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Morphogenesis of Denticles and Hairs in *Drosophila* Embryos: Involvement of Actin-Associated Proteins That Also Affect Adult Structures

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We sought components that function in morphogenetic events downstream from the segmentation pathway in *Drosophila* embryos, so we examined mutations that affect development of adult hairs and/or bristles to identify a subset that also affect hairs and denticles on the cuticle of first instar larvae. Mutations at 4 of 23 loci surveyed cause distinct abnormalities in these larval structures, and two other loci have more subtle, variable effects. In particular, *forked* and *singed* mutants produce complex, allele-specific phenotypes. These loci encode actin-associated proteins and, consistent with that information, mutations cause abnormalities in actin bundles that support nascent hairs and denticles in stage 14–16 embryos. We suggest that interactions between these and other actin-associated proteins are important in generating the diverse shapes of the cuticular specializations seen in both larvae and adults. Cell Motil. Cytoskeleton 38:9–21, 1997.

Key words: *Drosophila*; morphogenesis; cytoskeleton; actin; *forked*; *singed*

INTRODUCTION

Pattern formation and morphogenesis are connected but distinct aspects of development. Pattern formation specifies cell fates in an orderly array, and morphogenesis molds three-dimensional shape. These processes must be connected; most cell fates include morphogenetic events ranging from coordinated movements that generate large-scale morphology to individual changes in shape associated with differentiation. However, little is known about the linkage of patterning to morphogenesis. Because the genes and molecules that control segmentation in *Drosophila* embryos have been studied in considerable detail [Lawrence, 1992; Martinez Arias, 1993], the generation of specialized structures at specific positions on the cuticle of the first larval instar provides a potentially useful model for investigating those connections. Denticles and hairs whose arrangement is specified by the segmentation pathway are formed around cytoplasmic extensions supported by cytoskeleton [Poodry, 1980; Martinez Arias, 1993], and it seems likely that the diverse shapes represented among those features [Lohs-Schardin et al., 1979; Campos-Ortega and Hartenstein, 1985 Bejsovec and Wieschaus, 1993] are determined in part by proteins that organize cytoskeleton in characteristic patterns. Thus, it is reasonable that the genes encoding cytoskeletal proteins are one important link between patterning and morphogenesis.

As a step toward examining this hypothesis, we have begun to identify relevant genes and proteins. A strategy was suggested by the demonstration that at least some mutations that cause abnormal hair and bristle morphology in adult *Drosophila* identify genes encoding actin-associated proteins. For example, *singed* (*sn*) encodes a homologue of fascin, an actin-bundling protein first characterized in sea urchins [Bryan et al., 1993], and

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forked (*f*) encodes another protein that cross links actin in bundles present in developing bristles in pupae [Hoover et al., 1993; Petersen et al., 1994; Tilney et al., 1995]. In other systems, the number of actin-bundling proteins is modest [Matsudaira, 1991], and it is common for developmentally important genes to play roles in diverse contexts. Indeed, both the *Drosophila* fascin homologue and another actin-associated protein, profilin, are involved in oogenesis and in bristle formation [Verheyen and Cooley, 1994; Cant et al., 1994] Thus, mutations in some of these genes and in others producing abnormal phenotypes in adult hairs and bristles also may affect structures on the larval cuticle. In the present study, we confirm that hypothesis and show that, as expected, definitive phenotypes are foreshadowed by abnormal organization of actin cytoskeleton in embryonic epidermal cells.

MATERIALS AND METHODS

Stocks

Table I lists the *Drosophila* stocks examined and their sources. Relevant adult phenotypes were confirmed, and descriptions are in Lindsley and Zimm [1992]. Two stocks with *forked* deletions also were utilized: Df(1)ST985 Df(1)w^{67}c$^{23.2}$, *y*/In(1)sc^{S1L}sc^{8R}+dl49, and *sn^{X2} B* (Nancy Petersen, University of Wyoming) and Df(1)B$^{263\text{-}20}$, *B$^{263\text{-}20}$*/In(1)sc^{7} In(1)AM, and *sc^{7} car* (Mid America Drosophila Stock Center, Bowling Green State University).

Cuticle Preparations

Eggs were collected on apple-juice plates [Wieschaus and Nusslein-Volhard, 1986], washed, allowed to hatch in 0.7% NaCl, 0.02% Triton X-100 (NaCl-TX), fixed, and cleared in a mixture of 4 parts CMCP-10 mounting medium (Polysciences, Warrington, PA), 2 parts glacial acetic acid, and 1 part 85% lactic acid. Larvae were stored at 45–60° in this medium (a convenient substitute for Hoyer's medium) until it reached a viscosity approximating unmodified CMCP-10 (loosely covered for 1–2 h to allow acetic acid to evaporate). They were transferred to a slide in a drop of the remaining medium, covered, and placed overnight on a slide warmer at 45°C to complete clearing. Cuticles were examined with darkfield and phase-contrast optics. Photographs were made with a 100× (N.A. 1.3) Neofluar phase-contrast objective on a Zeiss photomicroscope III using Kodak technical pan film subsequently developed for high contrast (Kodak HC-110 developer, dilution D). All phenotypes were confirmed on multiple specimens from several independent preparations.

TABLE I. *Drosophila* Stocks

Locus of interest	Genotype	Stock[a] or source
Blunt short bristles	*Bsb^{1}*/TM6	1697
brevis	*bs^{1}*	455
Bristle	*BL^{1} L^{2}*/SM5	238
downy	*dow^{1}*/FM6	29
flare	*flr^{1}*/TM2	1132
forked	*f^{1}*	36
	w^{i} f^{3} bb^{N}	163
	v f^{3N} car	[b]
	w^{bf} f^{5}	157
	yvf^{X}	[b]
frizzled	*fz$^{1?}$*/TM1	1676
Hairless	*H^{1}*/In(3R)P	515
hook	*hk^{1}*	306
javelin	*jv^{1}*	521
javelinlike	*jvl^{1}*	523
Kinked	*Ki^{1}*	525
minus	*mi^{1}*/In(2LR)*bw^{V32g}*	350
multiple wing hairs	*mwh^{1}*	[b]
Pin	*Sp^{1} J^{1} L^{2} Pin^{1}*/SM5	408
prickle	*pk^{1} cn^{1}*	367
Prickly	*Pr^{1}*/In(3R)C, *e^{1}*	559
Stubble	*Sb^{1}*/In(3LR)Ubx^{101}	585
	ry Aldox^{n} sbd^{2}	[c]
singed	*w$^{1?}$ sn$^{1?}$; e$^{1?}$*	1870
	w^{co} sn^{2}	153
	sn^{3}	113
	sn^{4}	115
	sn^{34e}	116
	In(1)dl-49, *sn^{X2}*/In(1)Mud	[d]
smooth	*sm^{1} px^{1} pd^{1}*/SM5	400
spineless	*ss^{1}*	2973
tinylike	In(1)dl-49, *tyl^{1}*	778
tricorner	*ru^{1} jv^{1} trc^{1} ca^{1}*/TM3, *Ser^{1}*	1135

[a]Numbered stocks are from the *Drosophila* Stock Center, University of Indiana, Bloomington.
[b]From Dr. Kent Golic, University of Utah.
[c]Fromour own collection.
[d]From Dr. Lynn Cooley, Yale University.

Fluorescent Staining of Cytoskeleton

F-actin patterns were detected with rhodamine-phalloidin. Six-hour egg collections on apple-juice plates were incubated at 22°C for an additional 10–12 h and washed in NaCl-TX. Embryos were dechorionated, fixed, and released from vitelline membranes, basically as described by Tautz and Pfeifle [1989], except with 4% formaldehyde in phosphate buffered saline (PBS) as the fixative and 80% ethanol instead of methanol (which interferes with phalloidin binding) to ''pop'' vitelline membranes. We confirmed that embryos treated with ethanol had staining patterns and intensities comparable to hand-peeled embryos. Samples were refixed for 20 min in 4% formaldehyde–PBS, washed in PBS, 0.1% Triton X-100 (PBS-TX), and stored in that solution at 4°C for up to 2 weeks prior to staining. Embryos were rolled overnight at 4°C in rhodamine-phalloidin (Molecular

Probes, Eugene, OR) at 0.315 μM in PBS-TX (10 μl of reconstituted rhodamine-phalloidin stock solution in 200 μl), washed in PBS-TX (3 × 10 min at room temperature), and cleared and mounted in 70% glycerol. Embryos were staged by morphological criteria [Campos-Ortega and Hartenstein, 1985; Hartenstein, 1993]. Features allowing precise staging by phalloidin staining pattern are described below. Except for that shown in Figure 7, phalloidin staining patterns were analyzed and photographed by conventional epifluorescence on the Zeiss photomicroscope III with a 100× planapo objective. Tubulin was stained following the method of Foe [1989] using monoclonal anti-alpha tubulin (clone DM-1A from ICN Biomedicals) and a rhodamine-labeled affinity-purified goat secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Patterns were analyzed and recorded on a BioRad confocal microscope, as were the actin patterns shown in Fig. 7.

RESULTS

Cuticle Phenotypes

In wild-type larvae, denticles on the ventral surface of each abdominal segment except the first are arranged in six rows distinguished by size, shape, and polarity [Bejsovec and Wieschaus, 1993] (Fig. 1a). Nonsensory hairs cover much of the dorsal and lateral ectoderm in each segment and occur in a consistently arranged series of forms ranging from short, stout, and triangular to long, narrow, and gradually tapered [Lohs-Schardin et al., 1979; Campos-Ortega and Hartenstein, 1985] (Figs. 3a, 4a). A plane of polarity reversal, behind which hairs point forward, occurs in abdominal segments at a position that shifts anteriorly within the segment as one moves to more caudal segments. Longer, structurally distinct sensory hairs are spaced at regular positions on each segment. We examined these cuticular features in mutant larvae at each of 23 loci selected because they have adult phenotypes that include abnormal hairs and/or bristles of various sorts. At least some alleles at 4 of these 23 loci produced readily recognizable abnormalities in one or more larval features. These are *forked* (*f*), *singed* (*sn*), *multiple wing hairs* (*mwh*), and *Hairless* (*H*). Two other loci, *Stubble* (*Sb*) and *javelin* (*jv*), have subtle and somewhat variable effects and can overlap the wild type.

The *forked* Locus

Among the loci examined, *forked* has the most striking effects on larvae. Interestingly, the most extreme denticle and hair phenotypes are produced by different alleles, f^{3N} and f^{36a} respectively. With the former (Fig. 1b), denticles of all rows display normal arrangement and polarity, but they are thin and sinuous (''goose-necked'') compared with the stout hooks seen in the wild type. A more subtle but similar effect can be seen in the normally stout, triangular hairs near the anterior margins of segments and in ventral bands on the thoracic segments (Fig. 4b). Longer hairs are close to normal but typically are not as straight, uniformly tapered, and pointed as wild type. Altogether, hairs in f^{3N} have a slightly ''softer'' appearance than hairs in wild type (compare Fig. 4b with 4a). Several other *forked* alleles affect denticles in a similar fashion but to varying degrees: f^{3} is indistinguishable from f^{3N}, f^{X} and f^{5} are intermediate, and f^{1} is a weak allele in larvae.

In contrast, f^{36a}, one of the most extreme *forked* alleles in adults, has an intermediate denticle phenotype (Fig. 1c) but a striking and opposite effect on nonsensory hairs, particularly the longer ones (Fig. 3b). Hairs are short, fat, often spindle shaped, and sometimes apparently branched, fused, or duplicated laterally. The sensory hairs on both thoracic and abdominal segments also are affected but in a different way. They are approximately normal in length and thickness but display a variety of branched configurations (Fig. 4e–h), often with a wide range of variation within each specimen. The effect on denticles is somewhat specific by row (Fig. 1c). Second-row denticles are most noticeably thin and elongate; those in the fourth row have thin hooks that sometimes look ''floppy'' or curve backward; and those in the fifth row are smaller than normal. Rows 1 and 3 are almost normal, with hooks that are just a little thin and straight in most cases.

To investigate the null phenotype for *forked*, we used two different deletions that overlap the locus: $Df(1)B^{263\text{-}20}$ [Lindsley and Zimm, 1992] and an unnamed deletion in a background of $Df(1)w^{67}c^{23.2}$, recovered by Soichi Tanda (University of Maryland, personal communication, 1996) and temporarily designated Df(1)ST985. Although both are homozygous/hemizygous lethal, male escapers derived from heterozygous mothers are relatively common at the first larval instar. Identification is confirmed in the latter case by a *yellow* marker on the deletion chromosome. These males, which must lack any zygotic contribution to *forked* function, have the denticle phenotype of f^{3N} and the hair phenotype of f^{36a}. Likewise, female larvae heterozygous for either of those alleles over a deletion resemble the corresponding homozygote. Thus, absence of zygotic *forked* function produces the combined defects described for the two most severe alleles but not complete absence of hairs and denticles. Females homozygous for a deletion do not survive, so we cannot rigorously rule out some maternal contribution of forked product (mRNA or protein). However, the phenotypes of male escapers derived from mothers in which a deletion was heterozygous with f^{36a} or f^{3N} are not obviously more severe than those from mothers heterozygous for a wild-type allele, so a major maternal contribution seems unlikely.

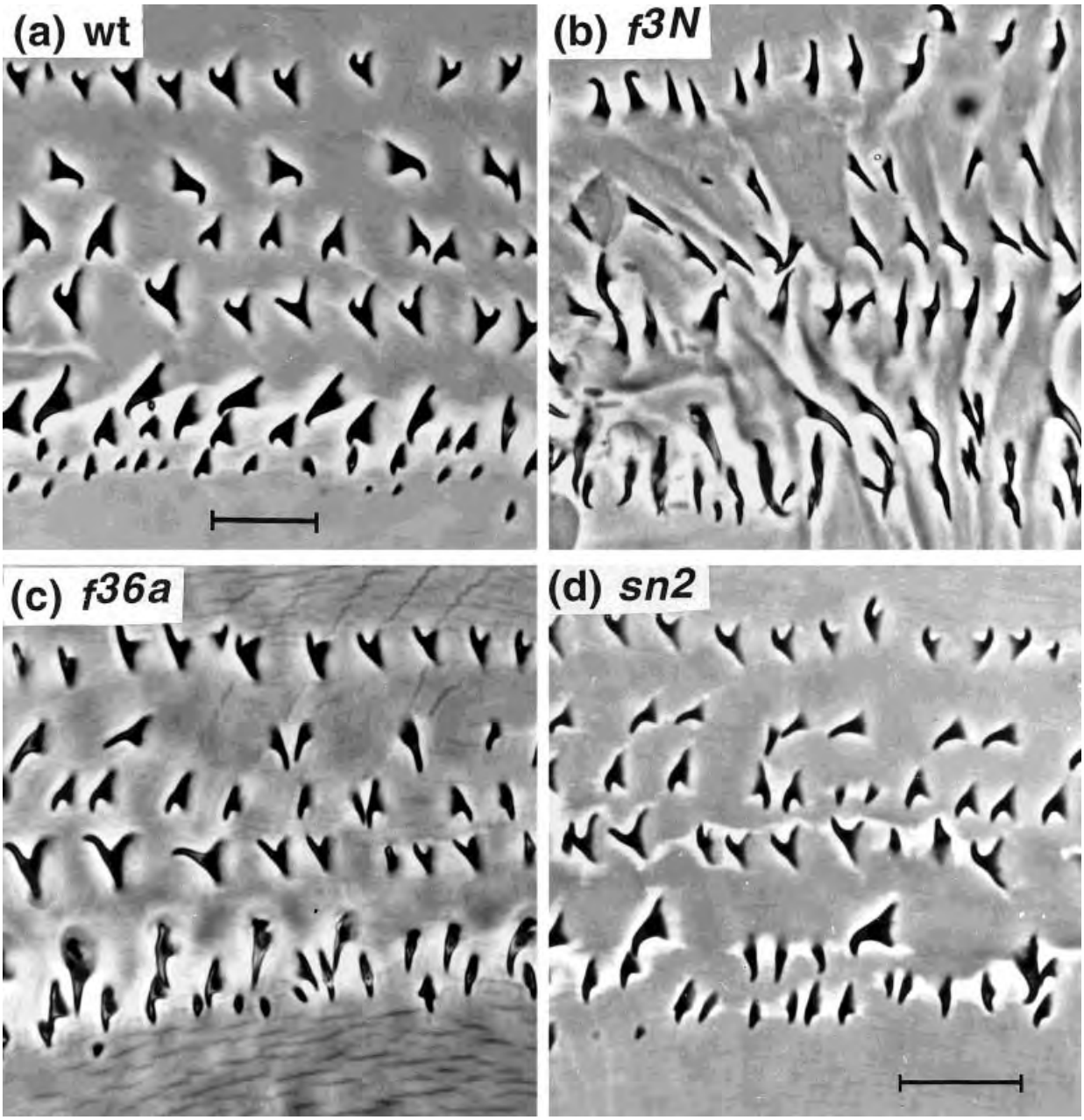

Fig. 1. Normal and mutant denticles. Each panel shows the ventral surface of a portion of the fifth abdominal segment of a first instar larva. In this and Figures 2–6, anterior is at the top. The specimens are wild type (**a**), f^{3N} (**b**), f^{36a} (**c**), and sn^{2} (**d**). Scale bars in all figures = 0.01 mm.

The *singed* Locus

Relative to *forked* mutations, the effects of *singed* mutations are more variable between individuals but less so across alleles. Denticles tend to be smaller and have thinner hooks than wild-type denticles (Fig. 1d). They also are less uniform and orderly along each row and sometimes have a ''weak'' or ''floppy'' appearance similar to f^{36a}. It is difficult to rank alleles precisely in terms of severity of the denticle phenotype, but sn^{X2}, sn^{2}, and sn^{3} are the strongest and sn^{34e} the weakest among those examined. Nonsensory hairs have an obvious (but variable) phenotype only in sn^{X2} among the alleles examined. In the most extreme cases (Fig. 3c), some hairs approach the spindle shape seen in f^{36a}, but often they are just somewhat shorter and thicker than normal (Fig. 3d). Other alleles have, at best, a subtle hair phenotype; longer hairs may be slightly thickened in the middle compared with the uniform taper of the wild type. Other alleles

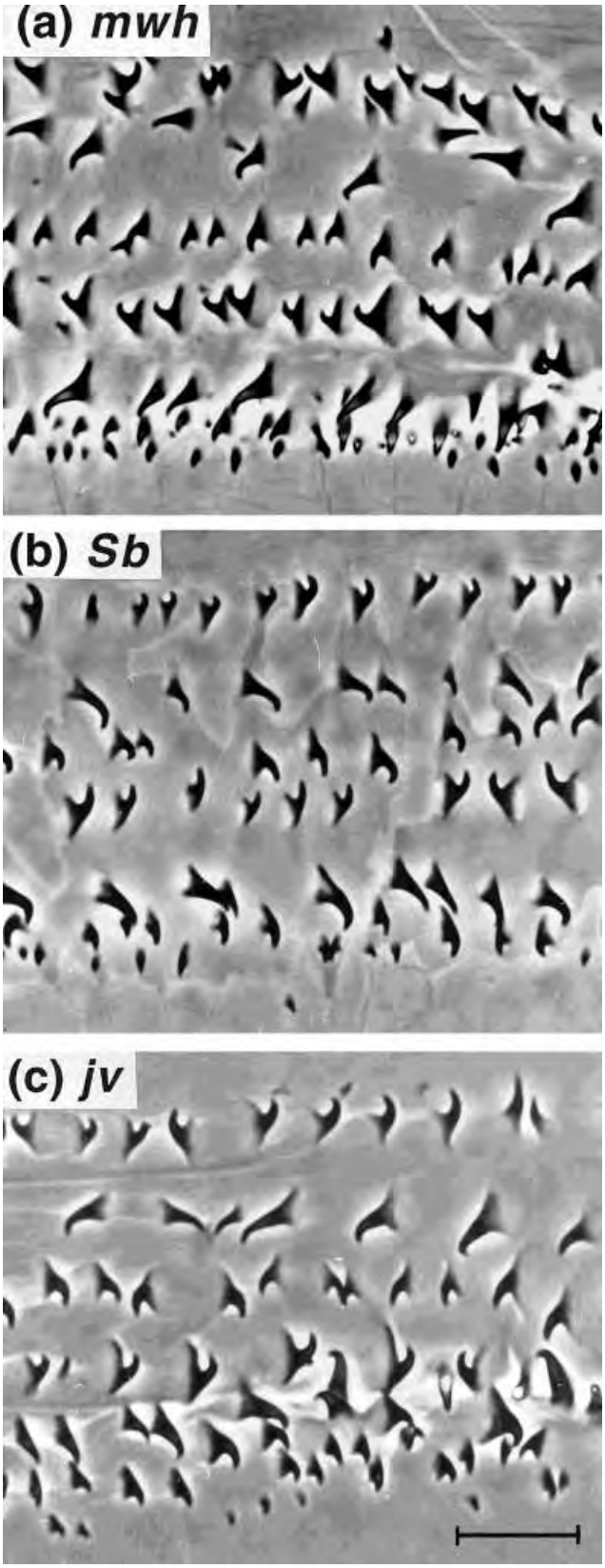

Fig. 2. Additional denticle phenotypes. The phenotypes represented are *mwh* (**a**), *Sb* (**b**), and *jv* (**c**). Compare these with the wild type in Fig. 1a.

clearly affect sensory hairs (Fig. 4i–k), producing variable branched forms resembling those seen with f^{36a}, although at a lower frequency.

It is more difficult to establish a null phenotype for the *singed* locus. The sn^{X2} is reported to be a null, at least in pupae [Cant et al., 1994], and it has the strongest larval phenotypes of any *sn* allele examined. However, like many *sn* alleles, it causes female sterility. The essential contribution occurs late in oogenesis [Cant et al., 1994], so mutant embryos obtained from heterozygous females may contain significant maternal product.

Other Loci

Larval phenotypes produced by *multiple wing hairs* are shown in Figures 2a, 3e, and 4c. Both hairs and denticles are smaller, more numerous, and less orderly than in those in the wild type. As with *forked* mutations, the effect changes somewhat with the particular ''target.'' For example, there are extra first-row denticles (recognized by hooks that point anteriorly), and they are not in a neat single row. The second row (hooks point posteriorly) also is poorly aligned (sometimes intermingled with row one) but contains few if any extra members. Rows three and four are fairly orderly but spaced more closely to accommodate extra elements. The *Hairless* mutation affects dorsal hairs but apparently not denticles. Hairs are sparse compared with those of the wild type (Fig. 3f), with all types of non-sensory hairs similarly affected. The Sb^{1} heterozygotes have denticles that are smaller, thinner, and more variable in size and shape than those in the wild type (Fig. 2b), but they are difficult to separate reliably; homozygotes for sbd^{2} (a recessive allele at the same locus) are similar. Hairs appear normal in larvae of both genotypes. The *javelin* zygotes have denticles that tend to be a little thin and elongate, particularly in rows 1 and 2 (Fig. 2c), but again rather variable and difficult to distinguish reliably from those in the wild type.

Actin Patterns

Staging criteria. Several features visible in phalloidin-stained specimens are convenient for recognizing equivalent developmental stages in mutants in which details of hair and denticle precursors might differ. (1) A band of F-actin marks the dorsal margin of the ectoderm during closure, allowing one to visualize progress as the converging marginal cells ''zip'' together during stages 14 and 15 [Martinez Arias, 1993; Young et al., 1993]. By early stage 16, chosen as a standard point of comparison, closure is just complete, but the position of the ''suture'' is still detectable (not shown). (2) In the gut at the same stage, distinct chambers [the ''three-part gut'' of Skaer, 1993] are clearly marked by constrictions that also stain

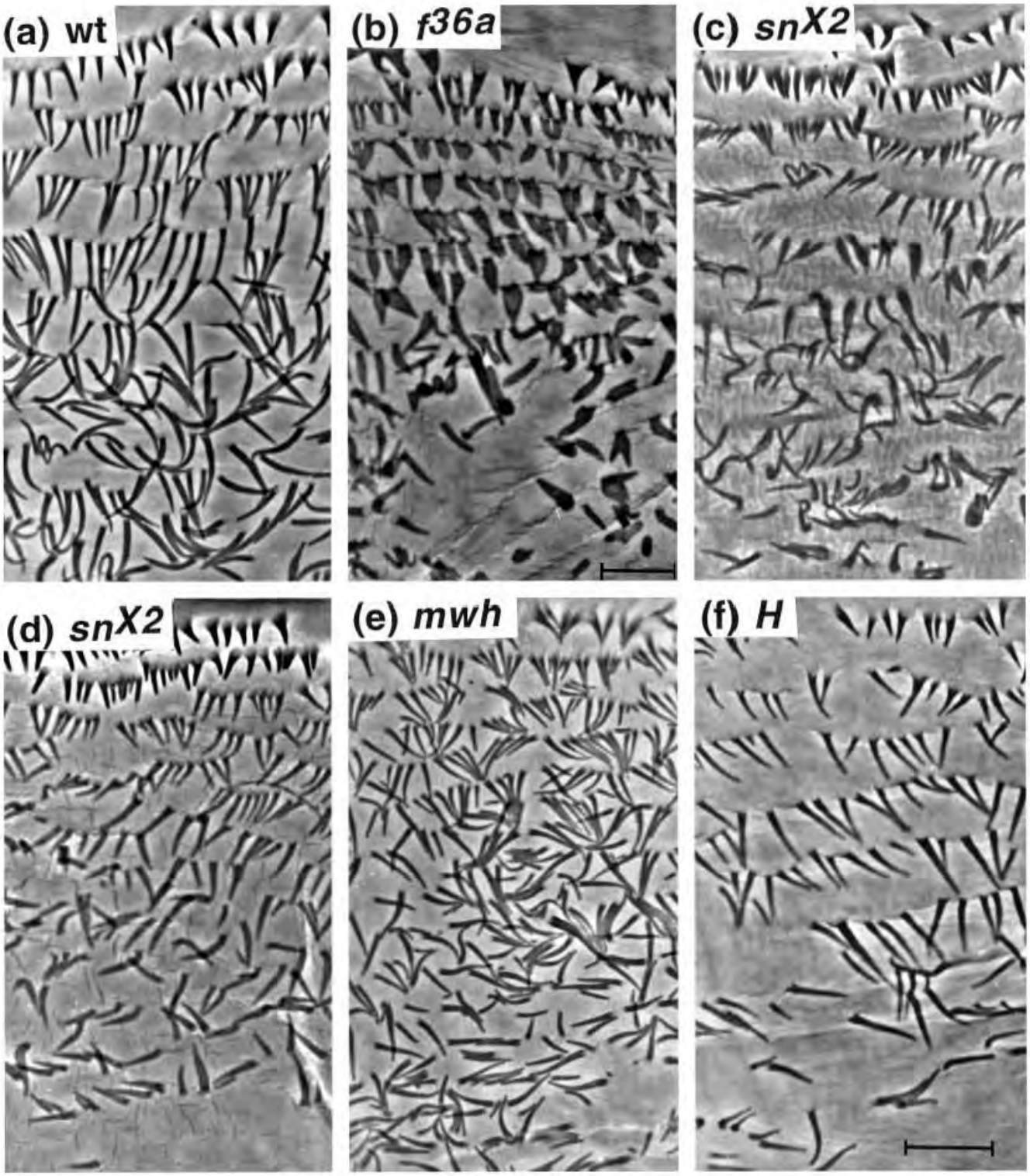

Fig. 3. Normal and mutant hairs. Each panel shows the dorsal surface of the first abdominal segment of a first instar larva. The specimens are wild type (**a**), f^{36a} (**b**), sn^{X2} (**c,d**), *mwh* (**e**), and *H* (**f**).

with phalloidin (Fig. 5a). (3) The ventral nervous system, strongly stained with phalloidin from stage 13, is undergoing condensation [Campos-Ortega and Hartenstein, 1985; Hartenstein, 1993] and by stage 16 forms a ladderlike pattern that is approximately 70–75% of the body length (Fig. 5b). Outgrowths from anterior ganglia (presumably axons) also are stained. (4) Differentiating muscle fibers are just beginning to stain, primarily near the ends (visible as a periodic pattern along the lateral body walls in Fig. 5a,b). In older embryos, the complete pattern of muscle fibers stains intensely.

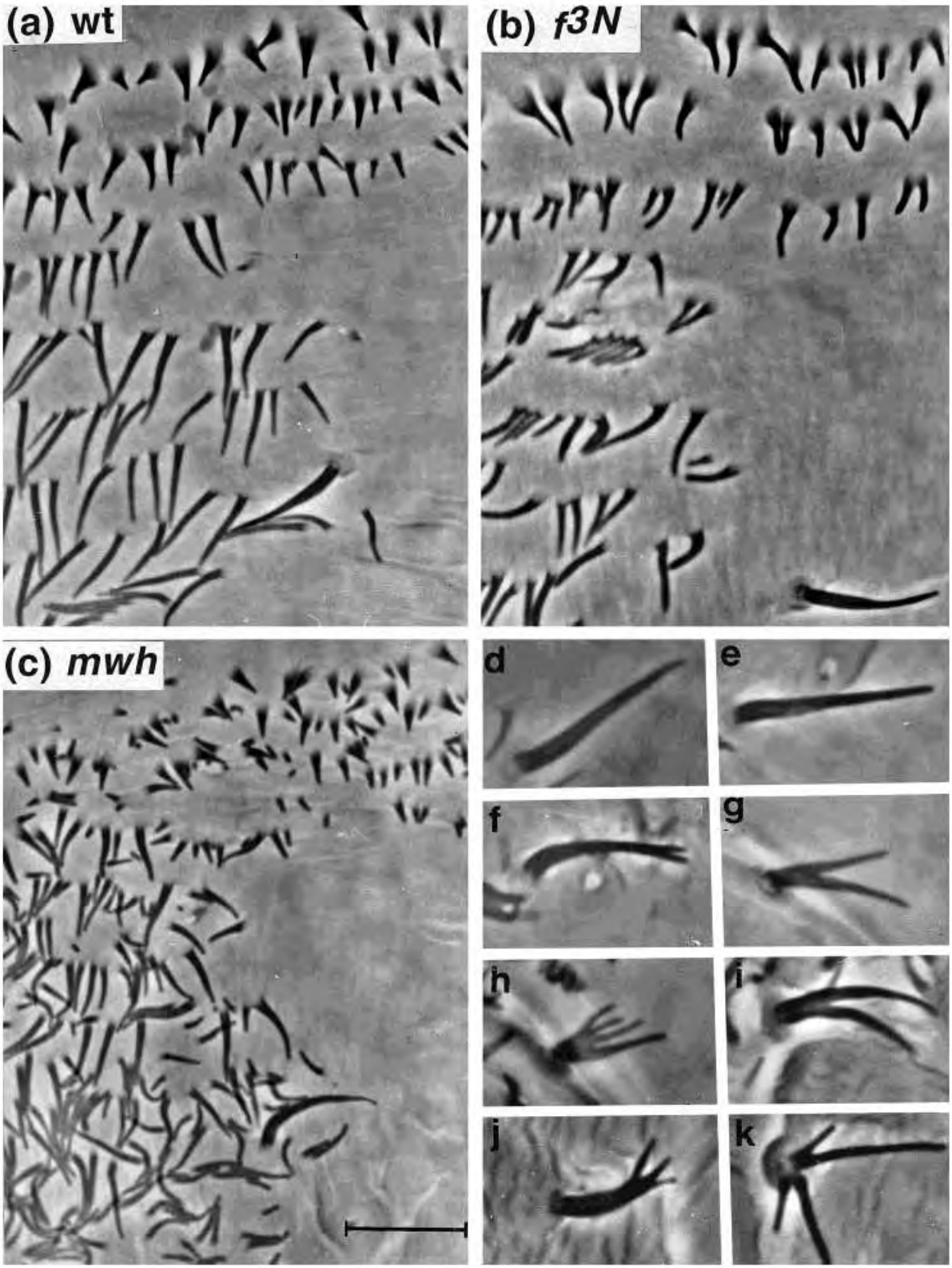

Fig. 4. Lateral and sensory hairs. The large panels are lateral views of the first abdominal segment in first instar larvae of wild type (**a**), f^{3N} (**b**), and *mwh* (**c**). Sensory hairs (larger than the others) can be seen in the lower right quadrant in each case. The small panels show sensory hairs (at approximately 1.5× higher magnification) from larvae of wild type (**d**) f^{36a} (**e–h**), and sn^{X2} (**i–k**).

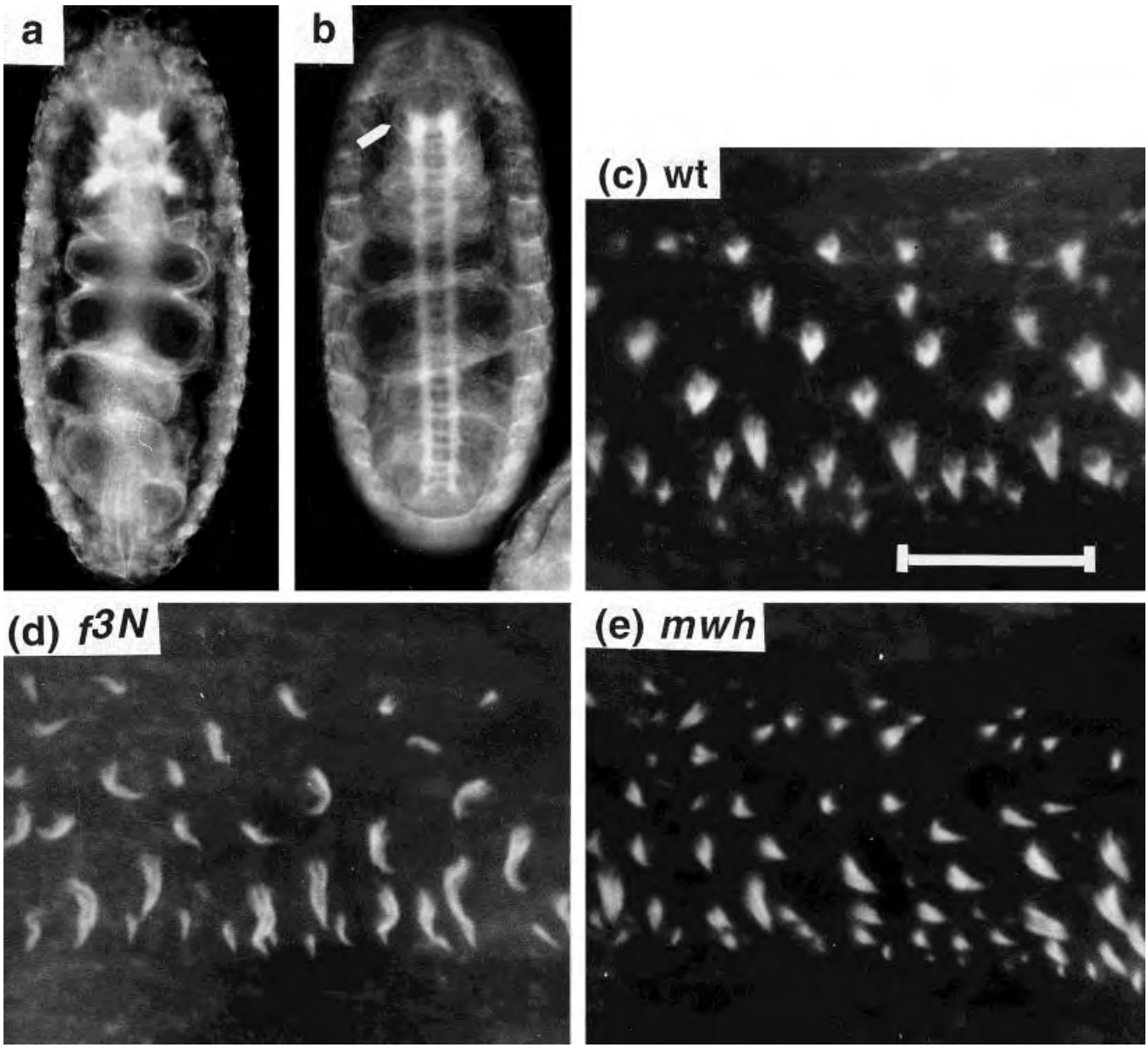

Fig. 5. F-actin patterns. The first two panels are dorsal (**a**) and ventral (**b**) low magnification views of phalloidin-stained embryos illustrating general features used to determine the stage of the embryos shown in the remaining three panels and those shown in Figure 6. The other three panels are views of the ventral surface (denticles) of the fourth or fifth abdominal segment in wild type (**c**), f^{3N} (**d**), and *mwh* (**e**) embryos.

Wild-type actin patterns. The cytoskeleton is involved in formation of hairs and denticles [Poodry, 1980; Martinez Arias, 1993], but available descriptions provide little information on developmental progressions or on differences related to the variety of hairs and denticles ultimately produced. The following brief account of key events and features revealed by phalloidin staining of wild-type embryos provides background for comparisons with mutants.

Orderly arrays of actin bundles are evident in both dorsal and ventral ectoderm during stages 14 and 15 (dorsal closure in progress). Nascent denticles are supported by prominent conical arrays of actin filaments (Fig. 5c) that elongate and become more sharply defined as development progresses. Differences in size between rows are evident, but, interestingly, polarity differences are not; all cones, including those in rows 1 and 4, point posteriorly. By early stage 16 (closure just completed), the dorsal actin bundles are spike shaped, and differences in shape, size, and polarity that correlate well with eventual differences in hair morphology are evident (Figs. 6a, 7e). For example, the bundles are stout and

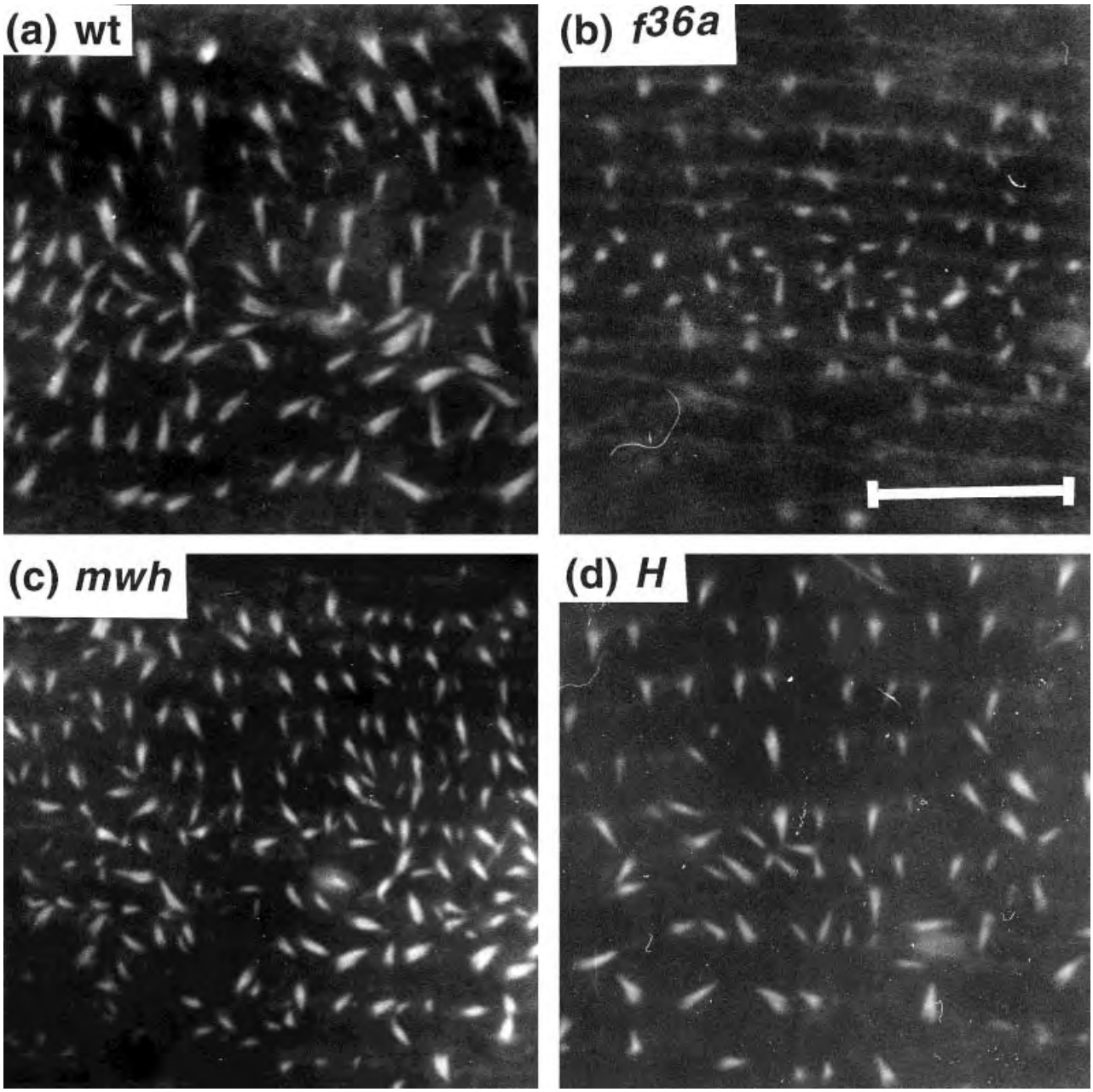

Fig. 6. Dorsal F-actin patterns. These are views of the dorsal surface (hairs) of the second or third abdominal segment of wild type (**a**), f^{36a} (**b**), *mwh* (**c**), and *H* (**d**) embryos.

triangular in the first couple of rows in each segment, longer and thinner in the rows behind those, and reversed in polarity in the posterior portion of abdominal segments. Further elongation and refinements in shape continue at least through stage 16, but early stage 16 is convenient for mutant comparisons because details in later stages are obscured by bright staining of muscle actin. For reference to other events, stages 14–16 cover approximately 10.3–16 h from egg laying at 25°C, with hatching at 21–22 h. Cuticle deposition begins near the stage 15–16 transition, and the major features of the first instar cuticle are in place by stage 17 [Martinez Arias, 1993; Hartenstein, 1993].

In developing adult bristles, a core of microtubules surrounded by actin bundles extends along the axis [Overton, 1967; Petersen et al., 1994]. Using indirect immunofluorescence on wholemounts, we have looked for evidence of a similar association in nascent larval hairs and denticles. At stages when protruding actin bundles are evident, we see no comparable pattern of

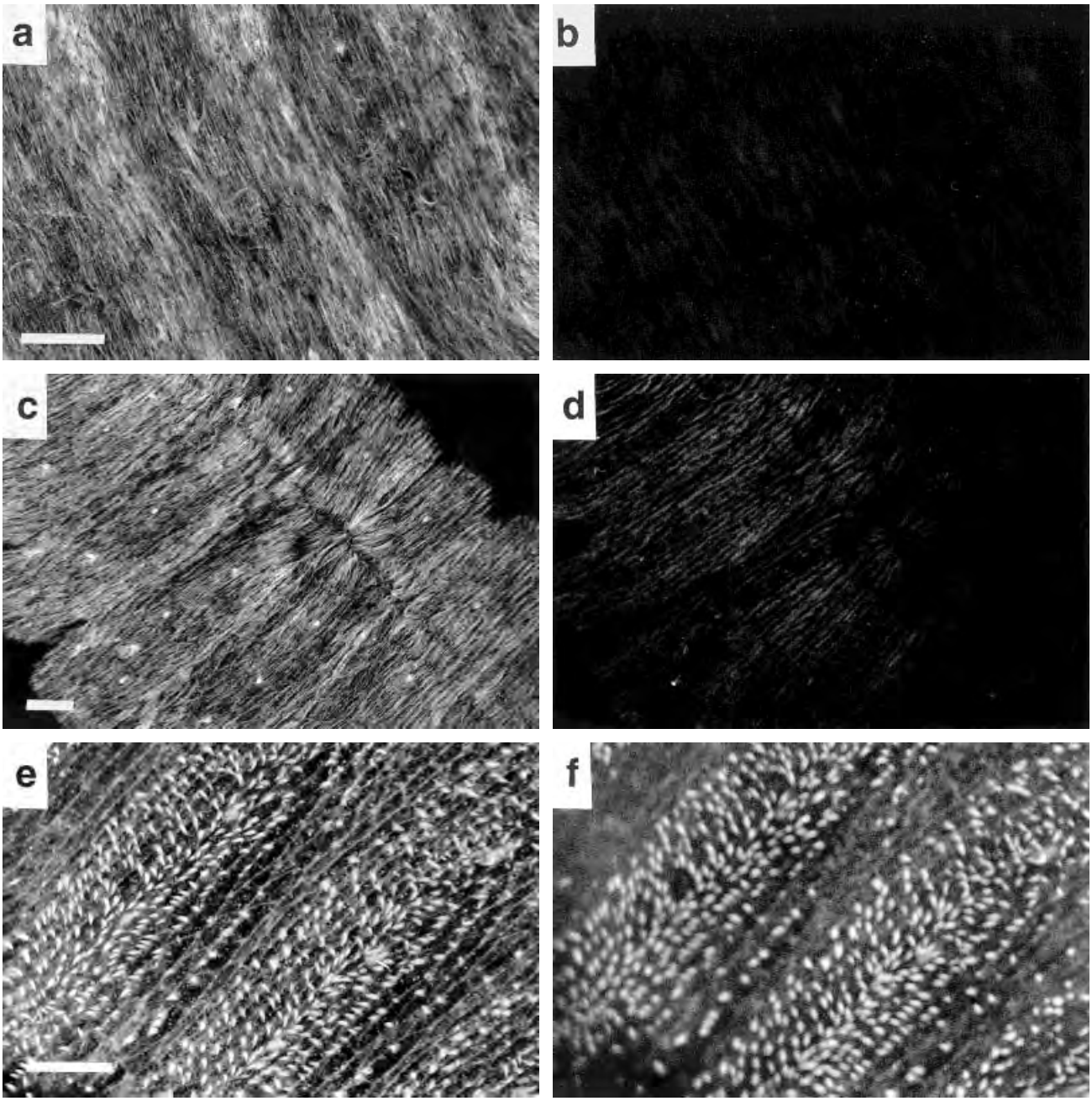

Fig. 7. Confocal views of wild-type tubulin and actin patterns. Panels **a, c, and e** show projections of two consecutive 1-μ optical sections just below the ventral (a) or dorsal (c,e) surfaces. Panels **b, d, and f** are grazing optical sections 1 μ above the same surfaces. Specimens shown in a–d are stained for tubulin, e and f for F-actin. All views are centered on abdominal segments 3–4. Anterior is to the lower left in a and d and to the upper left in c–f. The brighter bands in panel a underlie the denticle bands. Contrast the dorsal hairs clearly visible with actin stain above the dorsal surface (f) with the absence of corresponding tubulin stain either ventrally or dorsally (b,d). The regularly spaced bright spots in panel c mark the positions of sensory hairs. In favorable cases, tubulin stain can be traced into optical sections above the surface.

microtubules in denticles or nonsensory hairs (Fig. 7b,d). There are microtubules oriented in the long axis of the cell bodies (circumferential and, hence, perpendicular to the growth of hairs and denticles) (Fig. 7a,c). Microtubules are more concentrated ventrally in the anterior of each segment, corresponding to the position of denticle bands (Fig. 7a), but they do not extend into cytoplasmic protrusions (Fig. 7b,d). In contrast, microtubules are present along the axes of nascent sensory hairs, including a ''root'' extending below the surface (Fig. 7c).

Mutant actin patterns. In addition to the wild-type phenotype, we have examined actin patterns in f^{3N} f^{36a},

mwh, and *H*, which have the clearest mutant phenotypes. All reveal abnormalities that anticipate the relevant cuticle defects. For example, the denticle precursors in f^{3N} are thin and twisted as compared with more conical controls (Fig. 5d). The arrangement in the rows is normal, and, as in the wild type, polarity differences are not evident. In f^{36a}, hair precursors are small, faint, and poorly formed (Fig. 6b). It is difficult to see whether branched or laterally fused forms are present. In *mwh*, both hair and denticle precursors are smaller, more numerous than normal, and somewhat disorderly (Figs. 5e, 6c). The dorsal actin bundles in *H* are sparse and widely separated (Fig. 6d). In some preparations, we can see outlines of cells defined by cortical actin networks (e.g., Fig. 7e). These networks are fewer and larger in *H* than in wild type, so the basis of the phenotype appears to be a smaller number of hair-forming cells spread more thinly over the surface.

DISCUSSION

Overlap Between Genes That Affect Larval and Adult Cuticle

Among 23 loci chosen on the basis of adult hair and bristle phenotypes, 6 (26%) also affect morphology of denticles and/or hairs in first instar larvae. We believe this is a minimum estimate of the degree of overlap between the sets of genes that function in epidermal morphogenesis at those two stages. The larval structures are one to two orders of magnitude smaller than adult bristles, and a number of the mutations examined cause only subtle phenotypes, even in the adult bristles. Comparable abnormalities in, for example, larval dorsal hairs easily could be overlooked. In addition, larval cuticles are subject to variable effects of preparation. For example, hairs are rather flexible and can be bent or distorted by flow of the medium when mounted. Also, hairs normally display an almost continuous range of variation in size and shape, making it difficult to recognize small abnormalities. Perhaps most important, loci represented by single alleles probably have not been adequately sampled. Where multiple alleles were examined, the severity of phenotypes correlated poorly between larvae and adults, so it is quite possible that the commonly available alleles of some genes (known from adult phenotypes) have little or no effect in larvae even if their products play roles in both stages. Indeed, we initially scored sn^1 and f^1 larvae as normal; only with practice and insight gained from additional alleles did we learn to recognize their phenotypes. For mutations, like the sterile alleles of *singed*, maintained in balanced heterozygotes, perdurance of maternal gene products also may moderate or even eliminate embryonic and early larval phenotypes.

When specifically considering genes encoding actin-associated proteins, a category of particular interest if we view cuticle specialization as a model for the more general problem of morphogenesis, the degree of overlap between the sets functioning in larvae and adults may be even greater. Both genes known to belong to this class (*singed* and *forked*) have clear larval phenotypes. Moreover, it is reasonable to expect that genes in some other classes represented in the collection of adult mutants are more stage specific. For example, a number of mutants have small, fine bristles possibly due to nonspecific growth effects; these seem unlikely to influence the much smaller larval structures. We base this conjecture on *Minute* mutants. These usually are recognized by bristle phenotype, but the underlying cause is slow growth and development attributed to defects in ribosomal proteins [Kay and Jacobs-Lorena, 1987]. *Minutes* have a disproportionate effect on bristles presumably because those structures grow rapidly over a limited time [Lindsley and Zimm, 1992]. We have examined two relatively strong *Minutes* and see no defects in larval denticles or hairs (unpublished results). Likewise, it is clear that microtubules are present and important in nascent bristles [Overton, 1967; Petersen et al., 1994; Tilney et al., 1995], but we did not detect any comparable association of tubulin with either denticles or nonsensory hairs. Thus, bristle mutations associated with microtubule defects presumably would not affect the larval structures.

Do Common Elements Act in Combinations to Yield Varied Morphology?

The null phenotype of *forked*, and probably of *singed*, is misshapen hairs and denticles, not absence thereof. As with adult bristles [Tilney et al., 1995], even double mutants are not obviously more defective than severe *forked* alleles alone (unpublished observations). Thus, the components we have identified are not essential for formation of hairs and denticles. However, each has a role in forming a considerable range of more-or-less distinct structures. These observations are consistent with a combinatorial model; a modest number of molecular components may interact in overlapping sets to generate a larger number of distinct forms.

The observed allele specificity and divergent pleiotropy are consistent with that model. These features are particularly evident at the *forked* locus. Allele ''strength'' is poorly correlated between adults and larvae and even for different features on the larval cuticle. For example, f^3 and f^{3N} are weak alleles in adults, have the most extreme denticle phenotype we have seen, and have subtle effects on larval hairs. Conversely, f^{36a}, one of the strongest adult alleles, has only modestly abnormal denticles, particularly in rows 1 and 3 but has dramatic effects on both sensory and nonsensory hairs. Moreover, the ''direction''

of the effect is different for each type of larval structure; f^{36a} denticles are thin and ''floppy'', nonsensory hairs are short and fat, and sensory hairs are duplicated or branched. Effects even differ from row to row of denticles.

The *forked* locus is complex by both molecular and genetic criteria. It produces at least six distinct transcripts encoding overlapping proteins [Hoover et al., 1993]. Surprisingly, transcripts have been detected only in pupae. It is not clear how to reconcile that result with the larval phenotypes reported here. The severe effects seen in first instar males hemizygous for a deletion but derived from heterozygous, phenotypically normal females clearly demonstrate an important role for zygotic expression of *forked* during the embryonic period. The expression in embryos may be sufficiently restricted (spatially and/or temporally) to prevent detection in the mass RNA preparations from pooled embryonic stages that were used by Hoover et al. [1993]. Alternatively, there may be novel embryonic transcripts not detected by the fragments used as hybridization probes in those experiments. By indirect immunofluorescence, we did detect forked protein arranged in a pattern mirroring actin bundles (unpublished observations).

Genetically, *forked* alleles have been placed into left and right clusters and can be distinguished in terms of response to *suppressor of forked* and relative effects on macrochaetae, microchaetae, and tricomes [Green, 1955; Lindsley and Zimm, 1992], but these classifications do not correlate in any obvious way with larval phenotypes. Together, these observations suggest that *forked* products are involved in the formation of several distinct cuticular specializations in both embryos and pupae, perhaps with considerable specificity in the utilization the different forms of the protein. Alternatively, some of the mutant alleles may differentially affect regulation of the locus in various contexts. Indeed, at least four of the alleles we used contain transposable element insertions that change relative abundance of the transcripts detected in pupae [Hoover et al., 1993]. It will be interesting to look more closely for embryonic expression of *forked* and to examine in detail the patterns of expression of the different proteins (or transcripts) in wild-type embryos and in various *forked* mutants.

The divergent effects of individual alleles on different targets also suggest that the function of forked protein is very dependent on context. That is, because forked proteins interact with other components to produce diverse structures, even the ''direction'' of the effect of a given allele depends on the nature of those interactions (e.g., thin denticles, fat hairs, branched sensory hairs). The differential effects of *mwh* on denticle rows (disorderly in the first two rows, orderly but more closely spaced in the next two rows) suggest a similar picture. Allele and context specificity also are evident for *singed*, although less dramatic. Again, the ''strength'' of alleles as reported in adults is not a good predictor of strength in larvae, and the two phenotypes observed in larvae, thin denticles and branched sensory hairs, are not well correlated.

Actin Organization and Cuticle Morphogenesis

It is clear that patterns of actin organization that emerge in epidermal cells during stages 14–16 are important determinants of the final shape of diverse specializations present on the cuticle of the first larval instar. Patterns revealed by phalloidin staining anticipate many of the differences between structures present on normal cuticles. Moreover, these structures are altered in mutants in ways that are consistent with the definitive abnormal phenotypes. Because products of *forked* and *singed* are actin-associated proteins, it is likely that the observed effects on F-actin organization are direct. Wong and Adler [1993] proposed that the product of *mwh* also is directly involved in the formation of actin bundles in hair-forming cells of the wing, but the evidence is less compelling. The phenotypes produced by this mutation in larvae are generally consistent with the adult phenotype in that both stages form structures that are more numerous and smaller than normal, so it is likely that the mechanisms also are similar.

The product of the *Stubble*/*stubbloid* locus is a putative transmembrane serine protease postulated to transmit signals that influence organization of actin cytoskeleton in bristles [Appel et al., 1993]. Transcripts are detected in 12–18-h embryos, an interval that includes the stages in which we see actin bundles in developing denticles, but the subtlety and variability of the larval phenotype may make it difficult to confirm the mechanism. *Hairless* is thought to be involved in specification of cell type [Bang and Posakony, 1993], so the sparse distribution of dorsal hairs in larvae and the larger outlines of hair-forming cells in embryos probably reflect a decrease in the number of cells, assuming that fate rather than any direct effect on cytoskeleton.

CONCLUSION

Our results are consistent with the hypothesis that regulation of expression and/or function of cytoskeletal proteins is an important determinant of cuticle morphogenesis, making this a necessary link somewhere in the pathway between the pattern formation processes in the segmentation pathway and the morphological features whose arrangement is specified by that pathway. As additional *Drosophila* genes encoding cytoskeletal proteins are identified, particularly those for actin-associated proteins, it will be interesting to study the patterns of

expression relative to normal cuticle morphogenesis in larvae and to investigate the possibility that some of them are regulatory targets of the segmentation pathway. Because the cytoskeleton and associated proteins are involved in all morphogenetic movements, such studies may provide a useful model for the general problem of connecting pattern formation to morphogenesis.

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