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Mutations in the *rotated abdomen* locus affect muscle development and reveal an intrinsic asymmetry in *Drosophila*

(helical symmetry/muscle development/segmental staggering)

ENRIQUE MARTÍN-BLANCO* AND ANTONIO GARCÍA-BELLIDO

Centro de Biología Molecular "Severo Ochoa," Consejo Superior de Investigaciones Cientificas, Universidad Autónoma de Madrid, Cantoblanco, Madrid 28049, Spain

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ABSTRACT **In bilateral animals, the left and right sides of the body usually present asymmetric structures, the genetic bases of whose generation are still largely unknown [CIBA Foundation (1991) *Biological Asymmetry and Handedness*, CIBA Foundation Symposium 162 (Wiley, New York), pp. 1–327]. In *Drosophila melanogaster*, mutations in the *rotated abdomen* (*rt*) locus cause a clockwise helical rotation of the body. Even null alleles are viable but exhibit defects in embryonic muscle development, rotation of the whole larval body, and helical staggering of cuticular patterns in abdominal segments of the adult. *rotated abdomen* is expressed in the embryonic mesoderm and midgut but not in the ectoderm; it encodes a putative integral membrane glycoprotein (homologous to key yeast mannosyltransferases). Mesodermal cells defective in O-glycosylation lead to an impaired larval muscular system. We propose that the staggering of the adult abdominal segments would be a consequence of the relaxation of intrinsic rotational torque of muscle architecture, preventing the colateral alignment of the segmental histoblast cells during their proliferation at metamorphosis.**

Drosophila melanogaster, from embryo to adult, keeps a bilateral body plan with few exceptions. The most apparent handedness is manifested by the clockwise rotation of the male sex organ. Some loci can mutate to affect the development of the abdomen or genitalia, such that the adult structures are out of alignment with the rest of the body (1). The *rotated abdomen* (*rt*) locus was described first by Bridges and Morgan (2) as a poorly viable recessive mutation causing clockwise twisted abdomens through 60° to 90°.

Coiling and helical handedness anomalies are widespread in nature. Dextrality or sinistrality of the cleavages and the shell of *Limnaea peregra* are determined maternally and controlled by a single recessive locus called *sinistral* (3). In nematodes, rotational asymmetry of biological structures in *roller* mutants depends on interactions between sheets of helices (4). The cuticle has several layers of helical fibers arranged with a particular pitch. Mutations in a particular collagen gene locus can produce animals in which these sheets are shifted relative to each other. The animal itself may have a left-handed helical twist, for example, and move in an abnormal way.

The rotated abdomen phenotype could be due to the maintenance of the asymmetry of the internal structure of proteins in the higher levels that involve assemblies of protein molecules. Here, we describe the primary defects associated to this mutation. We cloned the *rt* gene, which codes for a mannosyltransferase, and analyzed its pattern of expression. We find that molecular defects in *rt* affect muscle structures and the alignment of adult cuticle.

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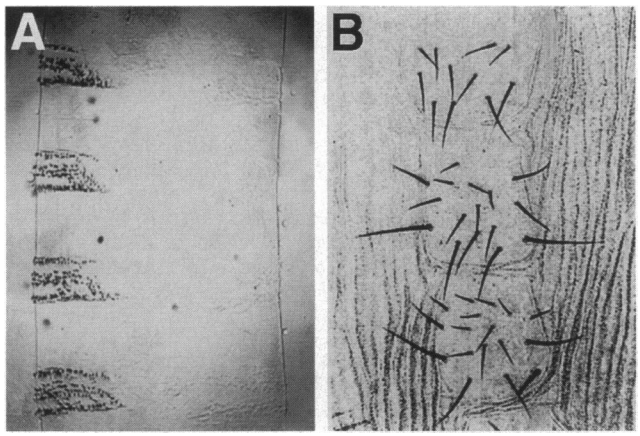

FIG. 1. Cuticular phenotypes of *rt* mutations. (*A*) Cuticle of a third instar rt^2 hemizygous larvae showing a correct alignment of cuticle landmarks. (*B*) Ventral view of abdominal segments of an adult fly of the same genotype showing the staggering of sternites along the anterior/posterior axis.

MATERIALS AND METHODS

Fly stocks were provided by Indiana and Bowling Green *Drosophila* Stock Centers. Established molecular techniques were used. DNA sequence was carried out on both strands using Sequenase (United States Biochemical). Sequence data were obtained by using synthetic oligodeoxynucleotides. DNA sequence and protein homology searches were conducted on the National Center for Biotechnology Information mail server using the BLAST program (5).

Hydropathy was compiled according to Kyte and Doolittle (30) using the GENEJOCKEYII program. A window of 19 amino acids was used. Positive values correspond to hydrophobic regions, and negative ones correspond to hydrophylic segments.

A digoxigenin-labeled *rt* cDNA was used as a probe in wild-type (Oregon-R) embryos. Whole mount *in situ* examinations were performed as in ref. 6. The anti-muscle myosin antibody was a gift of M. Bate (University of Cambridge) and was used as described (7). Fluorescein-phalloidin staining of larval muscles in flat preparations was performed according to established protocols (8). Adult abdomen muscle patterns were examined under polarized light using a Nikon polarizing microscope.

RESULTS

Available *rotated abdomen* alleles [rt^2 and rt^{P60} (9)] hemizygous over the deficiency $Df^{(vin)}4$ (10) cause partial lethality that is

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. X95956).

*Present address: Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, United Kingdom.

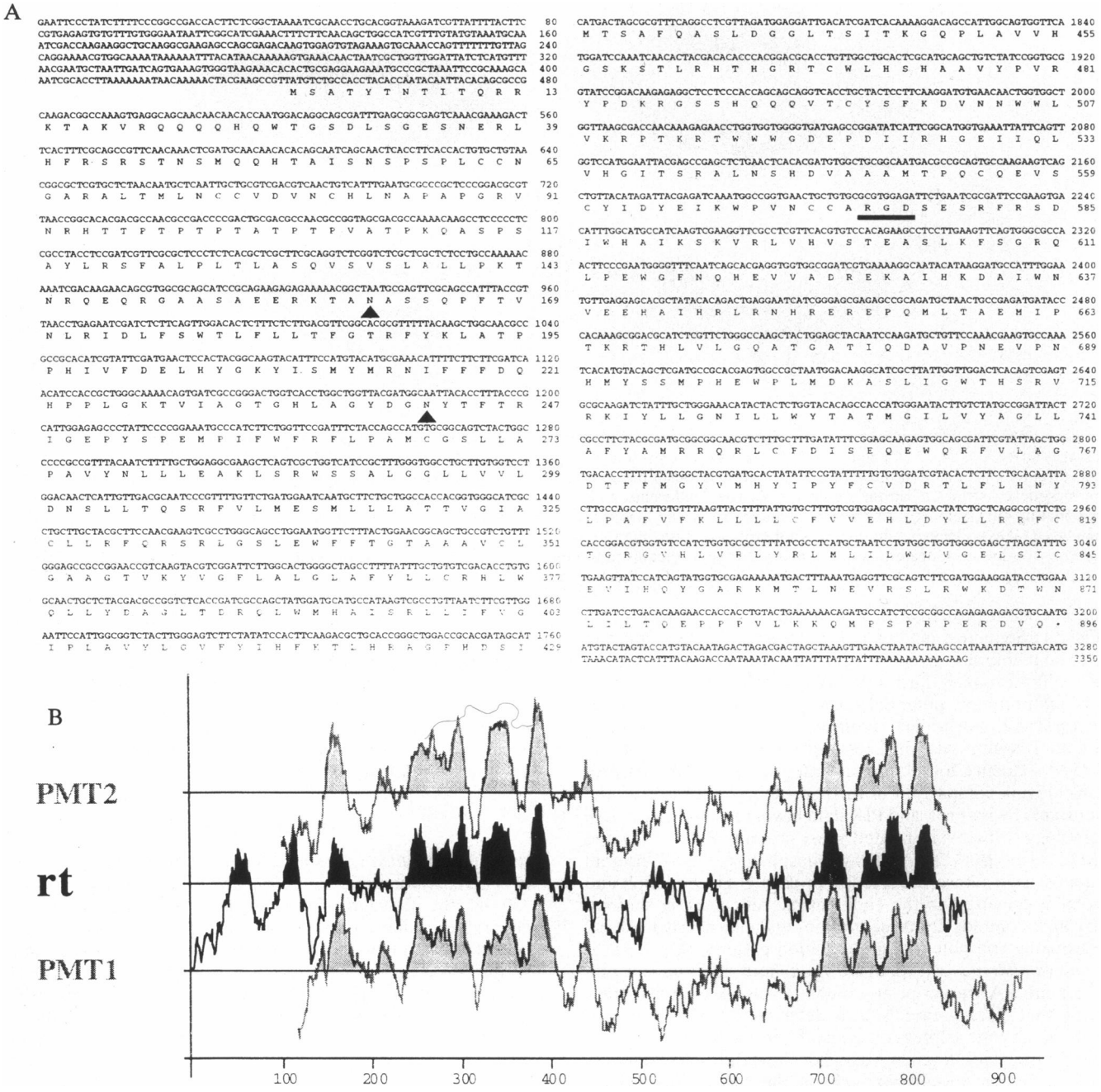

FIG. 2. *rotated abdomen* encodes a putative transmembrane glycoprotein related to *Saccharomyces cerevisiae* O-mannosyltransferases. (*A*) Nucleotide and predicted amino acid sequence of the *rt* gene. Putative asparagine linked glycosylation sites are marked by arrowheads. The putative Integrin binding site is underlined. (*B*) Hydropathy plot. PMT1, *rt*, and PMT2 were aligned according to their hydrophobic profile and sequences. The sequence and the overall structure of the three molecules are highly conserved.

not restricted to any particular developmental stage (data not shown). The *rotated abdomen* alleles have a zygotic recessive phenotype. The sterility component is associated to the abdominal rotation because homozygous females can occasionally give rise to normal progeny in outcrosses (data not shown). Phenotypic defects could be detected in first instar larvae, with their bodies rotated around its long axis, forcing the animals to move in a clockwise circle as they crawl, in a similar way to *roller* mutants of C. elegans (4). The larval cuticle presents a perfect segmental alignment of its characteristic landmarks (Fig. 1*A*). However, the adult abdominal cuticle segments appear staggered relative to each other (Fig. 1*B*).

rt maps at position 68D in the interval defined by the deficiencies $Df^{(vin)}6$ and $Df^{(vin)}7$ (11). We cloned the *rt* gene by screening a cDNA library (12) with a genomic walk probe kindly provided by E. Meyerowitz (California Institute of Technology, Pasadena). We confirmed the identity of the isolated cDNAs by comparison of their restriction maps and sequences with those from a rt^{P60} plasmid rescue (data not shown and ref. 9). Molecular mapping of rt^{P60} shows that this mutation disrupts the coding sequence by P-element insertion in the first exon (data not shown), possibly causing null condition. Southern blot analysis shows that the rt^2 spontaneous allele, which produce an undistiguishable phenotype, is caused by the insertion of a transposable element in the same genomic region (data not shown).

Following a long leader, *rt* cDNAs contain a complete large open reading frame (ORF) of 2688 nt. This ORF is followed by an untranslated region containing multiple stop codons and a poly(A) tail. The translation product would contain 896 aa

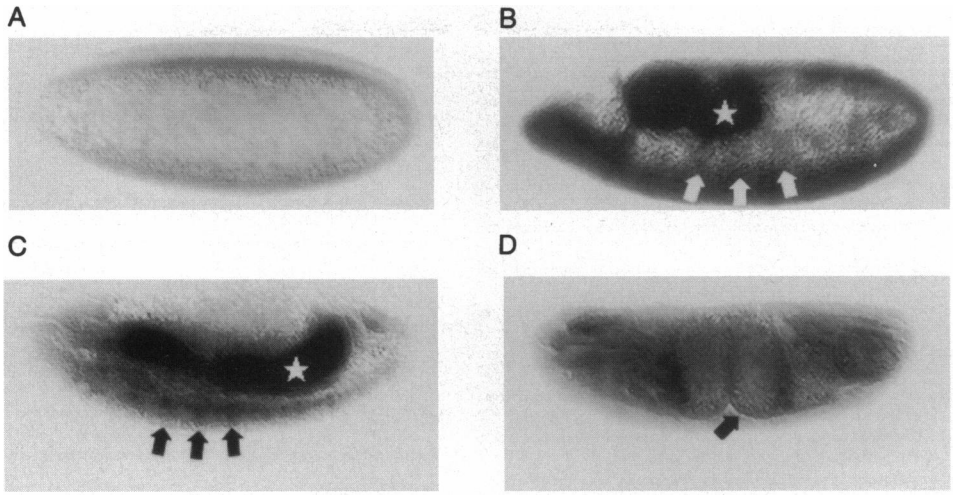

FIG. 3. Distribution of *rt* transcripts in wild-type *Drosophila* embryos. *In situ* hybridization of whole embryos with digoxigenin-labeled *rt* cDNA at stages 6 (*A*), 10 (*B*), 13 (*C*), and 16 (*D*) of Campos-Ortega and Hartenstein (16). (*A*) At the cellular blastoderm stage, *rt* is expressed uniformly and starts to accumulate in the ventrally located mesoderm primordium. (*B*) At germ band extension stage, the expression becomes periodic in the mesoderm; stripes of strong expression (arrows) alternate with stripes of weaker expression. A very strong signal is also detected in the invaginating gut (star). (*C*) As the germ band retracts, the mesodermal expression decays and becomes restricted to somatic muscle precursors (arrows). Gut expression is maintained as gut primordia fuse (star). (*D*) At late stages, after dorsal closure when the midgut constrictions are formed, *rt* expression has disappeared from the mesoderm and remains in the endoderm (arrow). Some expression is detected in a few cells of the head and the pharyngeal muscles. Anterior is always to the left and dorsal is up.

and encode a protein with a molecular mass of 102.000 Da (Fig. 2*A*). Sequence analysis and hydropathy profiles predict an integral membrane glycoprotein with a tripartite structure: (*i*) an amino-terminal third and (*ii*) a carboxyl-terminal third, both with multiple potential transmembrane helices, and (*iii*) a central hydrophylic part. Additionally, the protein has several potential N-glycosylation sites and an amino acid sequence (RGD) sequence found in many integrin-binding matrix proteins (13). A comparison of the *rt* protein sequence with the GenBank, Swiss-Prot, and PIR data bases reveals an extensive homology to two very related yeast proteins PMT1 (14) and PMT2 (15). Both are dolichyl-phosphate-mannose protein mannosyltransferases involved in protein O-glycosylation and probably localized to the endoplasmic reticulum. *rt* shows a very high homology to these proteins and also nearly identical hydropathy and potential glycosylation patterns (Fig. 2*B*).

rotated abdomen is expressed throughout development as a 3.2-kb mRNA transcript at variable levels. A maternal transcript that decays rapidly was detected by Northern blot analysis. Zygotic expression peaks between the 8th and the 12th hours of development (data not shown). RNA transcripts localize first in invaginating cells at the cellular blastoderm stage (Fig. 3*A*) and develop a periodic pattern of expression in mesodermal cells during extension (Fig. 3*B*) and retraction (Fig. 3*C*) of the germ band. The mRNA fades away from the mesoderm at later stages. This mesodermal pattern is accompanied by an early strong endoderm distribution in the midgut, more defined at later stages (Fig. 3*D*). Additional expression was detected in pharyngeal muscle precursors and conspicuous cells in the head (data not shown).

Embryonic nervous system and epidermis of *rt* mutants look perfectly normal and bilaterally symmetrical (data not shown). However, these embryos show a variable but almost fully penetrant muscular phenotype. Rare embryos have strongly disrupted myogenesis and fiber attachment abnormalities. More common defects are the lack of some muscles or the presence of very elongated and thin ones (data not shown and Fig. 4*A*). The most affected muscles are the lateral ones (muscles 4, 5, 8, 18, 23, and 24; see ref. 7). Less commonly, dorsal muscles 2, 3, 11, and 19 are also missing. These defects are not bilaterally biased. Abnormalities are found at the initial stages of myosin heavy chain expression, when muscles are not completely formed (data not shown) (17), suggesting that flaws initiate during muscle development and do not result from muscle degeneration. The musculature defects are still apparent in larvae that reach the third instar, with some larval muscles missing and others deranged and thinner with fewer nuclei (Fig. 4*B*). Reorientation of some adult muscles and absence of the normally persistent larval muscles (7) (Fig. 4*C*) are common defects in *rt* adults.

DISCUSSION

If *rotated abdomen* codes for a mannosyltransferase, involved in O-glycosylation of endodermal and mesodermal proteins, it will be the first protein of its class described in higher eukaryotes, where the initial steps of O-glycosylation are believed to be mediated by a different pathway (18). The gene seems not to be absolutely essential for fly viability. In yeast, the protein mannosyl transferase are members of an increasing family of enzymes, whose mutations do not lead to lethality (14). Defective glycosylation could affect conformational stability, protease resistance, protein targeting, and cell–cell interactions (for review, see ref. 19). Possible targets of *rt* dependent glycosylation could be *Drosophila* muscle integrins. Integrins are glycoproteins with a function in the formation of both sarcomeric cytoarchitecture (20) and muscle attachments (21). Alleles of both the *αPS2* and *βPS* integrin genes can produce a graded series of phenotypes in muscles, some of them reminiscent of *rotated abdomen* muscular defects (22) but they do not cause in viable alleles abdominal rotation, suggesting that this is not a constant result of muscle defects.

How could the molecular defects of *rt* alleles cause the observed systemic phenotypes of rotation, its clockwise character and the regularity of the adult epidermal staggering? One possibility is that the *rt* misfunction reveals an intrinsic left-right asymmetry as defined by other gene or genes (23). Our data show that in *rt* mutants most muscles (longitudinal and transverse) can be erratically affected. Thus, we favor the alternative hypothesis that the rt systemic helicoideal twist discovers the intrinsic angular tension of some muscles above the general flaws of the entire muscular lattice (Fig. 5*B*). Interestingly, there are three more genes, *twisted* (*tw*), *fringed* (*fr*), and *abdomen rotatum* (*ar*), known in *Drosophila* whose

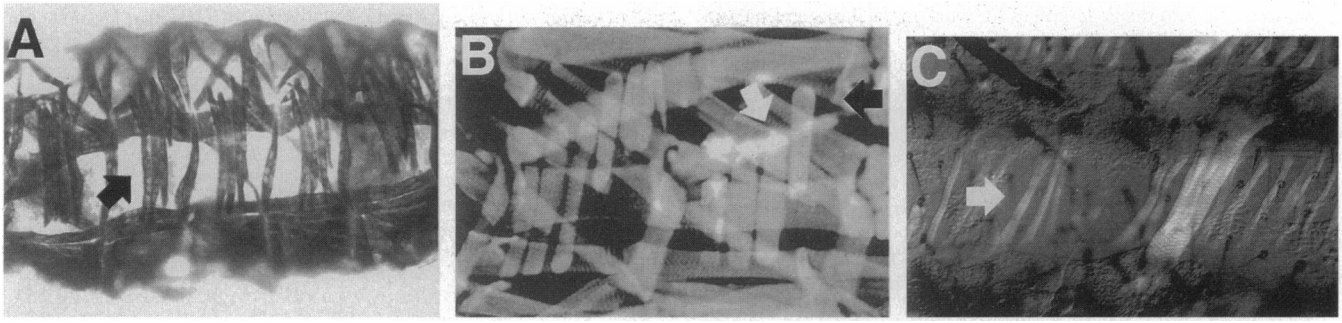

FIG. 4. Somatic muscle phenotypes of *rt* (rt^2) homozygotes. (*A*) Whole mount staining with anti-myosin antibody of embryos at stage 16 (16) to visualize the somatic muscle patterning. Mutant myotubes are narrow and in some cases missing (muscle 5, black arrow). (*B*) Fluorescein-phalloidin staining of larval muscles in flat preparations. In third instar larvae, some muscles are missing (muscle 18, black arrow) or deranged (muscle 3, white arrow). (*C*) Adult muscles and persistent larval ones visualize with polarized light in abdominal adult flat preparations. Notice that some persistent larval dorsal muscles are missing (muscle 1, white arrow). The orientation of the small adult abdominal muscles correspond to the staggered orientation of the imaginal cuticle.

mutations cause muscle defects and rotate abdomens (1). Like *rt*, *tw* and *fr* alleles cause clockwise rotations but in *ar* different alleles can cause either clock or counterclockwise rotations (24).

We propose that the resulting right-handed torque derives from the intrinsic helicity of one or several molecules in the architecture of the muscle fiber (Fig. 5*A*). Of the major structural proteins of muscle fibers, actin has been shown to exhibit a right-handed torque in its sliding force (25). Also, titins reveal a single globular head at one end that anchors in the M-line on a long and very thin rod about 1 μm long. They show a clear polarity and a convoluted appearance in micrographs that suggest an intrinsic angular tension. The elastic titin filaments might transmit this tension through relaxed sarcomeres (26). If (*i*) a right-handed torque along the anterior/posterior axis is consistently polarized in all fibers and in all segments, (*ii*) anchoring has no handedness in both left and right body sides and (*iii*) this intrinsic enantiomorphism is translated to higher levels of muscle architecture, then any failure resulting in muscle relaxation will cause the same twist at both sides of the body and hence consistent rotation of the whole muscular larval lattice (Fig. 5*B*). The resulting abdominal torque will affect first the larval shape (and locomotion) as in the *roller* mutants of *Caenorhabditis elegans* (4).

In *Drosophila*, the adult thoracic and abdominal segments are symmetrical because of the positional matching along the anterior/posterior axis of left and right imaginal cells during contralateral fusion in metamorphosis. Colateral fusion between segments, on the other hand, requires mediolateral positional matching. However, transplantation experiments have shown that whereas thoracic segments have mediolateral graded positional differences, the abdominal segments do not (27, 28). The abdominal segments of the adult result from proliferation of bilateral segmentally iterated histoblast nests growing on the overlaying larval cells at metamorphosis (29). The present results suggest that they join at the intersegmental boundaries and differentiate their cuticular pattern irrespective of the mediolateral positional values of either the larval segment or that of adjacent adult segments (Fig. 5*C*). In *rt* mutants, and possibly in mutants of the other genes, the muscular rotation of the larva causes shifts in the patterned matching of cells of adjacent segments and hence similar segmental epidermis rotation (Fig. 5*C*).

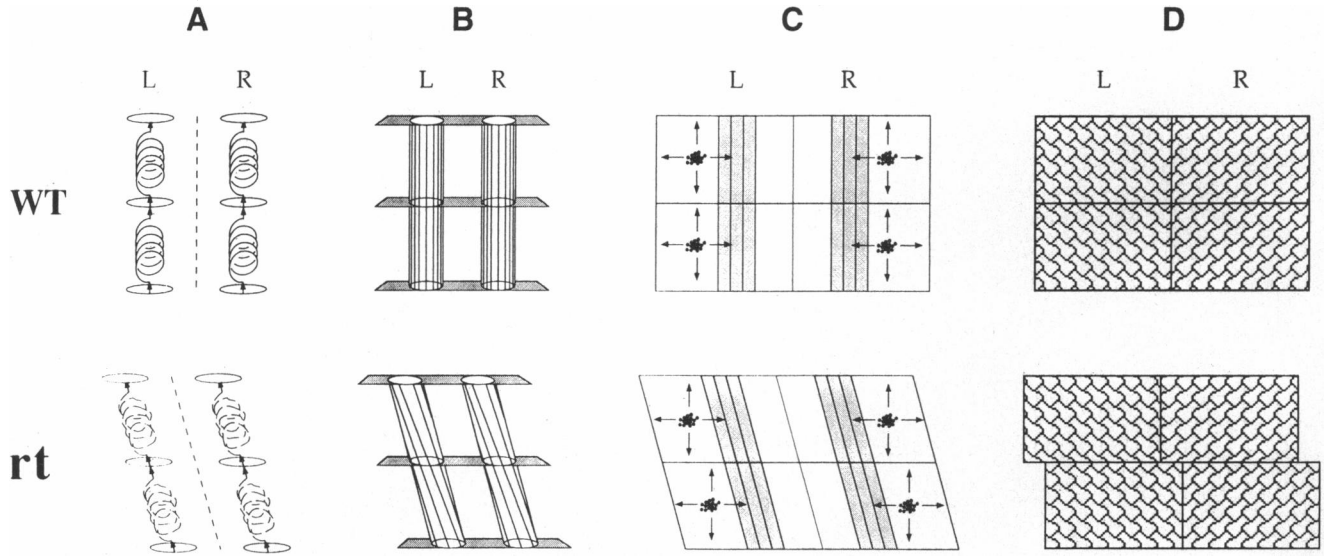

FIG. 5. A model for the translation of *rt* embryonic muscle defects into the helical staggering of the adult epidermis (two adjacent segments are represented). (*A*) Torque in individual fibers. The attachment of the fibers to the cell membrane (and this to the epidermal apodemes) are hypothetically of the same handedness at both sides of the body. In *rt*, cell membrane attachment is weakened and fiber relaxation ensues. (*B*) Morphology and pattern of longitudinal (as example) muscles. In *rt*, muscles are weaker and torque effects are unveiled. (*C*) Expansion below the larval epidermis of histoblasts from imaginal nests contacting in colateral fusions in metamorphosing pupae are in constant positions in wild type and in mediolaterally shifted ones in *rt*. (*D*) Alignment of adult cuticle after histoblasts growth and fusion. Mediolaterally aligned in wild type and staggered in the mutant condition.

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