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forked Proteins Are Components of Fiber Bundles Present in Developing Bristles of *Drosophila melanogaster*

Nancy S. Petersen,* Dirk-Henner Lankenau,†, Herschel K. Mitchell,‡ Patricia Young* and Victor G. Corces†

**Department of Molecular Biology, University of Wyoming, Laramie, Wyoming 82071, †Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218 and ‡Biology Department, California Institute of Technology, Pasadena, California 91125*

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ABSTRACT

The *forked* (*f*) gene of *Drosophila melanogaster* encodes six different transcripts 6.4, 5.6, 5.4, 2.5, 1.9, and 1.1 kb long. These transcripts arise by the use of alternative promoters. A polyclonal antibody raised against a domain common to all of the *forked*-encoded products has been used to identify *forked* proteins on two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and in Drosophila pupal tissues. The antibody stains fiber bundles present in bristle cells for about 15 hr during normal pupal development. Electron microscopy shows that these fibers are present from 40 to 53 hr in bristles of wild-type flies but are absent in the null f^{36a} mutant. The *forked* protein(s) thus appear to be an essential part of the bristle fibers. The phenotype of the f^{36a} mutation can be rescued by a 13-kb fragment of the *forked* locus containing the coding regions for the 2.5, 1.9, and 1.1-kb transcripts, suggesting that the proteins encoded by the three large *forked* RNAs are dispensable during bristle development. Increasing the copy number of a $P[w^+,f^+]$ construct containing the 13-kb fragment induces a hypermorphic bristle phenotype whose severity correlates with the number of copies of $P[w^+,f^+]$ present. These results indicate that alterations in the ratios among the *forked* proteins, or between *forked* products and other components of the fiber, result in abnormal assembly of the fibrillar cytoplasmic structures necessary for bristle morphogenesis.

forked (*f*) mutations affect bristle development in *Drosophila melanogaster* resulting in abnormalities of the large bristles on the head, thorax and scutellum of the adult. Phenotypes of *forked* mutations range from slightly bent bristles to grotesquely twisted bristles with branched ends which give the mutation its name. In the most extreme mutations such as f^{36a}, the machrochetes, microchetes and cell hairs (tricombs) are all affected (Poodry 1980). Because the abnormal bristles are easily visible in the adult, the *forked* locus has been a useful genetic marker since its discovery by Bridges in 1916 (Lindsley and Grell 1972).

Bristles represent the macroscopic external extensions of single cells, called trichogens, which are located on the epidermis. Early work on thoracic bristles has shown that the separation of the trichogen and the tormogen (socket-forming cell) in Drosophila occurs at about 16 hr after puparium formation at 25° (Lees and Waddington 1942; Lees and Picken 1945). The nuclei of these cells then grow considerably and become polyploid by about 40 hr. The formation of the bristle begins at about 30 hr and the bristle reaches half its final length at about 36 hr. At this time, a striking birefringence in the bristle-forming cell can be observed in a polarizing microscope (Lees and Picken 1945; Ribbert 1972). This reflects the formation of parallel intracytoplasmic fiber bundles associated with the cell surface which run parallel to the long axis of the bristle. The adult length of the bristle is reached by about 45 hr after puparium formation. The fiber bundles make up about 20% of the bristle cross section during this period, but completely disappear by 60 hr (Overton 1966, 1967).

Two mutants with abnormal bristle structure, *singed*^{3} (*sn*^{3}) and *Stubble* (*Sb*), have been shown to have abnormal fiber bundles. The fiber bundles in *singed* mutants are reduced in size, while those in *Stubble* are abnormal in both size and arrangement (Overton 1967). Similar fiber bundles have also been described in developing interomatidial and leg bristles (Reed, Murphy and Fristrom 1975; Perry 1968). These fiber bundles are completely absent in *forked*^{36a} bristles.

The *forked* gene encodes six overlapping transcripts 6.4, 5.6, 5.4, 2.5, 1.9 and 1.1 kb long which arise by the use of alternative promoters (Hoover, Chien and Corces 1993). Here we show that a polyclonal antibody raised against a domain common to all of the predicted *forked*-encoded proteins stains fiber bundles in wild-type but not f^{36a} bristles. Furthermore, a 13-kb *forked* fragment encoding the three smallest transcripts is capable of fully rescuing the phenotype of f^{36a} and f^{hd} mutants. Additional copies of the $P[w^+,f^+]$ construct result in higher than normal levels of the

encoded *forked* proteins, causing abnormal bristle morphogenesis. These phenotypic effects are compatible with a role for the *forked* proteins as structural components of the fiber bundles present in developing bristles.

MATERIALS AND METHODS

Fly stocks: Oregon-R (ORE) and f^{36a} stocks of *D. melanogaster* were obtained from the California Institute of Technology stock center and were maintained at 25° and 65–70% humidity. The f^{36a} mutant is the most extreme allele of *forked*. The phenotype of f^{36a} over a deletion is the same as that of the homozygous mutation, indicating that it behaves as a null. Developmental staging of animals was carried out by selection as white prepupae (0 time) and checking for pupation by 11 hr and very light yellow eye color at 48 hr (PETERSEN and YOUNG 1989).

Expression of the *forked* protein and antibody production: A 1.4-kb *Eco*RI fragment of the cDNA clone 22G for the 2.5-kb *forked* mRNA (HOOVER, CHIEN and CORCES 1993) was inserted into the pATH1 expression vector at the *Eco*RI site (SPINDLER, ROSSER and BERK 1984). This plasmid directs the synthesis of a β-galactosidase (β-gal)-*forked* fusion protein of about 87,000 kD under the control of the *trp* promoter. The translated *forked* protein includes most of the protein encoded by the longest open reading frame in the cDNA clone including amino acids 12–472 of the 637 amino acid predicted protein. The fusion protein was purified from gels and used to make a polyclonal antibody in rabbits using the RIBI adjuvant system (Immunochem Research Corp., Hamilton, Montana). Controls comparing preimmune serum with the *forked* immune serum showed that the antibody is specific for the fusion protein in *Escherichia coli* extracts.

Two-dimensional gel electrophoresis and antibody staining: Thoraces were dissected from 20 48-hr pupae. The proteins were dissolved in sodium dodecyl sulfate(SDS) buffer and run on two-dimensional acrylamide gels as described earlier (BUZIN and PETERSEN 1982). Gels were electroblotted onto poly(vinylidene fluoride) (PVDF) paper and the blots were stained with Coomassie, followed by destaining and restaining with the antibody to the β-gal-*forked* fusion protein at 1:2000 dilution. Antibody binding was visualized using goat anti-rabbit IgG conjugated horse-radish peroxidase with 3,3′-diaminobenzidine as a substrate. Reagents were purchased from Bio-Rad, and Bio-Rad protocols were followed.

Antibody staining of thoracic tissue: Pupae were dissected from their pupal cases at 48 hr after white prepupa formation. Dorsal thoracic tissue isolated from three pupae was placed on a coverslip. A second coverslip was placed on top of the tissue and the sandwich was pressed together and frozen in liquid nitrogen. On removal from liquid nitrogen the coverslips were split apart and tissue on each coverslip was fixed and stained according to a modification of the procedure of ZIPURSKY *et al.* (1984). Tissue was treated for 5 min with 3% formaldehyde in phosphate-buffered saline (PBS): 0.15 M NaCl, 0.04 M K_2HPO_4, pH 7.2), 30 min in 2% Triton X-100 in Tris-buffered saline (TBS) (0.02 M Tris, 0.15 M NaCl, 0.005 M KCl, pH 7.5), washed three times in TBS + 3% bovine serum albumin (BSA), and incubated with the *forked* antibody 1:500 for 4 hr in TBS + 3% BSA. Tissue was washed three times in TBS + 3% BSA and incubated for 4 hr with goat anti-rabbit fluorescein isothiocyanate, 1:200 dilution in TBS + 3% BSA. After three more washings in TBS, the stained tissue was examined at 400× magnification using a Nikon Optiphot microscope.

***P* element transformation:** A 13-kb *Not*I-*Sal*I fragment obtained from the cosmid clone CoSpeR-*forked*-12-2 was cloned into the plasmid CaSpeR4 (see Figure 5) to give a plasmid designated $P[w^+,f^+]$. DNAs of $P[w^+,f^+]$ and of a *P* element with a defective inverted repeat used as transposase source, pπ25.7wc (KARESS and RUBIN 1984), were then injected into $y\,w^{67c23(2)}f^+$ preblastoderm embryos as described (SPRADLING and RUBIN 1982; RUBIN and SPRADLING 1982).

Construction of lines with increasing number of $P[w^+,f^+]$ copies: Flies with six copies of the *forked* gene were obtained by crossing $w^{67c23(2)}\,f^+P[w^+,f^+]/w^{67c23(2)}\,f^+P[w^+f^+]$ females containing a wild-type copy of *forked* and an *X* chromosomal insertion of $P[w^+,f^+]$ obtained in the transformation experiments to $w^{67c23(2)}f^+/Y;\ P[w^+,f^+]$ males in which the $P[w^+,f^+]$ construct was inserted in an autosome. Three different autosomal insertions in combination with the same *X* chromosomal insertion were obtained for use in the experiments described below.

Scanning electron microscopy: For Figures 1 and 2, dissections of tissues and preparation for electron microscopy were carried out as described elsewhere (MITCHELL, ROACH and PETERSEN 1983). For Figure 6, flies were washed in 70% ethanol and fixed for at least 12 hr in 4% glutaraldehyde. Flies were washed in PBS, dehydrated in an alcohol series, soaked in hexamethyldisilazane for 20 min and dried and mounted on double stick tape on a mounting block. Samples were examined in a Joel scanning electron microscope. Photographs were taken with a Polaroid Positive/Negative 4×5 Black and White Professional Instant Sheet Film 55.

RESULTS

Comparison of bristle development in f^+ and f^{36a} flies: In f^{36a} the large bristles on the fly are twisted, gnarled and sometimes branched to give a *forked* appearance. An example of the gnarled and twisted appearance of *forked* scutellar bristles when compared to the straight bristles of normal flies is shown in Figure 1. In order to look for differences in bristle development which might account for this altered bristle morphology, we have examined sections of f^{36a} and wild-type (ORE) bristles in 36–57-hr pupae. Figure 2 shows the comparison between ORE and f^{36a} bristles at different times in development. At 36 hr, shortly after the start of bristle formation, the bristles of the wild type and mutant are similar. Both are embedded in extracellular matrix and filled with microtubules (Figure 2A). At 40 hr bristles are still embedded in the extracellular matrix. The cuticulin layer is forming around the outside of the bristle while the inside is filled primarily with microtubules organized along the axis of the bristle. By this time in the wild type, but not in the mutant, fiber bundles are formed at intervals around the perimeter of the bristle. The fiber bundles are completely missing in the f^{36a} bristles. A slight thickening on the outside edge of the bristle, however, can be seen where the fiber bundles would be in the wild type (Figure 2B). It could be that the fiber bundle construction in f^{36a}

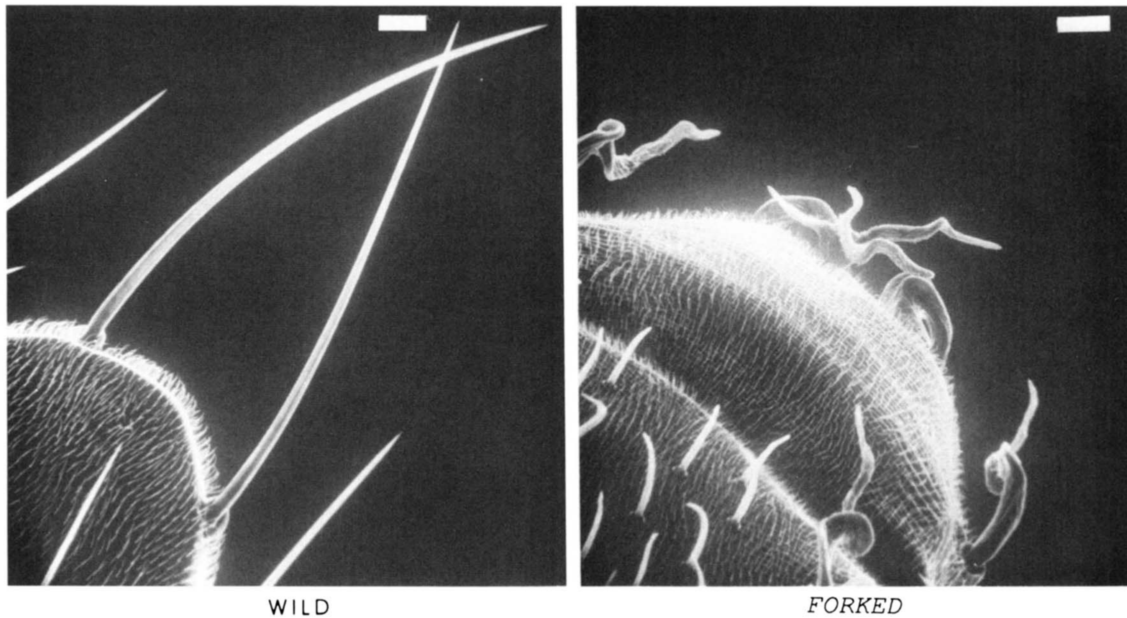

FIGURE 1.—Bristle phenotype of normal and *forked*^{36a} adult thoracic and scutellar bristles.

pupae is delayed relative to wild type, so sections of later stage bristles were also examined. In sections from 48- and 53-hr pupae (Figure 2, C and D), the fiber bundles are present in wild-type bristles, but f^{36a} bristles still have no fiber bundles. An oblique section of a 48-hr ORE bristle (Figure 2C) shows the fiber bundles in a more longitudinal view. Finally, at 57 hr (Figure 2E), there are no fiber bundles in either ORE or f^{36a} bristles, and a thick electron dense material has begun to form around the outside of the bristle. The cytoplasmic material in the interior portion of the bristle appears to be pulling away from this exterior bristle wall. Throughout this period no fiber bundles were seen in any of the sections from f^{36a} mutants. The absence of fiber bundles in f^{36a} bristles could account for the twisted, gnarled appearance of these bristles if the role of the fiber bundles is to straighten and provide support for the bristle during development.

Generation of *forked* antibody: From the above data it appears the *forked* gene plays a role in the synthesis, assembly, and/or the final structure of the fiber bundles. We have generated a polyclonal antibody to a recombinant *forked* protein in order to find out whether the *forked* gene product is a structural component of the fiber. This antibody should also be useful in following the synthesis and assembly of *forked* proteins into bristle fibers under phenocopy inducing and thermotolerant conditions.

A 1.4-kb *Eco*RI fragment of *forked* cDNA clone 22G (HOOVER, CHIEN and CORCES 1993) was inserted into the *Eco*RI site of the expression vector pATH1 to generate a β-gal-*forked* fusion protein. The construct was designed to express the longest open reading frame in the *forked* cDNA clone. This open reading frame encodes 461 amino acids from amino acid 12 to 472 of the protein encoded by the 2.5-kb transcript (see Figure 5). The fusion protein was purified from SDS-polyacrylamide gel electrophoresis (PAGE) gels and injected into rabbits to generate a polyclonal antiserum to the *forked* protein as described in MATERIALS AND METHODS. Further sequencing and characterization of *forked* cDNA clones has revealed that there are six major transcripts encoded by the *forked* gene, and that the open reading frame we have translated is common to all of the predicted proteins (see Figure 5; HOOVER, CHIEN and CORCES 1993).

Identification of *forked* proteins on two-dimensional gels: The *forked* anti-serum was used to detect proteins from 48-hr ORE and f^{36a} pupal thoraces (Figure 3). The proteins were run on two-dimensional isoelectric focusing/SDS-PAGE gels, electro-blotted onto PVDF paper, and antibody binding was detected using goat anti-rabbit horseradish peroxidase conjugated IgG as a second antibody. Two-dimensional gels were run to avoid interference by an extremely abundant pupal protein (MITCHELL and PETERSEN 1989) which runs at about the same molecular weight as the *forked* protein. Two major spots which are expected to be the products of the 2.5-kb transcript run at about 67 kD and 73 kD, respectively, close to the predicted longest open reading frame size of 71 kD. The two spots could be due to alternatively spliced forms of the mRNA, to the use of alternate translational start sites (HOOVER, CHIEN and CORCES 1993), or to posttranslational modification. Two much less abundant higher molecular weight proteins were also detected in ORE but not f^{36a} thoraces. These may be the products of the 5.4- and 5.6-kb transcripts which

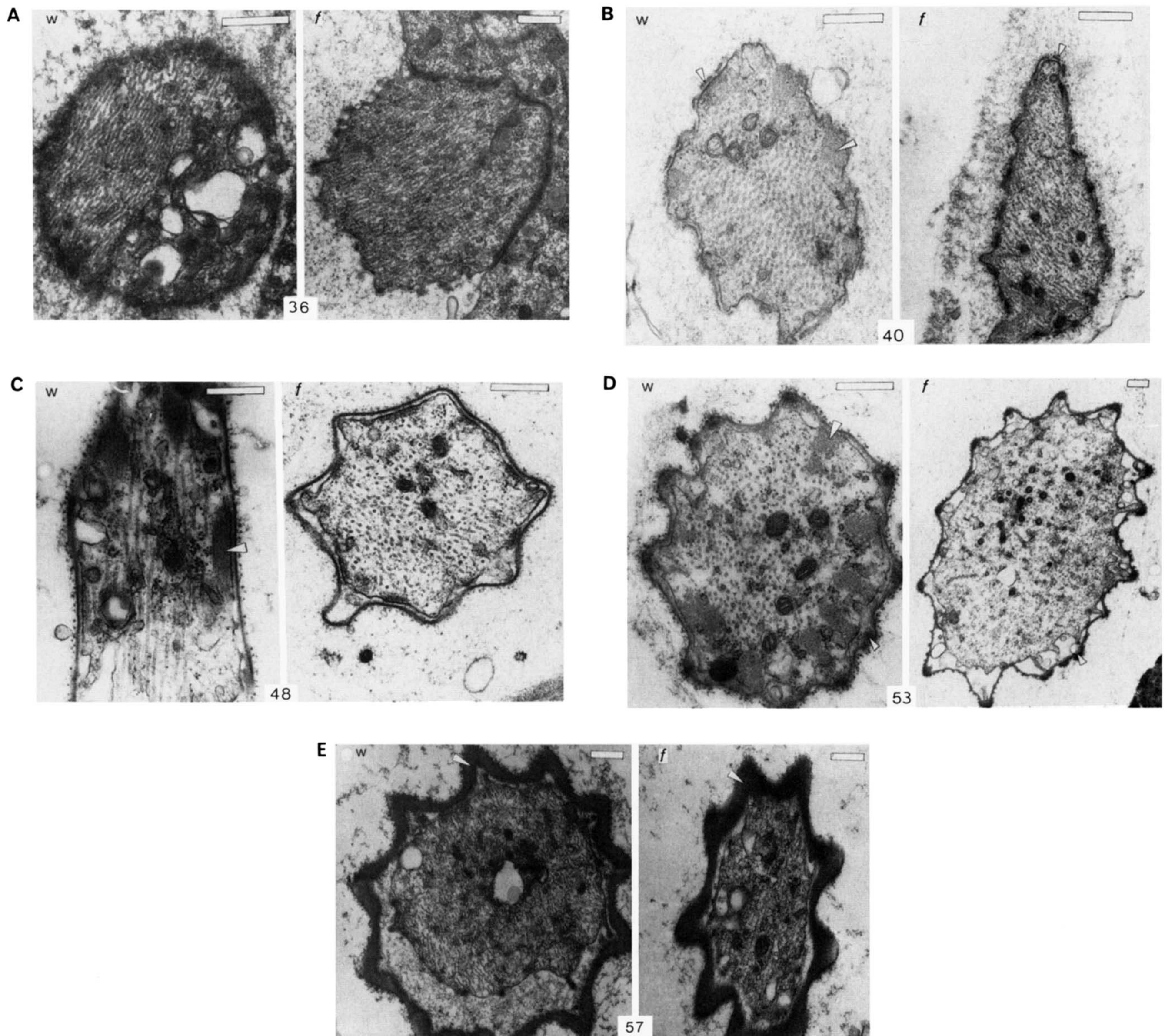

FIGURE 2.—*forked*^{36a} flies are missing fiber bundles normally present in bristles from 40–53 hr of development. Comparison of bristle development in normal and f^{36a} pupae. Electron micrograph sections of pupal bristles prepared at different times during pupal development. The numbers indicate the time when samples were dissected in hours at 25° after pupariation: A, 36 hr; B, 40 hr; C, 48 hr; D, 53 hr; E, 57 hr. Bars are 1 μm; w indicates ORE; *f* indicates f^{36a}. *forked*^{36a} flies are missing fiber bundles (indicated by arrows in B, C and D) normally present in bristles from 40 to 53 hr in wild-type pupae. By 57 hr electron dense material (indicated by arrowheads in E) has been formed around the outside of the bristle, and the fibers are absent in both mutant and normal bristles.

are predicted to encode 155- and 157-kD proteins, respectively.

***forked* antibody staining of pupal thoracic tissue:** The *forked* antibody was also used to stain epithelial tissue from ORE and f^{36a} pupae between 42 and 48 hr of age to determine whether *forked* proteins are a component of the fiber bundles which are missing in f^{36a} bristles of this age. In the 48-hr ORE bristles, the antibody stains what appear to be intertwined golden threads running the length of the intact bristle as seen in Figure 4A. In Figure 4B, a twisted bristle with clearly stained fiber bundles, as well as staining of smaller bristles, hairs and nuclei, is apparent. By contrast, the *forked* bristles have only pale, diffuse stain throughout as well as some cytoplasmic staining (Figure 4, C and D). All of the wild-type epithelial cells and especially the hairs (tricombs) seen in these preparations stain with *forked* antibody. This is not surprising since extreme *forked* mutations such as f^{36a} have phenotypic effects on hairs which are present on all epithelial cells. In fact there are structures in the hairs at about 33 hr which resemble the fiber bundles in bristles (MITCHELL and PETERSEN 1989). The antibody staining of nuclear and cytoplasm associated structures in ORE pupae seems to depend on the time of development. The faint staining in f^{36a} cytoplasm

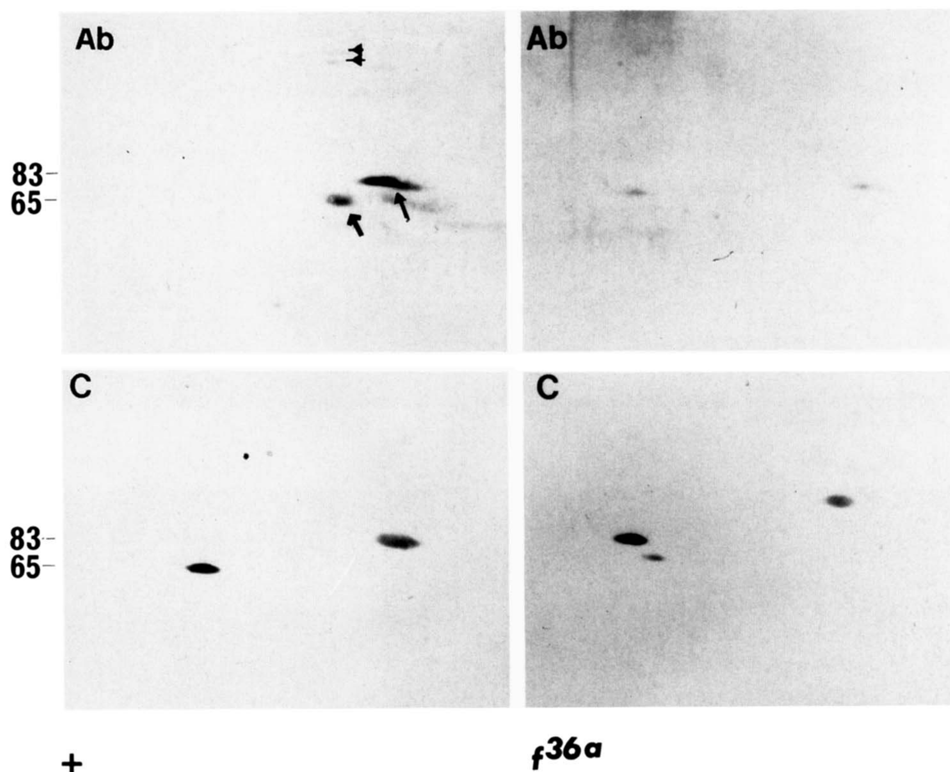

FIGURE 3.—Proteins recognized by an antibody to *forked* fail to accumulate in f^{36a} thoraces. Western blots of two dimensional gels showing that proteins which cross-react with the *forked* antibody are missing in developing f^{36a} thoraces. The blot of wild-type thoracic proteins is on the left; that of f^{36a} thoracic proteins is on the right. The pH gradient runs from 7.6 on the left to about 4.5 on the right. Thoraces were dissected from 48-hr pupae and the proteins were dissolved in SDS buffer, run on two-dimensional gels and blotted to PVDF paper. The blots were stained with Coomassie (C), followed by destaining and restaining with the antibody to the β-gal-*forked* fusion protein (Ab). The Coomassie stains primarily two previously described extremely abundant pupal proteins of 65 and 83 kD thought to be cuticle proteins (MITCHELL and PETERSEN 1989). The difference between these proteins in ORE and f^{36a} pupae maps to the autosomes and is therefore not due to the *forked* mutation. The proteins do serve as useful markers and as controls for equal loading of protein on the gel. On the antibody stained blot, arrows indicate the position of the protein which accumulates at 48 hr in wild type, but not in f^{36a} pupae. Arrowheads indicate two faint bands which may correspond to the proteins encoded by the 5.4- and 5.6-kb transcripts.

and bristles is likely to be due to the truncated protein synthesized from the prematurely terminated mRNA (HOOVER, CHIEN and CORCES 1993). Antibodies to tubulin and actin were used to stain tissue and bristles as controls for nonspecific binding. Neither of these antibodies stained fiber bundles (data not shown). However, APPEL *et al.* (1993) have stained the fiber bundles with phalloidin indicating that they do contain actin even though the antibody we used did not bind. Clearly, the fiber bundles in the ORE bind the antibody strongly, suggesting that one role for the *forked* proteins is as a structural component associated with the bristle fibers.

Establishment of $P[w^+,f^+]$ transformant lines: Antibodies directed against a domain common to all six *forked*-encoded products detect the presence of one or more of these proteins in fibrillar cytoplasmic structures present in the bristle cells, suggesting that all or at least some of these proteins are structural components of this fiber. The f^3 mutant resulting from the insertion of the *412* retrotransposon into the first intron of the 5.4-kb and 6.4-kb RNAs shows only a weak bristle phenotype (HOOVER, CHIEN and CORCES 1993). This implies a less important role for the proteins encoded by the large *forked* transcripts with respect to those made from the 2.5-, 1.9- and 1.1-kb RNAs during bristle secretion. To test whether the large *forked* RNAs encode proteins that are dispensable for *forked* function, we constructed transgenic flies carrying a 13-kb *Not*I-*Sal*I fragment containing the 2.5-, 1.9- and 1.1-kb RNA coding regions plus 6 kb of upstream sequences. This fragment does not overlap the entire first exon of the 5.6-kb RNA (Figure 5). The 13-kb fragment was cloned into the *Not*I and *Xho*I sites of the CaSpeR4 vector, yielding a vector called $P[w^+,f^+]$, and was subsequently transformed into w^{67c23} f^+ embryos. Eighteen transformed lines were obtained, six with insertions in the *X* chromosome, nine with insertions in the second chromosome and three in the third chromosome. Complete rescue of the mutant f^{36a} can be achieved when the $P[w^+,f^+]$ construct is homozygously present on an autosome (see below).

Expression of the small *forked* proteins can rescue the null phenotype: The phenotype of the null mutant f^{36a} is shown in Figure 6A. f^{hd}, which is a *P*

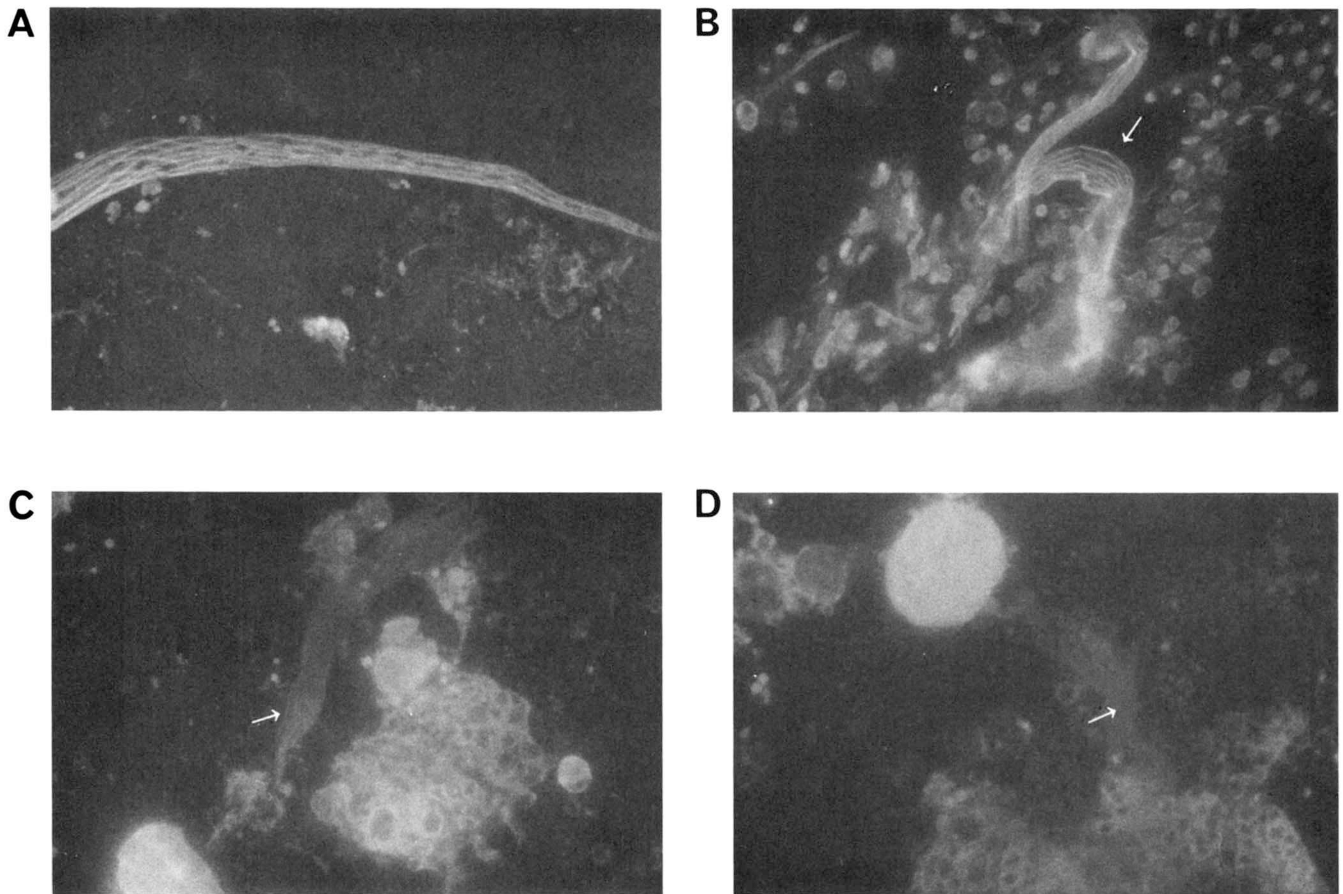

FIGURE 4.—Antibody staining of developing bristles from wild-type and f^{36a} flies. Thoracic epithelium was prepared from 48-hr wild-type and f^{36a} pupae. A and B are 48-hr ORE thoracic tissue. C and D are f^{36a} 48-hr thoracic tissue. In A, this primary image is a large bristle; arrows indicate positions of bristles in B, C and D.

element insertion into the second exon of the 2.5-kb *forked* transcript, shows the same phenotype. These phenotypes are caused by the absence of *forked* RNAs (HOOVER, CHIEN and CORCES 1993). The phenotype of the f^{36a} mutant is the most extreme bristle phenotype and is indistinguishable from that of a deletion of the *forked* region. The construct $P[w^+,f^+]$ used in this study is sufficient to rescue the phenotype of f^{36a} and f^{hd} flies completely (Figure 6B). This, however, is dependent on the number of copies of the $P[w^+,f^+]$ construct present in the genome. For example, 35% of males of the genotype $f^{36a}/Y; P[w^+,f^+]/+$ which contained one autosomal copy of the $P[w^+,f^+]$ construct, had exclusively wild-type bristles, 60% had one or two hypomorphic macrochaetae, and 5% of these males had three to four hypomorphic bristles. When females of the genotype $f^{36a}/f^{36a}; P[w^+,f^+]/P[w^+,f^+]$ were examined, 42% had wild-type bristles, 51% had 1–2 hypomorphic bristles and 7% of these females had three to four hypomorphic macrochaetae. None of the hypomorphic bristles in these flies was phenotypically as severely affected as the ones in the original f^{36a} mutant (Figure 6A). Increasing the copy number of the $P[w^+,f^+]$ construct by one, to obtain males of the genotype $f^{36a}/Y; P[w^+,f^+]/P[w^+,f^+]$, caused all bristles to look wild type. These males have the same autosomal copy number of $P[w^+,f^+]$ as their sisters; however, 58% of the females exhibit at least one hypomorphic bristle, suggesting that the transformed *forked* gene is subject to dosage compensation (Figure 6C). Assuming that the 6 kb of 5′ information present in the $P[w^+,f^+]$ construct are enough to drive proper expression of the 2.5-, 1.9- and 1.1-kb *forked* transcripts at normal levels, these results suggest that the proteins encoded by these three RNAs are sufficient to rescue the *forked* null phenotype. Therefore, the proteins encoded by the three large *forked* transcripts might be dispensable for *forked* function during bristle morphogenesis, as long as the three small proteins are made at levels approximately twice those present in wild-type flies.

A new bristle phenotype is caused by overexpression of the small *forked* proteins: The established transformed $P[w^+,f^+]$ lines carry the normal wild-type *forked* gene on the *X* chromosome. Therefore, we expected all transformed flies to possess wild-type bristles. Nevertheless, the males in some transformed lines show a strong *forked* phenotype whereas their

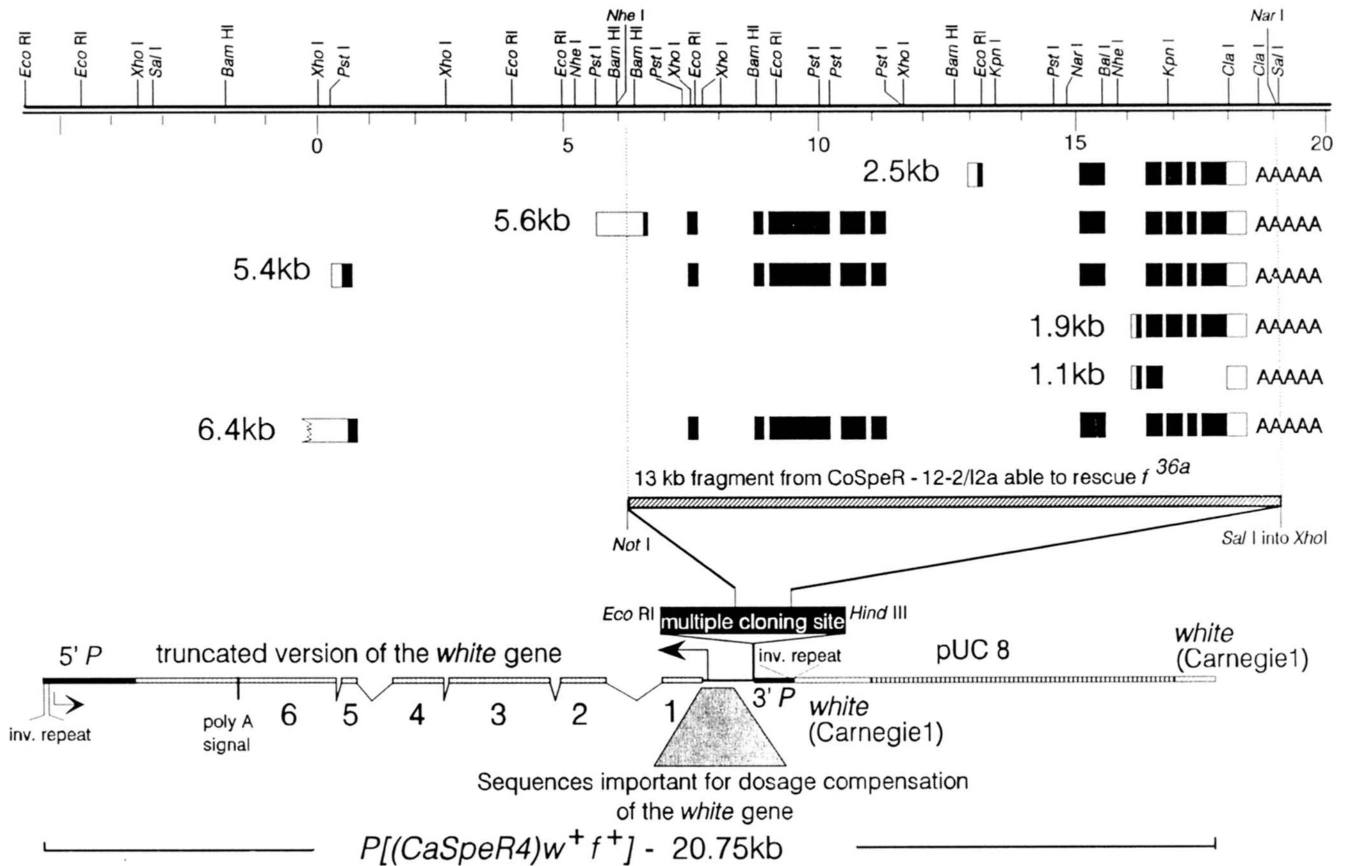

FIGURE 5.—Structure of the *forked* gene and encoded transcripts, and the $P[w^+,f^+]$ construct. The upper part of the figure represents the restriction map of the *forked* gene. The coordinates below give distance in kilobases. Position 0 is as defined by HOOVER, CHIEN and CORCES 1993. Exons are denoted by black boxes and introns by empty spaces between boxes. Unfilled boxes represent transcribed untranslated RNA. The 13-kb fragment included in the $P[w^+,f^+]$ construct is aligned with the *forked* gene (vertical lines). The sequence organization of the CaSpeR4 vector is given in the lower part of the figure. Sequences involved in dosage compensation of the *white* gene are indicated (PIRROTTA, STELLER and BOZZETTI 1985). The *white* sequences flanking the pUC DNA do not possess coding potential. The 5′ and 3′ sequences of the *P* element, into which a *white* gene with a truncated first intron is inserted, are indicated. Inverted repeats of the *P* element are marked (inv. repeat). The polyadenylation signal of the *white* gene is also indicated.

female siblings show normal bristles. This result can be rationalized if higher levels of *forked* expression due to dosage compensation in the males are responsible for the bristle phenotype. In support of this, the severity of the *forked* phenotype in males correlates with the intensity of the eye coloration. Flies that show a darker eye color due to higher level of expression of the *white* gene contained in the $P[w^+,f^+]$ construct also show a more pronounced bristle phenotype. The effect is most striking in homozygous males of a transformed line containing two independent $P[w^+,f^+]$ insertions on the second chromosome. Because these males have five copies of the *forked* gene, we conclude that the bristle phenotypes are a consequence of increased levels of *forked* gene products. This hypothesis was tested by successively adding more copies of the $P[w^+,f^+]$ construct to a strain carrying one copy of the transformed *forked* gene in the *X* chromosome. Adding one autosomal copy of $P[w^+,f^+]$ to such males, represented by the genotype $f^+ P[w^+,f^+]/Y; P[w^+,f^+]/CyO$, results in all individual males exhibiting at least one hypermorphic bristle. Increasing the number of $P[w^+,f^+]$ further in males leads to a higher number of hypermorphic bristles per individual fly and also the phenotype of individual bristles becomes more severe (Figure 6D). As predicted from the assumption that the *forked* gene is roughly expressed twice as much in males as in females, the first hypermorphic macrochaetae in females were observed in flies of the genotype $f^+ P[w^+,f^+]/f^+ P[w^+,f^+]; P[w^+,f^+]/+$ containing five copies of f^+. The bristle phenotypes due to over expression of the small *forked* proteins are hypermorphic, in contrast to the phenotypes observed in lack of function *forked* alleles. To confirm that the phenotypic effects are due to altered levels of *forked* expression, we performed northern analysis of transformed lines with different numbers of the $P[w^+,f^+]$ construct. The amounts of the *forked* 2.5-, 1.9- and the 1.1-kb RNAs increased proportionately to the copy number of $P[w^+,f^+]$ (data not shown). Scanning electron micrographs of hypermorphic bristles are shown in Figure 6, D–F.

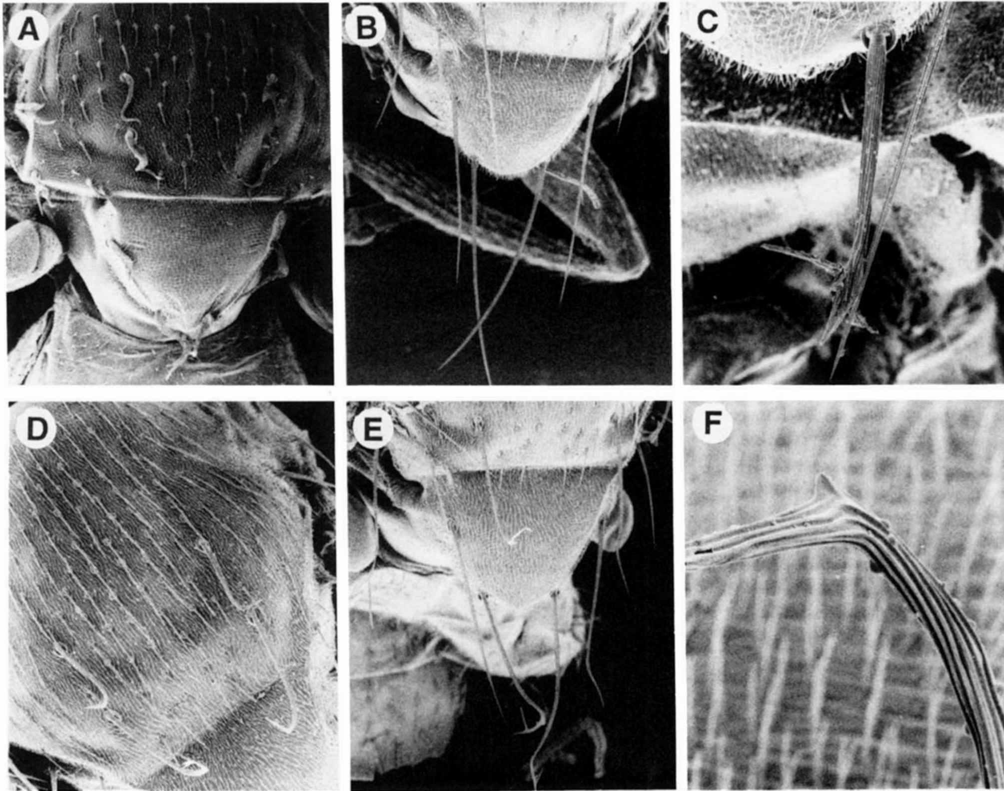

FIGURE 6.—Scanning electron micrographs of hypomorphic and hypermorphic bristle phenotypes and the rescue of the f^{36a} null mutant by the $P[w^+,f^+]$ construct. A, Scutellar bristle of the f^{36a} null mutant. B, Bristles of a male of the genotype f^{36a}/Y; $P[w^+,f^+]/^+,f^+]/2P[w^+,f^+]$ showing complete rescue of the null f^{36a} phenotype. C, Bristles of a female of the genotype f^{36a}/f^{36a}; $P[w^+,f^+]/P[w^+,f^+]$; some mutant bristles are still present due to incomplete rescue of the f^{36a} phenotype. D, Scutellar bristles in a male of the genotype f^+ $P[w^+,f^+]$; $P[w^+,f^+]/P[w^+,f^+]$; $P[w^+,f^+]/P[w^+,f^+]$, containing six copies of the *forked* gene. All males in this stock show the hypermorphic bristle phenotype. E, Scutellar bristles from a female of the genotype f^+ $P[w^+,f^+]/f^+$ $P[w^+,f^+]$; $P[w^+,f^+]/P[w^+,f^+]$; $P[w^+,f^+]/P[w^+,f^+]$. Only 37% of the females in this strain show the hypermorphic bristle phenotype. F, Photograph of a mutant bristle due to *forked* overproduction. Note the curve present at the branching node of the bristle shaft. Branching of the bristles occurs especially at the tip where the bristle shaft becomes increasingly thin.

The results discussed above suggest that over expression of the small *forked* proteins in flies carrying a wild-type copy of the *forked* gene results in abnormal bristle morphogenesis, probably due to improper assembly of the cytoplasmic fibrillar structures observed in the bristle cells. This could be caused by an incorrect ratio between the small and large *forked* proteins or, alternatively, by an abnormal ratio of small *forked* proteins to other components of the fibrillar structure not encoded by the *forked* gene. To discern between these two alternatives, we constructed flies containing increasing numbers of the $P[w^+,f^+]$ construct in the background of the f^{36a} mutation which lacks all *forked*-encoded products. We were able to induce the same hypermorphic bristle phenotype in flies carrying the f^{36a} allele as in flies carrying a wild-type *forked* gene. The presence of the hypermorphic bristle phenotype was first observed in males of the genotype f^{36a}/Y; $P[w^+,f^+],P[w^+,f^+],/P[w^+,f^+],P[w^+,f^+]$, indicating that whereas only three copies of the *forked* gene are sufficient to induce a hypermorphic phenotype in f^+ males, four copies of the $P[w^+,f^+]$ construct are required to cause the same effect in f^{36a} males. These results agree with the hypothesis that the hypermorphic bristle phenotype is caused by higher than normal levels of the small *forked* proteins. In addition, the results suggest that this phenotype must be a consequence of an altered ratio of the small *forked* proteins to components of the fiber other than *forked*, since f^{36a} flies lack the *forked* proteins encoded by the large transcripts.

DISCUSSION

Several lines of evidence indicate that the protein(s) encoded by the *forked* gene are components of fiber bundles which are transiently present in bristles during mid-pupal development. We have shown that fiber bundles which normally are present in bristles from 40–53 hr of pupal development are missing in f^{36a} mutant bristles. In wild-type flies, but not f^{36a} mutant flies, these fiber bundles are stained with a polyclonal antibody to a common region in all of the predicted *forked* proteins. A portion of the *forked* gene which encodes the most abundant 2.5-kb transcript as well as two smaller transcripts is sufficient to rescue the *forked* phenotype; however, overexpression of this

construct leads to an abnormal hypermorphic phenotype. All of these observations are consistent with a structural role in bristle fibers for the *forked* proteins.

The complete absence of fiber bundles in f^{36a} bristles is the most extreme fiber bundle phenotype described. Several mutations with bristle phenotypes similar to f^{36a} have reduced or altered fiber bundles. Fiber bundles in sn^3 bristles are reduced in size and flattened, and *Sb* bristles also have reduced amounts of irregularly organized fiber bundles (OVERTON 1967; APPEL *et al.*, 1993). The phenotype of adult sn^3 bristles is twisted or wavy bristles which are quite similar to bristles in the milder alleles of *forked*. It would appear that the fiber bundles transiently present during development are involved in straightening the bristles.

The antibody staining of bristle fibers also suggests a role for the *forked* protein(s) as a structural part of the bristle fiber rather than a role in the processing or transport of fiber proteins. The role of the fibers in hair development is not clear, but they may also play a role in straightening these structures as hairs on adults are slightly wavey in both sn^3 and *forked* flies.

The antibody we have generated is likely to cross-react with all six of the *forked* proteins based on the cDNA sequence data in the accompanying paper (HOOVER, CHIEN and CORCES 1993). The cDNA used to generate the antibody includes exons which are common to all six of the major transcripts and which are predicted to be translated in the same reading frame. However, most of the antibody staining we are seeing is likely to be due to 2.5-kb transcript encoded protein because the 2.5-kb transcript is the most abundant of the *forked* transcripts at this time in development. Furthermore, the 71-kD protein it encodes appears to be the most abundant protein recognized by the antibody on two-dimensional gels. We expect that this, along with the data indicating that the 2.5-kb transcript is essential for normal bristle development, means that the 71-kD protein is a part of the bristle structure. Besides being found in bristle fiber bundles, antibody cross-reacting material is seen in the cytoplasm and nucleus of the cell at different times in development as well as in cell hairs. To further investigate these effects as well as to determine conclusively which of the proteins is part of the bristle fiber, we are making specific antibodies to individual *forked* proteins.

Results presented here show that a portion of the *forked* gene encoding the 2.5-, 1.9- and 1.1-kb RNAs is sufficient to rescue the *forked* null phenotype. A complete rescue with this fragment, however, is dependent on its copy number in the genome. The same copy number of the $P[w^+,f^+]$ construct as in wild-type flies does not fully rescue the *forked* phenotype of f^{36a}, and additional copies of the transformed gene are needed to obtain normal bristle morphology. This suggests an important although dispensable role for the proteins encoded by the 6.4-, 5.6- and 5.4-kb *forked* transcripts in fiber bundle formation in developing bristles. These are the only transcripts disrupted in the mutation f^3 which is a mild allele of *forked*. The proteins encoded by these RNAs also contain several ankyrin domains that might mediate interactions between *forked* products and other cellular proteins (HOOVER, CHIEN and CORCES 1993). One possible role for the ankyrin repeats is to mediate interactions between the fiber bundles and membrane proteins. These interactions might not be necessary for proper bristle morphogenesis, but they might be required for other functions such as the transmission of touch-sensitive information between the environment and the sensory nerve cell.

Overexpression of the *forked* proteins encoded by the 2.5-, 1.9- and 1.1-kb RNAs results in defects in bristle morphogenesis. This is not surprising given the prediction that these proteins form part of the fibrillar cytoplasmic structure that is necessary for proper bristle formation. Overexpression of other proteins that form similar supramolecular structures also gives rise to abnormalities in their assembly. For example, high levels of β-tubulin expression in yeast interfere with normal microtubule formation (BURKE, GASDASKA and HARTWELL 1989; WEINSTEIN and SOLOMON 1990). This hypermorphic phenotype supports evidence indicating a structural role for the *forked* proteins in the cytoplasmic bundle structures of the bristle cell obtained by the immunolocalization experiments using antibodies against *forked* proteins. Since this phenotype is observed in flies carrying the normal copy of the *forked* gene, it could be a consequence of an altered ratio between the ankyrin-containing proteins encoded by the 6.4-, 5.6- and 5.4-kb RNAs and those synthesized from the 2.5-, 1.9- and 1.1-kb transcription units present in the $P[w^+,f^+]$ construct used in the transformation experiments. Nevertheless, the fact that the same phenotype can be observed in f^{36a} mutants, which lack any of the proteins encoded by the three large transcripts, suggests that the defects in bristle morphology are caused by an imbalance between the small *forked* proteins and other components of the fiber bundles distinct from those encoded by the *forked* gene. This observation thus suggests the presence of other structural proteins in the fiber bundles such as actin (APPEL *et al.* 1993). Biochemical analysis of other mutations which affect bristle morphogenesis may result in identification of additional proteins involved in this structure.

The molecular analysis of the *forked* gene also provides the basis for further study of the induction of the f^{36a} phenocopy by heat shock. This phenocopy is

an ideal model system for studies of phenocopy induction and thermotolerance (MITCHELL and PETERSEN 1985; PETERSEN and MITCHELL 1991). In pupae which are heterozygous for the f^{36a} gene there is a sensitive period during which a heat shock will induce the mutant phenotype in 100% of the pupae (MITCHELL and PETERSEN 1985). Furthermore, when the pupae are given a thermotolerance inducing treatment before the phenocopy inducing heat shock, the *forked* bristle defects are prevented (PETERSEN and MITCHELL 1988). This phenomenon is a model for environmentally induced defects in vertebrates, such as spina bifida and cleft palate, which can also be prevented by thermotolerance inducing treatments (PETERSEN 1990).

To understand the molecular mechanism for forked phenocopy induction and thermotolerance it is first necessary to know what *forked* gene products are required for normal bristle development and where and when they are expressed. The *forked* protein(s) appears to be a structural protein which is made in sufficient amounts so that its synthesis, processing and transport can be followed under phenocopy inducing and thermotolerance inducing conditions.

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