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Expression Patterns of a twist-Related Gene in Embryos of the Spider Achaearanea tepidariorum Reveal Divergent Aspects of Mesoderm Development in the Fly and Spider

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ABSTRACT—We cloned an Achaearanea tepidariorum (Chelicerata, Arachnida) gene related to Drosophila twist (twi), which encodes a basic helix-loop-helix transcription factor required to specify mesoderm fate in the Drosophila embryo. The cloned spider gene was designated At.twist (At.twi). We examined its expression by whole-mount in situ hybridization. At.twi transcripts were first detected in cells located at the polar and equatorial areas of the spherical embryo when the cumulus reached the equator. As the extraembryonic area expanded, more cells expressed At.twi transcripts. The At.twi-expressing cells became distributed nearly uniformly in the embryonic area. At these stages, some At.twi-expressing cells were found in the surface epithelial cell layer, but other At.twi-expressing cells were at slightly deeper positions from the surface. When the embryo was transformed into a germ band, all At.twi-expressing cells were situated just beneath the surface ectoderm, where they became metamerically arranged. Although little expression was observed in the caudal lobe of the elongating germ band, new stripes of At.twi expression appeared beneath the ectoderm in accordance with the posterior growth. These observations suggested that the cells expressing At.twi were most likely mesoderm. We propose that At.twi can be used as a molecular marker for analyzing mesoderm development in the spider embryo. Moreover, comparison of the expression patterns of twi and At.twi revealed divergent aspects of mesoderm development in the fly and spider.

Key words: chelicerate, arthropod, mesoderm development, twist, snail

INTRODUCTION

The mesoderm is the third germ layer that emerges between the ectoderm and endoderm during the development of bilaterians. Since the mode of mesoderm development among bilaterian taxa varies markedly, it seems almost impossible to reconstruct the evolution of mesoderm development based solely on morphological data.

In Drosophila, the molecular mechanisms that specify the fate of the primary mesoderm and regulate the development of the mesoderm have been extensively studied. In the syncytial blastoderm, maternal gene products establish morphogen gradients that define the anterior-posterior (AP) and dorsal-ventral (DV) axes (St Johnston and Nüsslein-Volhard, 1992). The nuclear gradient of Dorsal protein along the DV axis directly regulates the transcription of zygotic genes in a concentration-dependent manner, resulting in region-specific gene expression (Chasan and Anderson, 1993). At the ventral one-fourth region of the blastoderm, transcription of twist (twi) and snail (sna) is activated, which are essential for mesoderm formation (Simpson, 1983; Leptin and Grunewald, 1990). The twi gene, encoding a basic helix-loop-helix (bHLH) transcription factor (Thisse and Thisse, 1992), activates the transcription of mesoderm-specific genes (Leptin, 1991; Furlong et al., 2001). The sna gene, encoding a zinc-finger transcription factor (Boulay et al., 1987), mainly represses the transcription of ectodermal genes (Leptin, 1991). Such positive and negative regulation by twi and sna specifies the primary mesoderm at the ventral region. The twi gene continues to be expressed in all cells of the mesoderm until they extend over the whole of the inner surface of the ectoderm (Thisse et al., 1988). During the patterning of the mesoderm, twist expression is reg-
ulated by wingless, hedgehog, and Notch signaling (Lee and Frasch, 2000; Riechmann et al., 1997; Tapanes-Castillo and Baylies, 2004). sna expression disappears from the mesoderm soon after the internalization (Alberga et al., 1991). Signals from the surface ectoderm also contribute to mesoderm development (Staehling-Hampton et al., 1994).

Characterization of the dorsal, twi and sna homologs in another insect, Tribolium, revealed a conserved mechanism of mesoderm formation in long- and short-germ insects (Sommer and Tautz, 1994; Chen et al., 2000). Even in nonarthropod metazoans, twi homologs have been identified and shown to be expressed in mesoderm- or endoderm-derived tissues (Hopwood et al., 1989; Yasui et al., 1998; Harfe et al., 1998; Imai et al., 2003; Nederbragt et al., 2002; Spring et al., 2000; Martindale et al., 2004). However, none of these non-arthropod twist genes appear to function in the specification of the primary mesoderm. Moreover, in non-insect arthropods, few molecular data concerning mesoderm development are available.

To investigate the variation in the mode of mesoderm development among arthropods, we employed embryos of the spider Achaearanea tepidariorum. This animal belongs to Chelicerata, which is phylogenetically distant from Insecta within Arthropoda (Friedrich and Tautz, 1995; Boore et al., 1998; Giribet et al., 2001; Cook et al., 2001). Although many previous works have described the embryonic development of spiders and other chelicerales (Montgomery, 1909; Holm, 1940, 1952; Seitz, 1966; Anderson, 1973; Yoshikura, 1975; Suzuki and Kondo, 1995), it is largely unclear how the mesoderm is formed and patterned during chelicerate development due to technical limitations in identifying mesoderm cells. The stages of Achaearanea embryogenesis that have been defined on the basis of morphological characteristics in living embryos (Akiyama-Oda and Oda, 2003) are illustrated in Fig. 1. In the Achaearanea embryo, several morphologically distinct cell types have been observed beneath the surface ectoderm at the cumulus-migrating and later stages (Montgomery 1909; Akiyama-Oda and Oda, 2003).

Fig. 1. The stages of Achaearanea embryogenesis. Stages 1 to 8 have been defined in the previous study (Akiyama-Oda and Oda, 2003). (A) Stage 2. The energids (shaded circles) appear at the periphery of the egg, and undergo synchronous cleavages. (B) Stage 3. The energids shift toward one pole of the egg along the surface. This pole is designated the embryonic (Em) pole, and the opposite pole the abembryonic (Ab) pole. (C) Stage 4. The energids settle and form a germ disc (shaded area). Cells are internalized at the embryonic pole to form the primary thickening. (D) Stage 5. Some of the internalized cells, which are the cumulus mesenchymal (CM) cells, migrate in a cluster toward the rim of the germ disc (thick arrow). (E) Stage 6. Germ disc cells migrate in directions indicated by thick arrows, with the extraembryonic area (Ex) formed and expanded. During this stage, the radially symmetrical germ disc is converted into a fan-like shape with the AP and DV axes. (F) Stage 7. The fan-shaped embryo is transformed into a germ band with metameric morphologies. The previous central region of the germ disc develops into the caudal lobe (CL). (G) Stage 8. The germ band continues to extend (as indicated by thick arrows in F), and nascent limb buds appear. (H) Stage 9. The limb buds extend distally. L1 to L4 indicate the limb buds for the first to fourth walking legs, and Ch and Pp indicate those of the chelicera and pedipalp, respectively. The dotted line indicates the boundary between the prosoma and the opisthosoma.
These include cumulus mesenchymal (CM) cells, that are characterized by At.dpp expression, and some At.fkh expressing cells (Akiyama-Oda and Oda, 2003). However, it is not known whether the Achaearanea mesoderm can be characterized molecularly in the same manner as the mesoderm in the Drosophila embryo. In this study, we cloned A. tepidariorum genes related to Drosophila twist and sna, designated At.twi and At.sna, respectively, to examine whether these genes can help to identify the mesoderm cells in developing embryos of the spider. Whole-mount in situ hybridization revealed that At.twi, but not At.sna, appears to be expressed in likely mesoderm cells. Thus, we propose that At.twi can be used as a molecular marker for analyzing the development of the spider mesoderm. Comparison of the expression patterns of twi and At.twi revealed divergent aspects of mesoderm development in the fly and spider.

MATERIALS AND METHODS

Animals

The spider Achaearanea tepidariorum was used in our experiments. Embryos were obtained as described previously (Akiyama-Oda and Oda, 2003). Stages 1 to 8 of embryonic development have been defined in the previous study (Fig. 1; Akiyama-Oda and Oda, 2003). Stage 9 is the period during which the nascent limb buds extend distally. Stage 10 is the period during which the extending limbs become segmented and the germ band splits to shift laterally.

cDNA cloning

To isolate Achaearanea homologs of the Drosophila twist and sna genes, we initially performed polymerase chain reactions (PCR) using degenerate primers. The following primers were used: twist forward primer, 5′ tngcnaa(t/c)gt(g/a)gnga(g/a)(c/a)tg 3′ (for the amino acid sequence M/LANVRER); twist forward primer for nested PCR, 5′ gtn(a/c)gnga(a/g)(a/c)gnca(a/g)(a/c)gncac 3′ (for the amino acid sequence VRERQRT); twist reverse primer for nested PCR, 5′ a(a/g)tlt(g/a)ngt(t/c)tg(a/g)tat(t/c)tt 3′ (for the amino acid sequence RTHTLPC); sna forward primer, 5′ aa(a/g)atgc(t/c)at(a/g)cncna 3′ (for the amino acid sequence KMHIRTH); sna reverse primer, 5′ aang(t/c)tt(t/c)tcncngt(a/g)tg 3′ (for the amino acid sequence FPKEGTH); sna forward primer for nested PCR, 5′ gncnc(t/c)an(t/c)ntcngt 3′ (for the amino acid sequence TTHRIHG). cDNA prepared from embryos and larvae were used as the template.

To isolate cDNA clones, digoxigenin (DIG)-labeled probes for the amplified fragments were prepared using a PCR DIG Probe Synthesis Kit (Roche). Two cDNA libraries of embryos and hatched prelarvae (Akiyama-Oda and Oda, 2003) were screened with the probes. Three clones for At.twi and one for At.sna were obtained. Both strands of the representative At.twi and At.sna clones were sequenced, revealing that these clones cover the entire or nearly entire coding regions of the mRNAs. However, the translation start site for At.twi mRNA was not determined with confidence since no in-frame stop codon was identified upstream of the first methionine in the isolated cDNA clones. In the At.sna clone, a potentially artificial deletion of one nucleotide was detected. The DNA fragments covering the site were amplified by RT-PCR to determine the authentic sequence of At.sna. The At.twi and At.sna sequences are available from the DNA data bank of Japan (DDBJ) with the following Accession numbers: At.twi, AB167807; At.sna, AB167392.

Molecular phylogenetic analysis

The deduced amino acid sequences of the bHLH domains of At.twi and other twi-related genes as well as the zinc finger-containing regions of At.sna and other sna-related genes were aligned manually. Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) using PHYLIP (Felsenstein, 1993). Confidence in the phylogenies was assessed by bootstrap resampling of the data sets.

Whole-mount in situ hybridization

For whole-mount in situ hybridization, spider embryos were fixed as described previously (Akiyama-Oda and Oda, 2003). RNA probes were prepared using T7 RNA polymerase (Stratagene) and DIG RNA Labeling Mix (Roche). The first 916 bp of At.twi cDNA including the entire bHLH domain, and the 1692 bp region of At.sna cDNA (nt180-1871), which contains all the zinc finger motifs, were used as templates. Hybridization, washes and detection were performed as described previously (Akiyama-Oda and Oda, 2003). Some embryos were counterstained with the anti-pMAD antibody PST1 (Penson et al., 1998).

Sectioning

Stained embryos were dehydrated in a graded ethanol-xylene series, embedded in TissuePrep (Fisher Scientific), and then serially sectioned at 5 µm thickness.

RESULTS AND DISCUSSION

Cloning of A. tepidariorum genes related to twi and sna

A. tepidariorum cDNAs related to twi and sna were cloned. The details of each cDNA sample are described below and in the Materials and Methods.

At.twi

The isolated cDNA clone encoded a polypeptide of 221 or more amino acids with a bHLH domain and a WR motif characteristic of Twist proteins (Fig. 2A). The bHLH domain displayed 61–69% amino acid identity with those in the Twist proteins of Drosophila, jellyfish, mollusk, leech, lancelet and mouse, compared to the 54%, 47% and 43% identity that it shared with the bHLH domain of the Drosophila non-Twist-class bHLH proteins Hand, Fer1 and Dimm, respectively. Based on the topology of a phylogenetic tree constructed using the bHLH domain sequences (Fig. 2C), the isolated gene was found to belong to the twist family. Accordingly, the gene was designated At.twist (At.twi).

At.sna

Analysis of the isolated cDNA clone and amplified cDNA fragments revealed a polypeptide of 303 amino acids with five zinc finger motifs. The amino acid sequences of these motifs were highly similar to those found in Drosophila Sna, Escargot and Worniu and other animal Snail/Slug-class proteins (Fig. 2B). The isolated gene was classified into the snail/slug family, based on phylogenetic analyses using the sequences of the regions encompassing zinc finger II-V motifs (Fig. 2D). Accordingly, the gene was designated At.snail (At.sna). At.sna was most closely related to Cs.sna, a snail homolog in Cupiennius salei, another spider species (Weller and Tautz, 2003).
At.twi transcripts are expressed in mesoderm cells

We examined the expression patterns of **At.twi** transcripts in spider embryos by whole-mount in situ hybridization. **At.twi** transcripts were not detected before late stage 5, when the CM cells reached the periphery of the germ disc (Akiyama-Oda and Oda, 2003). At early stage 6, **At.twi** transcripts were detected in cells located at the polar and equatorial areas of the embryo (Fig. 3A). In the initial pattern of **At.twi** expression, no reproducible asymmetry could be recognized with respect to the embryonic-abembryonic axis. As the extra-embryonic area expanded, more cells expressed **At.twi** transcripts (Fig. 3B). The **At.twi**-expressing cells became distributed nearly uniformly in the embryonic area. At these initial stages, some **At.twi**-expressing cells were found in the sur
Fig. 3. Expression patterns of *At.twi* transcripts as revealed by whole-mount *in situ* hybridization. Signals of the *At.twi* transcripts are presented in purple. (A) An early stage 6 embryo viewed from the top of the germ disc. This embryo was also stained for pMAD (brown) to determine its orientation. The asterisk indicates the site of the germ disc rim at which the cumulus arrived. (B) A mid-stage 6 embryo viewed from the prospective caudal side. The emerging extra-embryonic area (Ex) shows little *At.twi* expression. (C) A stage 7 embryo viewed from the ventral side. Anterior is to the top; CL, caudal lobe; L1 to L4, first to fourth walking legs. (D–G) Flat preparations of embryos at stage 7 (D), stage 8 (E) and stage 9 (F, G). Anterior is to the left. One anterior domain and the following four stripes of *At.twi* expression are seen in the stage 7 embryo (D). The four stripes correspond to the segments bearing walking legs (L1–L4). The anterior domain was subdivided to give rise to two additional stripes (arrowheads) that correspond to the chelicerae (Ch) and pedipalps (Pp). In opisthosomal segments produced from the caudal lobe (CL), stripes or patches of *At.twi* expression are seen (arrows). (H, I) Close-up images showing the polar area of an embryo at early stage 6. The focus was adjusted to the surface epithelial layer for the first image (H), and shifted a little deeper into the interior of the embryo for the second image (I). Arrows indicate the *At.twi*-expressing cells in focus. y, yolk. (J, K) Longitudinal section of a stage 7 embryo. Anterior is to the left. The area boxed in J is magnified in K. *At.twi*-expressing cells are situated just beneath the surface ectoderm. (L, M) A stage 9 embryo viewed from the lateral side. The area boxed in L is magnified in M. Expression stripes that appeared in the opisthosomal region (arrows) are situated just beneath the surface ectoderm. Expression is not detectable at the caudal lobe (CL). Scale bar: 250 µm in A–C; 100 µm in D–G, L, M; 25 µm in H–K.
face epithelial layer but other *At.twi*-expressing cells were located a little deeper than at the surface (Fig. 3H, I).

The stage 7 embryo, which is transforming into a germ band, showed one anterior domain and four stripes of *At.twi* expression (Fig. 3C, D). Sectioning of stained stage 7 embryos revealed that all the *At.twi*-expressing cells were situated just beneath the ectoderm (Fig. 3J, K). The four stripes were persistently seen at later stages (Fig. 3D–F), and probably correspond to the segments bearing walking legs. The fourth stripe displayed higher levels of *At.twi* expression than the remaining three stripes (Fig. 3C–E). The anterior domain of *At.twi* expression was sequentially subdivided to produce two stripes (Fig. 3D–F). These two stripes probably correspond to the chelicerae and pedipalps. Each of the six, in total, *At.twi* expression stripes at the prosomal region was laterally split into two large patches. These changes in the shapes of the *At.twi* expression domains occurred in accordance with the formation of the limb buds (Fig. 3E, F), although the expression gradually declined during limb bud extension (Fig. 3G). In embryos whose limb buds were extending, relatively high levels of *At.twi* expression remained detectable at the anterior terminal region (Fig. 3G).

At the formed caudal lobe, little *At.twi* expression was observed (Fig. 3C–E). However, in the segments produced from the caudal lobe, expression stripes appeared (Fig. 3F, G, arrows). Like the prosomal stripes, these opisthosomal stripes were also situated just beneath the ectoderm (Fig. 3L, M). At later stages, each was laterally split into two patches (Fig. 3G).

We speculated that the cells expressing *At.twi* are mesoderm cells for four reasons, as follows. First, at least in stage 7 and older embryos, all the *At.twi*-expressing cells were situated just beneath the surface ectoderm. Second, the *At.twi*-expressing cells, unlike typical endoderm cells, became metamERICALLY arranged. Third, in stage 9 embryos, most of the *At.twi*-expressing cells present in the prosomal segments were associated with the forming limb buds, where somatic and visceral mesoderm cells have been observed morphologically (Montgomery, 1909). Fourth, in *Achaearanea*, the central nervous system develops from the surface ectoderm at stage 9 and later in a way similar to that described in *Cupienius* (Stollewerk et al., 2001; Y. A. and H. O., unpublished observations). It is likely that the *At.twi*-expressing cells are distinct from the internalized neural cells. The distributions of the *At.twi*-expressing cells pre-

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**Fig. 4.** Expression patterns of *At.sna* transcripts as revealed by whole-mount in situ hybridization. Signals of the *At.sna* transcripts are presented in purple. Embryos shown in A–C and E were also stained for pMAD (brown). (A) An early stage 5 embryo viewed from the top of the germ disc. The asterisk indicates the center of the germ disc. (B) An early stage 6 embryo viewed from the top of the germ disc. *At.sna* expression is detected in a broad ring of the germ disc area. Around the polar area, lower levels of *At.sna* expression are observed. The asterisk indicates the site of the germ disc rim at which the cumulus arrived. (C) A mid-stage 6 embryo viewed from the prospective caudal side. The bracket indicates the extra-embryonic area, which displays little or no *At.sna* expression. (D) A stage 7 embryo viewed from the ventral side. Anterior is to the top. Metameric patterns of expression are observed. (E, F) Longitudinal section of a stage 5 embryo. The area boxed in E is magnified in F. (G, H) Longitudinal section of a stage 7 embryo. Anterior is to the left. The area boxed in G is magnified in H. *At.sna* expression is detectable in surface cells (arrowheads) but not in internal cells (arrows). Scale bar: 250 μm in A–D; 50 μm in E, G; 25 μm in F, H.
presented here are similar to those of cells that Montgomery has described as mesentoblasts and mesoblasts (Montgomery, 1909). We propose that \textit{A.twi} can be used as a marker for analyzing mesoderm development in the spider. Moreover, our observations suggest that \textit{twi} and \textit{A.twi} play conserved roles in the mesoderm development of the fly and spider.

\textbf{\textit{At.sna} transcripts are expressed in part of the ectoderm}

The expression patterns of \textit{At.sna} transcripts were also examined by whole-mount \textit{in situ} hybridization. \textit{At.sna} transcripts were detected in a broad ring near the margin of the germ disc from late stage 4 or early stage 5, and similar patterns persisted up to the beginning of stage 6 (Fig. 4A–C). Sectioning of stained stage 5 embryos revealed that the \textit{At.sna}-expressing cells were located only in the surface cell layer (Fig. 4E, F). As the extra-embryonic area expanded, this area displayed lower levels of expression compared to the embryonic area (Fig. 4C). At stage 7, \textit{At.sna} expression was seen in stripes (Fig. 4D). Although this expression pattern was, at a glance, similar to that of \textit{A.twi} at the corresponding stage, the \textit{At.sna} expression stripes were located in the surface ectoderm with no expression in the inner layer (Fig. 4G, H). At later stages of embryogenesis, \textit{At.sna} transcripts were detected in the central nervous system (data not shown), consistent with the expression pattern of \textit{Cs.sna} (Stollewerk \textit{et al}., 2001; Weller and Tautz, 2003). These observations indicate that \textit{At.sna} is expressed in part of the ectoderm, but not in the \textit{twi}-expressing mesoderm. Our data do not suggest that the role for \textit{snail} in the mesoderm formation of the fly is conserved in the spider.

\textbf{Divergent aspects of mesoderm development in the fly and spider}

Comparison of the expression patterns of \textit{twi} and \textit{A.twi}
revealed divergent aspects of mesoderm development in *Drosophila* and *Achaearanea* (Fig. 5). In the fly embryo, all the mesoderm arises from the future ventral side (Fig. 5A). In contrast, in the spider embryo, the initial distribution of *At.twi* transcripts was not ventrally localized; instead, this distribution was rather symmetric with respect to the embryonic-abembryonic axis (Fig. 3A). The emerging asymmetry of *At.twi* expression during stage 6 (Figs 3B, 5B) appeared to be associated with the formation of the extra-embryonic area. The *At.twi* expression pattern within the embryonic area showed little unevenness along the emerging DV axis until the limb buds began to form. These observations indicate that the relationship between DV pattern formation and mesoderm formation differs in the fly and spider. In the *Drosophila* embryo, all prospective mesoderm cells are internalized from the ventral side within a short period, and this process can be observed (Sweeton *et al*., 1991; Kam *et al*., 1991; Oda and Tsukita, 2001). In the spider embryo, however, it was not so easy to determine from where and how the mesoderm was internalized. This difficulty may be at least partially due to asynchronous internalization of prospective mesoderm cells, as *At.twi*-expressing cells were found both at the surface and inner layers in the same germ discs (Fig. 3H, I). However, we could not reconstruct the dynamic processes of cell behavior and gene expression with any certainty. Nonetheless, the initial patterns of *At.twi* expression (Figs 3A, 5B) may suggest that the central and peripheral areas of the germ disc are the sites of mesoderm internalization in the *Achaearanea* embryo. Invagination of the individual cells that have stochastically started to express *At.twi* at the surface might explain the mesoderm internalization. The mechanism by which the *At.twi* expression is initiated is an interesting issue to be studied in the future, which will clarify the difference between the fly and spider.

In the *Drosophila* embryo, *twi* transcription is initiated simultaneously in all prospective mesoderm cells, and mesoderm differentiation proceeds synchronously in all segments. Apparently, this is not the case in the *Achaearanea* embryo. The number of *At.twi*-expressing cells increased progressively during early and mid stage 6 (Fig. 3A, B). This increase may be due to initiation of *At.twi* transcription as well as to cell division. The four expression stripes of *At.twi* corresponding to the segments bearing walking legs emerged during the formation of the germ band, when *At.sna* and many other genes showed striped expression in the surface ectoderm (Fig. 4D; our unpublished data). Thus, it appears that the patterning of the prosomal mesoderm is accompanied with that of the surface ectoderm. The appearance of new *At.twi* expression stripes at the growing opisthosomal region during stages 8 and 9 (Fig. 5B) is the result of initiation of *At.twi* transcription rather than subdivision of cell populations with persistent *At.twi* expression, since we have not observed intermediate states of such subdivision. Interestingly, in contrast to the opisthosomal region, the *At.twi* expression stripes corresponding to the chelicerae and pedipalps appeared to be formed as a result of sequential subdivision (Fig. 3D–F). In the prosomal and opisthosomal regions, different mechanisms may regulate the initiation of *At.twi* transcription. What cellular and molecular mechanisms cause the segmentation of the mesoderm in the spider embryo remain to be elucidated.

We suggest that there are crucial differences in the way the mesoderm develops in the fly and spider. Investigation of the cellular and molecular mechanisms underlying these differences will contribute to a better understanding of the ancestral mode of mesoderm development in the phylum Arthropoda. Although possible variations in the mode of mesoderm development among spiders as well as chelicerates will be considered, we believe the *twi* family genes will be useful in comparative analyses that aim to reconstruct the evolution of the arthropod mesoderm.

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