotonal organs which are observed are the result of fusions of adjacent segments rather than overproliferation. One possible explanation for these fusions may be that as normal segmentation breaks down in *zw3* and *nkd* embryos, the cells of the PNS are no longer confined within their segmental boundaries.

The ectopic *en* expression detected in *nkd* mutant embryos (Fig. 3B) is consistent with the results of Martinez-Arias *et al.* (1988). Using *in situ* hybridization, Martinez-Arias *et al.* (1988) have shown that in *nkd* embryos the pattern of *en* transcription is first changed shortly after gastrulation and that the *en* stripes become broader as each covers approximately one-half, rather than one-third to one-quarter, of a metameric unit. Interestingly, Martinez-Arias *et al.* (1988) have shown that the cells that ectopically express *en* are localized posteriorly to the regular *en*-expressing cells in wild-type embryos. Our data show that the segmental furrow in both *nkd* and *zw3* embryos is still posterior to the *en* domain (Figs. 3B3, 3C3) suggesting that in both *nkd* and *zw3* embryos the segmental furrow is shifted posteriorly. These results are consistant with the shift of the parasegmental grooves in *nkd* embryos detected at an earlier developmental stage by Martinez-Arias *et al.* (1988). It is worth noting that mutations in other segment polarity loci are also associated with defects in the parasegmental and segmental grooves. In segment polarity mutations that do not show late expression of *en* (i.e., *wingless* and *dishevelled;* Perrimon and Mahowald, 1987; DiNardo *et al.*, 1988; Klingensmith *et al.*, 1989), there is no segmental furrow formation. In *patched* embryos, in which *en* is expressed in additional stripes, extranumerary furrows are observed (see Figs. 1-6 in Perrimon and Mahowald, 1988; Martinez-Arias *et al.*, 1988). These results support the hypothesis that it is the juxtaposition of en^+ cells with the most posteriorly located en^- cells that defines the final position of the segmental furrow.

In both *nkd* and *zw3* embryos segment fusions are observed after germ band retraction, which are responsible for the late pair rule appearance of *zw3* (see the fusion of ganglia in Fig. 5F) and *nkd* (Fig. 4E) embryos. These fusions may be caused by the juxtaposition of en^+ cells with abnormal neighbors, which causes a breakdown of the cell-cell interactions required to maintain normal segmentation. An alternate explanation for the naked phenotype is that the most anterior cells of each segment die in both mutants and are replaced by regeneration of en^+ cells.

Segment Polarity Loci Represent a Diverse Group of Genes: Role of zw3?

As indicated in the Introduction, the segment polarity genes can be subdivided into four phenotypic classes: *naked*-like, *patched*-like, *wingless*-like, and *engrailed*-like. There are at least 14 loci, all zygotically required, which have been identified as belonging to one of these classes; these are listed along with what is known of their maternal requirements (Table 3). *zw3* is the first locus to exhibit a maternal effect similar to the zygotic effect of *nkd.*

A few of the segment polarity genes have been cloned and their spatio-temporal expression patterns determined via *in situ* hybridization to embryos. Some of them, such as *en* (Weir and Kornberg, 1985; Fjose *et al.*, 1985), *wg* (Baker, 1987) and *gooseberry* (Bopp *et al.*, 1986; Cote *et al.*, 1987), have transcripts that accumulate in segmental stripes. The coding sequences of *en* and *gsb* reveal characteristics of DNA binding proteins. In contrast, transcripts from the *armadillo* locus are seen throughout the embryo (Riggleman *et al.*, 1989). No information is available yet on the spatial pattern of expression of *zw3* or *nkd.*

It is likely that at least some members of the segment polarity class will belong to one of two types of genes:

TABLE 3
MATERNAL AND ZYGOTIC REQUIREMENTS OF SEGMENT POLARITY GENES

	Maternal	Zygotic	Reference
naked-like			
naked	?	+	
zeste-white 3	+	+	This work
patched-like			
patched	?	+	
costal-2	+	+	Grau and Simpson, 1987
wingless-like			
armadillo	+	+	Wieschaus and Noell, 1986
dishevelled	+	+	Perrimon and Mahowald, 1987
porcupine	+	+	Perrimon *et al.*, 1989
fused	+	+	Counce, 1956
wingless	−	+	Baker, 1988
gooseberry	?	+	
hedgehog	?	+	
Cell	−	+	Orenic *et al.*, 1987
Cubitus-interruptus^D	−	+	Orenic *et al.*, 1987
engrailed-like			
engrailed	−	+	Lawrence *et al.*, 1983

Note. Mutations have been grouped into four groups on the basis of the embryonic mutant phenotype. References are provided only for the maternal requirement.

those which regulate or transmit intercellular signals, expected to be nonautonomous, and those which receive or transduce such signals, expected to be autonomous. For instance, there is both genetic and molecular evidence to suggest that the *wg* gene product acts as a secreted signal (Morata and Lawrence, 1977; Babu and Bhat, 1986; Baker, 1987, 1988; Rijsewijk *et al.*, 1987; Cabrera *et al.*, 1987; Papkoff *et al.*, 1987; R. Nusse, personal communication). Other genes (e.g., *armadillo*), which have phenotypes identical to *wg*, are cell autonomous (Wieschaus and Riggleman, 1987) and are possibly involved in transducing the *wg* signal (Riggleman *et al.*, 1989).

The function of *zw3* is cell autonomous in the adult; since it is not yet known if the requirement for *nkd* is cell autonomous, it is possible that the function of the *zw3* gene product is to receive or transduce the *nkd* signal. Genes such as *zw3*, which exhibit maternal effect phenotypes that resemble zygotic lethal phenotypes, may not be required to initiate, but rather to realize or maintain specific zygotic functions (Perrimon and Mahowald, 1986). It is not clear how the late neurogenic phenotype is related to the embryonic phenotype, but there is precedent for genes important early in development being used later for related but different functions. Other mutations, in particular mutations at the *hairy* locus, are known to affect both embryonic segmentation and development of wing sensory structures (Ingham *et al.*, 1985b). If, in fact, *zw3* is required to receive or transduce some intercellular signal, then this function may be required for the normal development of hair cells in the adult epidermis as well. Molecular characterization of the *zw3* and *nkd* loci will distinguish these possibilities.

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