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Genetics

Different genetic requirements for anterior RNA localization revealed by the distribution of Adducin-like transcripts during *Drosophila* oogenesis

(cytoskeleton/development/embryo/maternal-effect genes/pattern formation)

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ABSTRACT **The proteins encoded by polar-localized mRNAs play an important role in cell fate specification along the anteroposterior axis of the *Drosophila* embryo. The only maternally synthesized mRNA known previously to be localized to the anterior cortex of both the oocyte and the early embryo is the bicoid mRNA whose localization is required to generate a homeodomain protein gradient that specifies position along the anteroposterior embryonic axis. We have identified and characterized a second mRNA that is localized to the anterior pole of the oocyte and early embryo. This mRNA encodes a *Drosophila* homolog of mammalian adducin, a membrane-cytoskeleton-associated protein that promotes the assembly of the spectrin–actin network. A comparison of the spatial distribution of bicoid and Adducin-like transcripts in the maternal-effect RNA-localization mutants exuperantia, swallow, and staufen indicates different genetic requirements for proper localization of these two mRNAs to the anterior pole of the oocyte and early embryo.**

The asymmetric distribution of developmentally important molecules is a general phenomenon thought to contribute to proper cell and organismal polarity (reviewed in refs. 1 and 2). A number of polar-localized RNAs have been identified in the *Drosophila* egg and/or early embryo. The bicoid, Bicaudal-D, *fs(1)K10*, and yemanuclein α RNAs are localized to the anterior of the oocyte (3–8). The last three of these RNAs become delocalized before the end of oogenesis; only the bicoid RNA is anterior-localized through oogenesis and into early embryogenesis.

To identify additional molecules that might play a role in anteroposterior axis specification in the oocyte and early embryo, we have focused on identifying maternal RNAs localized to either the anterior or the posterior pole of the *Drosophila* egg and early embryo. Here, we report the identification of a *Drosophila* RNA encoding a homolog of mammalian adducin (9–11), which is localized to the anterior pole of the oocyte and early embryo. We refer to the corresponding gene as Adducin-like. The Adducin-like RNA represents the second RNA that is localized to the anterior pole through these developmental stages. A comparison of the distribution of Adducin-like RNA with that of the other such RNA, bicoid, in mutants previously shown to result in delocalization of bicoid transcripts has revealed different genetic requirements for anterior localization of these two RNAs.

MATERIALS AND METHODS

Identification of an Anterior-Localized RNA. While examining the embryonic expression patterns of different *Drosophila* retrotransposons by whole-mount RNA tissue *in situ* hybridization (unpublished results), we serendipitously detected an anterior-localized RNA in the early embryo by using a 2.2-kb *Eco*RI–*Pst* I genomic fragment adjacent to the insertion site of a *mdg3* retrotransposon (from clone pUC6ORC) (12). This *Eco*RI–*Pst* I fragment was used to screen a 0- to 4-hr embryonic cDNA library (13) and an ovarian cDNA library (D.D. and H.D.L., unpublished results). Ten cDNA clones were isolated, ranging in length from 1.3 to 4.3 kb.

DNA Sequence Analysis. Double-stranded DNA sequencing was carried out on three near-full-length cDNAs‡ by using a series of internal oligodeoxynucleotide primers according to D. Mathog's (California Institute of Technology) modification of a published protocol (14). Both strands were sequenced completely by using Sequenase version 2.0 (United States Biochemical). The Genetics Computer Group sequence analysis package was used to assemble and analyze the DNA sequence. Similarity searches were carried out with the FASTA and the BLAST programs, and the BESTFIT and GAP programs were used to compare and align sequences found to be similar.

RNA Tissue *in Situ* Hybridization. Whole-mount RNA tissue *in situ* hybridization was based on the method of Tautz and Pfeifle (15). Ovaries from adult females were dissected in phosphate-buffered saline (PBS), fixed for 25 min in 10% paraformaldehyde or formaldehyde/50 mM EGTA/10% dimethyl sulfoxide in PBS, and washed several times in PBS plus 0.1% Tween 20. Ovaries were then rubbed gently between two frosted microscope slides to break apart the ovarioles and devitellinize the late egg chambers (a method suggested by Stephen Cohen, Baylor College of Medicine). Subsequent postfixation, proteinase K digestion, and refixation were as described (15). Embryos were fixed according to this protocol, with minor modifications. Digoxigenin probes were labeled by random priming of DNA synthesis according to instructions from the manufacturer (Boehringer Mannheim) or by single-sided PCR amplification according to a protocol provided by Nipam Patel (Carnegie Institution of Washington). Hybridization and detection were as described (15), using the alkaline phosphatase substrate kit II (Vector Laboratories). Ovaries and embryos were mounted in JB4 plastic mountant for microscopy (Polysciences).

Computer-Assisted Image Analysis. Images of embryos visualized for Adducin-like RNA by whole-mount RNA tissue *in situ* methods were captured and digitized for computer analysis by using a Dage–MTI CCD-72 series solid-state camera (Dage-MTI, Michigan City, IN) and an Image

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. L07617).

Grabber NUBus digitizer board (Neotech, Eastleigh, Hampshire, U.K.) installed in a Macintosh II computer. Initial processing of the image was carried out by using Image Grabber software (version 2.01). Subsequent processing and production of a pseudocolor image representing the concentration distribution of Adducin-like transcripts was carried out by using Color Image public domain software [version 1.27; written by J. Ayers and G. Fletcher; available via anonymous file transfer protocol (FTP) from sumex-aim.stanford.edu].

Mutant Strains. Adducin-like RNA distribution during oogenesis and embryogenesis was examined in a number of maternal-effect mutants. The loci (and alleles) studied were exuperantia (exu^{XL1} and exu^{VL57}) (16), staufen ($stau^{D3}$ and $stau^{RY}$) (17), swallow (sww^{1}) (18, 19), cappuccino ($capu^{HK3}$) (20), and spire ($spir^{RP48}$) (20).

RESULTS

Anterior Localization of *Drosophila* Adducin-Like RNA During Oogenesis and Embryogenesis in Wild Type. Adducin-like RNA expression is detectable during early oogenesis in region 2B of the germarium (Fig. 1*A*) and continues throughout oogenesis (stages of oogenesis according to ref. 21). From stage 6 of oogenesis onward, the RNA can be seen to be localized to the anterior pole of the oocyte (Fig. 1 *A–D*). We consistently observe that, by stage 10, the RNA is concentrated anterodorsally and extends somewhat more posteriorly along the dorsal side of the oocyte than along the ventral side (Figs. 1 *B* and *D* and 2). These maternally synthesized transcripts maintain their anterodorsal position during the early cleavage stages of embryogenesis (Fig. 1*E*), then appear to be released and to diffuse posteriorly, thus establishing an anteroposterior RNA gradient (Fig. 1*F*) (stages of embryogenesis according to ref. 22). Maternal transcripts are lost by the syncytial blastoderm stage (Fig. 1*G*). Zygotic expression commences in the neuroblasts at stage 9 of embryogenesis, reaching high levels throughout the central nervous system by stage 15 (Fig. 1*H*).

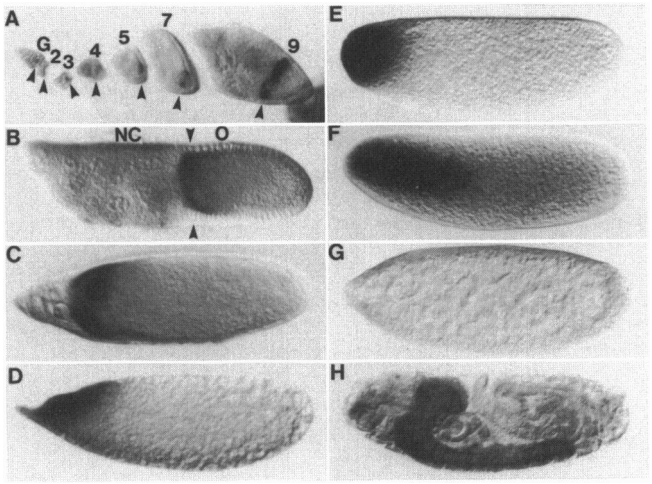

FIG. 1. Distribution of Adducin-like RNA during wild-type oogenesis and embryogenesis visualized by whole-mount RNA tissue *in situ* hybridization (15). Stages are according to King (21) (oogenesis) and Campos-Ortega and Hartenstein (22) (embryogenesis). (*A–D*) Oogenesis. (*A*) Arrowheads point to the RNA. It can first be readily detected in region 2B of the germarium (G). In stage 2–5 egg chambers, the RNA fills the most posterior cell, the oocyte. From stages 6 to 12, the RNA is localized to the anterior margin of the oocyte. This can be seen in the follicles labeled "7" and "9" in *A*, in a stage 10 egg chamber in *B* (NC, nurse cells; O, oocyte), and in a stage 12 egg chamber in *C*. The RNA consistently extends more posteriorly along the dorsal side of the oocyte than along the ventral side and is present at its highest levels anterodorsally from as early as stage 10 (also see Fig. 2*A*). (*D*) During stages 13/14, the RNA becomes even more concentrated at the anterodorsal tip of the mature egg (also see Fig. 2*B*). During oogenesis, most of the RNA is concentrated at the anterior of the oocyte; however, low levels of RNA can also be seen in the nurse cells throughout oogenesis (e.g., see *B*, which is focused on the nurse cells to show the RNA), suggesting that some or all of the RNA might be synthesized in the nurse cells. (*E–H*) Embryogenesis. (*E*) In early cleavage stage embryos (stage 1–2), the RNA is localized anterodorsally. (*F*) Slightly later (stage 2–3), the RNA appears to diffuse posteriorly, establishing an anteroposterior gradient. (*G*) By the syncytial blastoderm stage (stage 4), maternal RNA is no longer detectable. (*H*) Zygotically transcribed RNA reaches high levels in the central nervous system by stage 15/16 of embryogenesis. For all oocytes and embryos (except *C*), anterior is to the left and dorsal is up; *C* is a dorsal view.

The Anterior-Localized RNA Encodes a *Drosophila* Homolog of Mammalian Adducin. Northern blot analyses detected a 4.4-kb poly(A)$^+$ band in RNA purified from ovaries and early embryos (data not shown). Several cDNAs between 4.2 and 4.3 kb in length were isolated and sequenced (Fig. 3*A*). A single long open reading frame was found that encodes a 1156-aa protein with a high degree of overall similarity to mammalian adducin, a membrane-cytoskeleton-associated protein that is involved in regulating the association of spectrin and actin in a variety of cell types (9–11) (Fig. 3*B*). Human adducin is composed of heterodimers of α and β subunits, 737 and 726 aa long, respectively, which exhibit 49% amino acid identity and 66% similarity to each other (10). The presumptive *Drosophila* protein identified here exhibits 38% identity and 58% similarity to human α-adducin and 36% identity and 56% similarity to human β-adducin, preventing us from definitively identifying it as either a *Drosophila* α or β homolog. The presumptive *Drosophila* Adducin-like protein is, respectively, 419 and 430 aa longer than the human α- and β-adducin subunit proteins. We refer to this *Drosophila* gene as Adducin-like-56D (*Add*) based on its homology to mammalian adducin and its cytological map position. Reduced stringency genomic Southern blot analysis indicates that there are no additional *Drosophila* Adducin-like genes

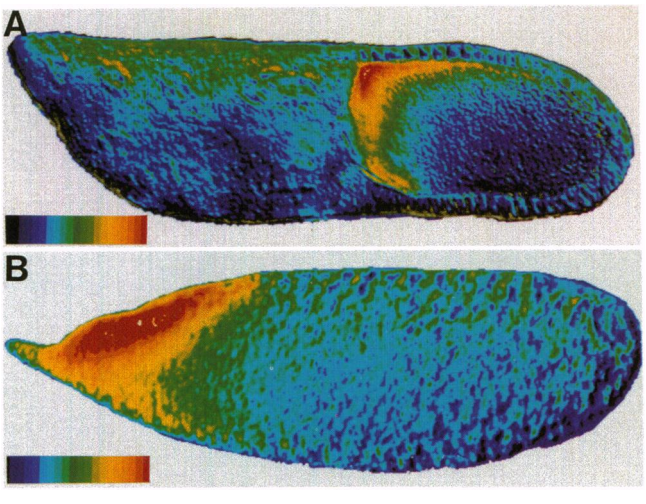

FIG. 2. Pseudocolor images of Adducin-like RNA during oogenesis. (*A*) Image of the stage 10 egg chamber shown in Fig. 1*B*. Highest concentrations of RNA are represented by red, and lowest concentrations of RNA are represented by blue. The peak concentration of Adducin-like RNA is anterodorsal, and the RNA extends more posteriorly along the dorsal side of the oocyte than along the ventral side. (*B*) Pseudocolor image of the stage 13/14 egg chamber shown in Fig. 1*D*. Highest concentrations of RNA are represented by red, and lowest concentrations of RNA are represented by blue. The peak concentration of Adducin-like RNA is anterodorsal. See *Materials and Methods* for details about the computer hardware and software used in the production of these images.

with greater than 63% similarity to the *Add* gene (data not shown). Preliminary sequence analysis of additional cDNAs suggests that variant *Drosophila* Adducin-like proteins are produced by differential splicing of the transcripts produced by the *Add* gene (data not shown). Since the probes used in our *in situ* hybridization analysis recognize common portions of the Adducin-like transcripts, we conclude that all forms of Adducin-like RNA present in the oocyte and early embryo are anterior-localized.

Effects of Maternal Mutants on Localization of Adducin-Like RNA. For bicoid to carry out its normal developmental functions, its RNA must be localized to the anterior cortex of the egg (3–5, 23). Localization of bicoid RNA is accomplished in at least four distinct phases and requires the products of the exuperantia, swallow, and staufen genes (3, 4). It has been suggested that the products of the exuperantia, swallow, and staufen genes may play an accessory role in anchoring bicoid RNA to the cytoskeleton (19, 24). Since Adducin-like mRNA is also anteriorly localized in both the *Drosophila* oocyte and early embryo, we examined its distribution in embryos derived from females homozygous for the exuperantia, swallow, or staufen mutations and compared it with that of bicoid mRNA (Fig. 4).

exuperantia activity is required prior to stage 9 of oogenesis to localize bicoid mRNA to a perinuclear cap on the apical side of each nurse cell nucleus and in a ring at the anterior end of the oocyte (3, 4, 24). In contrast to bicoid, we find that Adducin-like mRNA localization proceeds normally in exu-

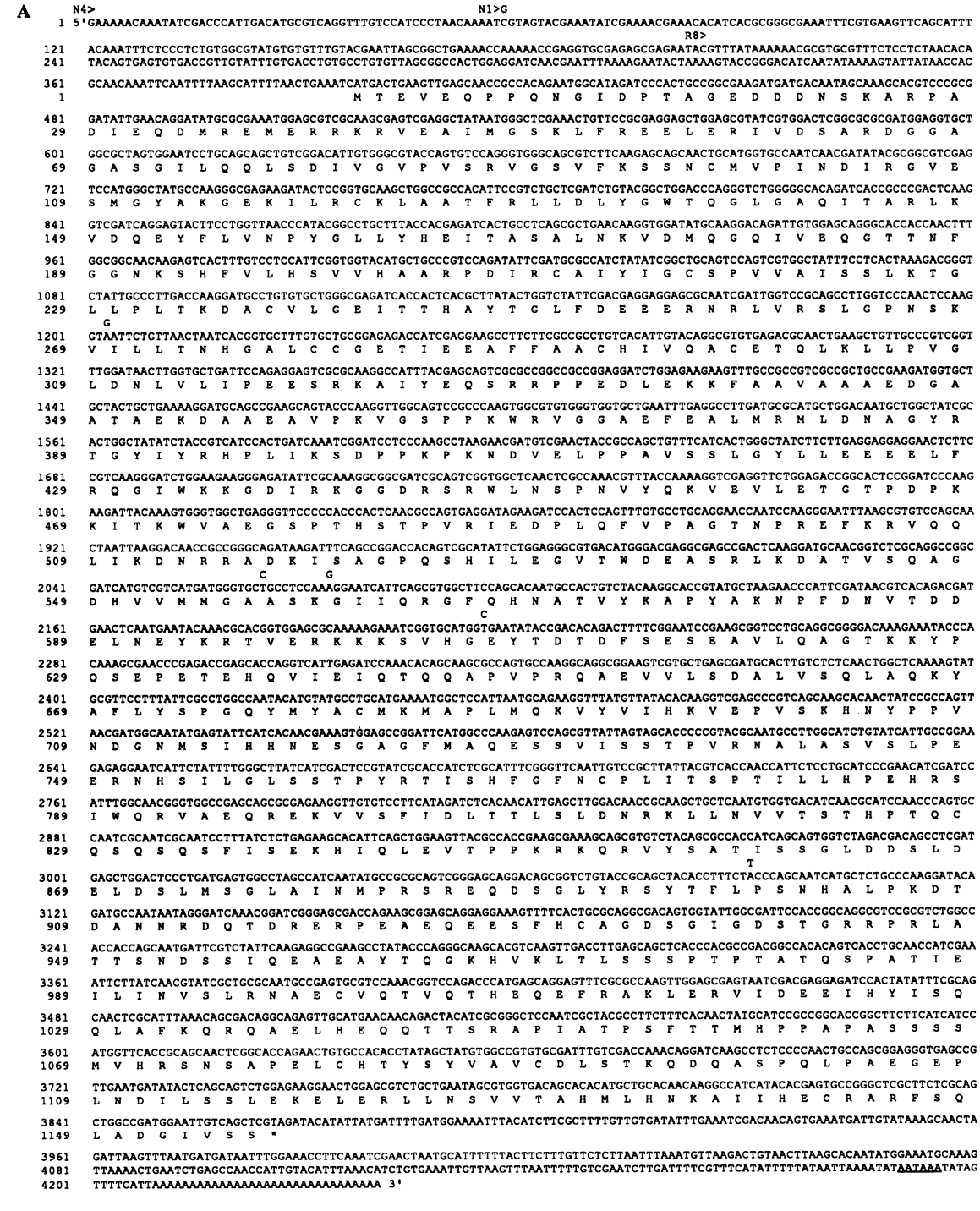

FIG. 3. (*Figure continues on the opposite page.*)

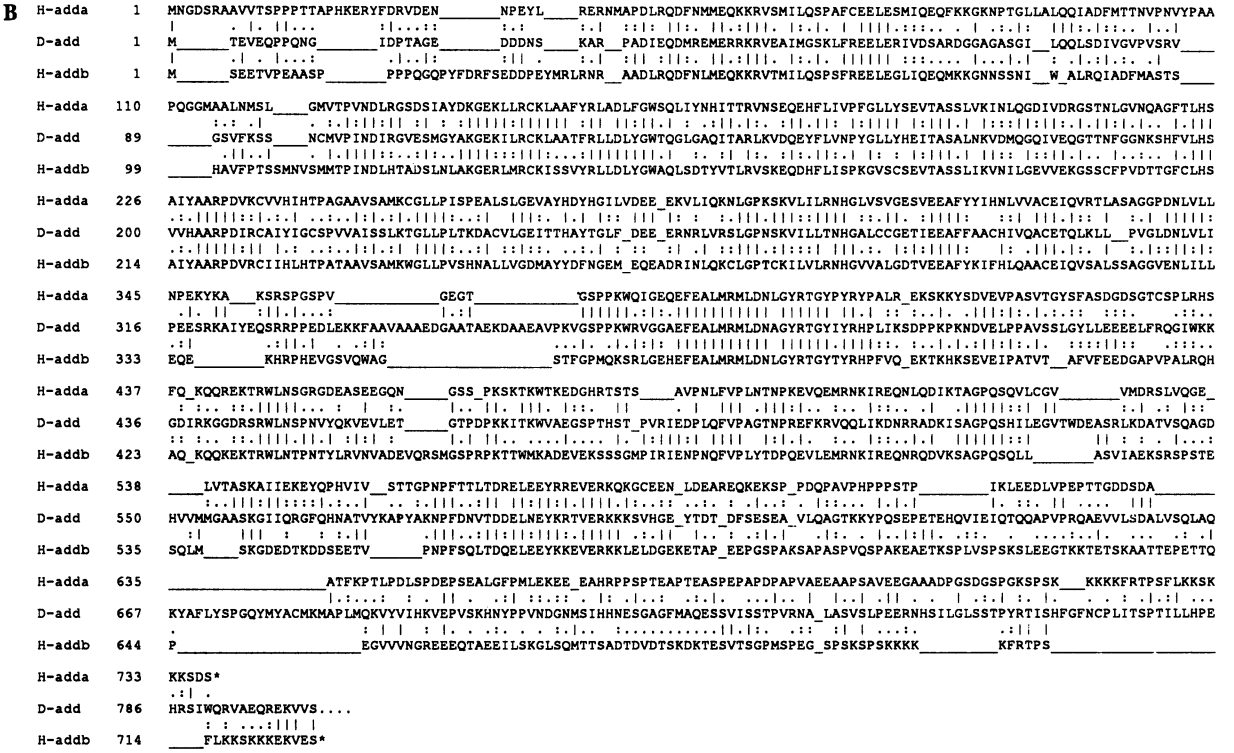

FIG. 3. Sequence of *Drosophila* Adducin-like cDNAs and comparison of *Drosophila* Adducin-like protein with human adducins. (*A*) Three near-full-length cDNAs (N1, N4, and R8), each >4.2 kb long, were sequenced, revealing an open reading frame of 3468 bases that encodes a 1156-aa protein. ">" indicates the 5′ end of each cDNA. Several minor sequence variations among the cDNAs were found: one occurs in the 5′ untranslated region (DNA sequence position 57), whereas all the others occur in the third base of codons and thus do not affect the protein sequence (DNA sequence positions 1203, 2064, 2073, 2214, and 3090). The encoded protein sequence is shown below the DNA sequence. Following the open reading frame is a 345-base 3′ untranslated region. A potential polyadenylylation signal (AATAAA) preceding the 3′ end is underlined. (*B*) Amino acid sequence comparison of *Drosophila* Adducin-like protein (D-add) and human α-adducin (H-adda) and β-adducin (H-addb) (10). Identities are represented by vertical bars; similarities are represented by colons or periods. Gaps are indicated by horizontal lines.

perantia mutants (Fig. 4*A*), showing that exuperantia plays no role in the initial stages of Adducin-like mRNA localization. In parallel experiments on embryos produced by the females from the same mutant stock, we confirmed that bicoid RNA is delocalized as expected (data not shown) (3, 4).

swallow activity is required for the next phase of bicoid mRNA localization where nurse cell localization disappears and bicoid RNA becomes localized to the anterior cortex of the developing oocyte (3, 4, 19). In the case of bicoid mRNA, embryos from swallow mutant mothers first exhibit delocalization effects at stage 10B of oogenesis when the nurse cells are contracting and transferring their cytoplasm into the oocyte (3, 4, 19). In oocytes and embryos derived from swallow mutant females, Adducin-like mRNA is also delocalized (Fig. 4 *C–F*). However, Adducin-like mRNA delocalization commences by stage 8 of oogenesis (Fig. 4*C*), 12 hr earlier than delocalization of bicoid mRNA. We consistently observe that delocalized Adducin-like mRNA first spreads posteriorly along the dorsal side of the oocyte (Fig. 4*D*), only later becoming uniformly distributed along both its dorsoventral and its anteroposterior axes (Fig. 4 *E* and *F*).

The last of the loci shown to be required for maintenance of bicoid mRNA localization is staufen, which acts to maintain bicoid mRNA in an anterodorsal position after stage 12 of oogenesis (3, 4). Neither of two strong staufen alleles had any effect on Adducin-like mRNA localization (Fig. 4*B*). In contrast, bicoid mRNA was delocalized as expected (data not shown) (3, 4).

In addition we have found that cappuccino and spire mutations, which have been shown to result in delocalization of the *fs(1)K10* RNA (25), have no effect on anterior localization of Adducin-like RNA (data not shown).

DISCUSSION

An Anterior-Localized RNA in the *Drosophila* Oocyte and Early Embryo Encodes a Homolog of a Membrane-Cytoskeletal Protein. The similarity between mammalian adducin and the protein encoded by the *Drosophila* RNA identified here extends over the entire length of the mammalian protein, indicating that we have identified a *Drosophila* homolog of mammalian adducin. This *Drosophila* Adducin-like RNA is localized to the anterior pole of both the oocyte and the early embryo; thus, to our knowledge, it is the first such polar-localized RNA to encode a homolog of a known cytoskeletal protein. The Bicaudal-D protein was previously shown to exhibit similarity to myosin heavy chain and related proteins; however, this similarity is limited to the myosin tail domain (7). Further, the Bicaudal-D RNA is only transiently localized anteriorly during oogenesis (7).

Mammalian adducin is a membrane-cytoskeletal protein that promotes association of spectrin with F-actin at actin–spectrin junctions in a calcium/calmodulin-regulated reaction (9–11). Adducin isoforms are present in erythrocytes, brain, liver, kidney, lung, testes, epithelial tissues, and various cultured cell lines (9–11). In epithelia and cultured cells, the protein is localized to sites of cell–cell contact (11). It has been suggested that adducin functions in the assembly of the spectrin-based membrane cytoskeleton, which plays a key role in orchestrating the topographic relations of integral membrane proteins within the membrane as well as in organizing integral membrane protein interactions with cytoplasmic proteins (9–11). An asymmetric distribution of the *Drosophila* Adducin-like protein might be expected to participate in the establishment and/or maintenance of an asymmetric

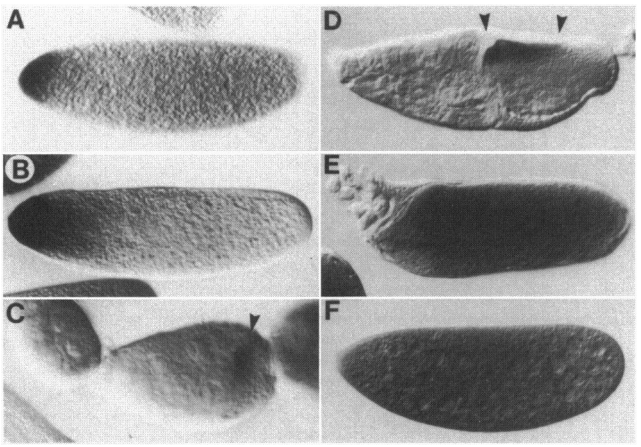

FIG. 4. Distribution of Adducin-like RNA during oogenesis and embryogenesis in mutants, visualized by whole-mount RNA tissue *in situ* hybridization (15). Anterior localization of Adducin-like mRNA occurs normally in oocytes and embryos from exuperantia (*exu*) (*A*) and staufen (*stau*) mothers (*B*) but not in oocytes and embryos from swallow (*sww*) mothers (*C–F*). (*A*) Adducin-like RNA distribution in an embryo produced by a female homozygous for exu^{XL1}, a strong *exu* allele (16). The distribution is indistinguishable from that seen in wild-type embryos (compare with Fig. 1 *E* and *F*). In parallel experiments, bicoid RNA was assayed in embryos from these females and was found to be delocalized as expected (3, 4) (data not shown). The Adducin-like mRNA distribution was also examined in ovaries of exu^{VL57} mothers and found to be normal (data not shown). (*B*) Adducin-like RNA distribution in an embryo produced by a female homozygous for $stau^{D3}$, a strong *stau* allele (17). The distribution is indistinguishable from that seen in wild-type embryos (compare with Fig. 1 *E* and *F*). In parallel experiments, bicoid RNA was assayed in embryos from these females and was found to be delocalized as expected (3, 4) (data not shown). The Adducin-like mRNA distribution was also examined in ovaries of $stau^{D3}$ and $stau^{RY}$ mothers and found to be normal (data not shown). (*C–F*) Adducin-like RNA is delocalized in oocytes and embryos produced by females homozygous for sww^{1}, a strong swallow allele (18, 19). (*C*) By stage 7/8 of oogenesis, Adducin-like RNA begins to delocalize from the anterior pole of the oocyte (arrowhead). (*D*) A stage 10 oocyte exhibits partial delocalization of Adducin-like RNA along the dorsal side (arrowheads). (*E*) By stage 12 of oogenesis, Adducin-like RNA is completely delocalized. This delocalized RNA persists through oogenesis and the cleavage stages of embryogenesis (*F*). In all panels, the orientation is with anterior to the left and dorsal up.

membrane-cytoskeletal network in the nurse cell–oocyte complex as well as in the oocyte and early embryo. Further analysis of Adducin-like protein functions will help to assess the role played by such a cytoskeletal network during oogenesis and embryogenesis.

Adducin-Like and bicoid RNAs Differ in Their Genetic Requirements for Anterior Localization. Despite the similarity of the anterior localization patterns for the bicoid and Adducin-like RNAs, the genetic requirements for their proper localization differ. While exuperantia, swallow, and staufen are required at different stages for the localization of bicoid RNA, only swallow functions in the localization of Adducin-like RNA to the anterior pole of the oocyte and early embryo. The initial anterior localization and the later anterior maintenance of Adducin-like RNA must, therefore, depend on some as yet to be identified components. Further studies will be needed to explore the molecular and genetic basis for the differences between bicoid and Adducin-like RNA localization mechanisms, particularly whether or not Adducin-like RNA carries a localization "tag", as is the case for bicoid RNA (26), and the identification of additional loci required for RNA localization.

Embryos from swallow mutant females exhibit abnormalities in addition to those predicted solely on the basis of delocalization of bicoid RNA. These include defects in nuclear cleavage and cellularization as well as in abdominal development. Consequently the swallow protein has been proposed to play a more general role in cytoskeletal organization during oogenesis and early embryogenesis (19, 24). Whether any of the developmental abnormalities in embryos from swallow mutant females are attributable to delocalization of Adducin-like RNA remains to be determined.

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