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Identification of a *Drosophila* activin receptor

(protein-serine/threonine kinase/receptor kinase/transforming growth factor β superfamily)

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ABSTRACT **Activins are cytokines of the transforming growth factor β superfamily that control various events during vertebrate embryo development and cell differentiation in the adult, and act through transmembrane receptors that contain a cytoplasmic protein-serine/threonine kinase domain. We describe the identification, deduced primary structure, and expression pattern of Atr-II, a receptor serine/threonine kinase found in *Drosophila*. With the exception of the spacing of 10 cysteine residues, the extracellular domain of Atr-II is very dissimilar from those of vertebrate activin receptors, yet it binds activin with high affinity and specificity. The kinase domain sequence of Atr-II is 60% identical to those of activin receptors from vertebrates, suggesting similarities in their signaling mechanisms. Maternal Atr-II transcript and its product are abundant in the oocyte. During development, the highest levels of Atr-II transcript and protein are observed in the mesoderm and gut. The possible role of an activin signaling system in *Drosophila* development is discussed.**

The transforming growth factor β (TGF-β) family of growth and differentiation factors control cell proliferation, differentiation, and tissue inductive interactions in many organisms (reviewed in refs. 1–4). Most known members of this family are from vertebrates. However, the identification of the *Drosophila* factors dpp (5) and 60A (6, 7), both related to mammalian bone morphogenetic proteins (BMPs), confirms the presence of this family in invertebrates. Many members of this family, including dpp, activins, and some of the BMPs, have been implicated in controlling various aspects of development (1–4, 8–10). The evolutionary conservation of these factors and their role in intercellular signaling suggest that they are part of a fundamental strategy by which development is regulated in metazoan organisms.

Understanding the function of these factors requires structural information about their receptors and signaling elements. Several types of membrane receptors for TGF-β, activins, and BMPs have been identified, among which the type II receptors are of particular significance (11). Two distinct activin type II receptors (12–14) and one TGF-β type II receptor (15) have been cloned from vertebrates and shown to encode membrane proteins containing a cytoplasmic protein-serine/threonine kinase domain. Another member of this receptor family is encoded by the *Caenorhabditis elegans daf-1* gene, which controls dauer larva development in response to an unknown ligand (16).

To further characterize potential developmental roles of TGF-β-related signaling pathways, we have searched for receptor serine/threonine kinase family members in *Drosophila*. Here we describe the identification of such a receptor, Atr-II, and demonstrate that it binds activin with high specificity. The identification of Atr-II suggests the presence of an activin signaling system in *Drosophila*. The sequence¶ and binding characteristics of this receptor indicate that specification of activin binding requires a surprisingly small number of conserved amino acids.

MATERIALS AND METHODS

Genomic Cloning of Atr-II and Isolation of cDNAs. Digestion of the mouse ActR-II activin receptor cDNA (12) with *Kpn* I and *Hin*cII yielded an ≈550-bp *Kpn* I–*Kpn* I fragment, specific for the N-terminal half of the kinase domain, and an ≈440-bp *Kpn* I–*Hin*cII fragment, specific for the C-terminal half. Using these fragments as ^{32}P-radiolabeled probes under low-stringency hybridization conditions (17), we obtained crossreacting genomic clones from a Canton-S λEMBL3 library kindly provided by R. Blackman and W. Gelbart (Harvard University). To obtain cDNA clones, a 1.2-kb *Bgl* II genomic fragment that cross-hybridized with both mouse kinase probes was hybridized to a 0- to 4-hr embryonic cDNA library (18). One of the 17 positive cDNAs obtained, 9a, was subcloned in both orientations into pBluescript II (Stratagene) as a 3.1-kb fragment obtained by partial digestion with *Eco*RI. A series of nested deletions was generated for each strand by using the Erase-a-Base kit (Promega), and sequenced by A.L.F. sequencing (Pharmacia).

***In Situ* and Northern Hybridization Analyses.** Polytene chromosomes were prepared from salivary glands of third-instar larvae according to standard protocols (19), probed with random-primed digoxygenin-labeled probe, and immunochemically visualized (Genius kit; Boehringer Mannheim) after the reactions were stopped by dilution in phosphate-buffered saline and the chromosomes were counterstained with Giemsa stain.

Total RNA was prepared by the hot phenol method (20). Poly(A)$^+$ RNA was purified on oligo(dT)-cellulose (Collaborative Research), electrophoresed (2 μg per lane) in formaldehyde/1% agarose gels, and transferred to Nytran (Schleicher & Schuell) by capillary blotting and UV crosslinking. Hybridization with random-primed probes was carried out under standard conditions (17).

For *in situ* hybridization, embryos from a *y,w* stock were dechorionated with 50% bleach and fixed for 20 min in phosphate-buffered saline/heptane, 2:1 (vol/vol), containing 4% (wt/vol) formaldehyde. The embryos were devitellinized by washing in methanol and stored at −20°C in 100% ethanol until use. Sense and antisense Atr-II RNA probes were labeled with digoxigenin-UTP (Boehringer Mannheim) by transcription from either the T3 or T7 promoter of pBluescript II. The RNA was hydrolyzed in 30 mM Na_2CO_3 (pH 10.2) for 10 min prior to use. Hybridization and detection were done under the conditions of Tautz and Pfeifle (21), with

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Abbreviations: BMP, bone morphogenetic protein; TGF, transforming growth factor.

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. L22176).

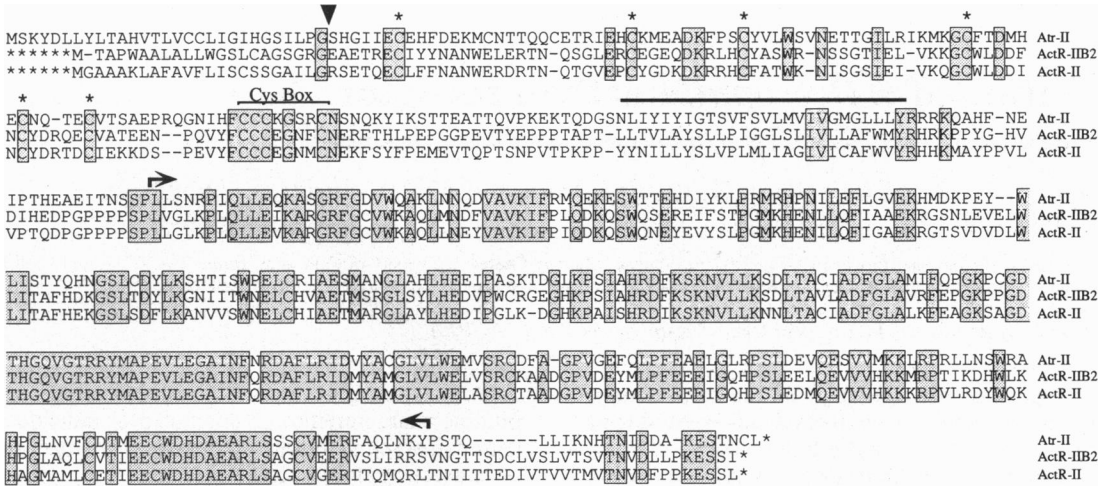

FIG. 1. Alignment of the amino acid sequence of Atr-II with the ActR-II (12) and the ActR-IIB2 (13) mouse activin receptor sequences. Amino acids conserved in all three proteins are boxed. Indicated are the potential signal-peptidase cleavage site (arrowhead), the 10 conserved cysteines (stars), the cysteine box (bracket overline), the putative transmembrane region (heavy overline), and the limits of the kinase domain (arrow brackets).

post-hybridization washes for 48 hr. Embryos were mounted on slides in 30% (vol/vol) phosphate-buffered saline/70% glycerol. Embryonic stages are as described (22).

Transfections, Binding, and Affinity-Labeling Assays. To subclone the Atr-II cDNA from clone pNB40-88D-19b into pCMV5 vector (13), *Sac* II-digested pNB40-88D and *Mlu*

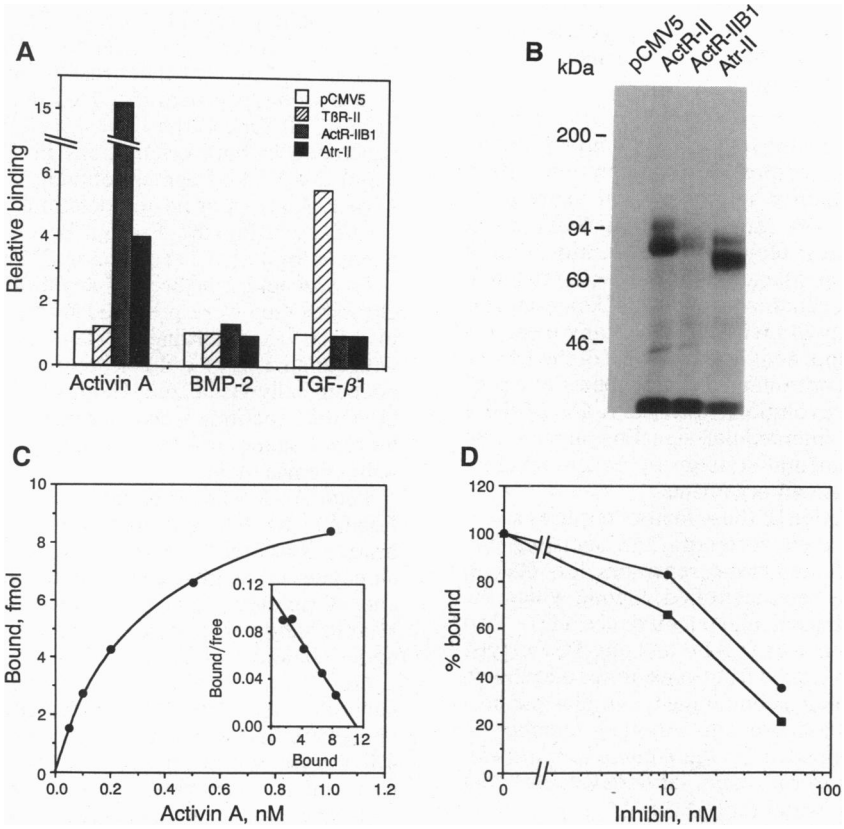

FIG. 2. Binding and affinity labeling of *Drosophila* and mouse activin receptors. (*A*) Binding of activin A, BMP-2, and TGF-β1 to receptors. COS-1 cells transfected with Atr-II (black bars), ActR-IIB2 (gray bars), TβR-II (hatched bars), or pCMV5 vector alone (open bars) were affinity-labeled with ^{125}I-activin, ^{125}I-BMP-2, or ^{125}I-TGF-β1, and total binding was determined with a γ counter. (*B*) Receptor affinity labeling. COS-1 cells transfected with Atr-II, ActR-II, ActR-IIB1, or pCMV5 vector alone were affinity-labeled with ^{125}I-activin A and disuccinimidyl suberate. Detergent extracts from these cells were subjected to SDS/PAGE and autoradiography. Equivalent amounts of radioactivity were loaded in each lane. Position and molecular mass of protein markers are indicated. (*C*) Equilibrium binding assays of Atr-II. COS-1 cells transfected with the Atr-II expression vector were incubated with various concentrations of ^{125}I-activin A for 3 hr at 4°C. Specific binding was then determined, and the mean of triplicate values is presented. (*Inset*) Scatchard analysis of the data. (*D*) Competition of activin A with inhibin A. COS-1 cells transfected with Atr-II (○) or ActR-IIB2 (■) were incubated with 250 pM ^{125}I-activin A and various concentrations of human inhibin A. Specific binding was determined in duplicate samples, and the mean values are plotted.

I-digested pCMV5 ends were blunted with T4 DNA polymerase and then digested with *Hin*dIII, and the Atr-II fragment was ligated into pCMV5. This vector and pCMV5 vectors containing the ActR-II and ActR-IIB cDNAs (13) were transfected into COS-1 cells and subsequently assayed for ligand binding with radioiodinated human recombinant activin A and BMP-2 (gifts from Y. Eto, Ajinomoto and J. Wozney, Genetics Institute, respectively) and TGF-β1 (R & D Systems) as described (13). Affinity-labeled samples were analyzed by SDS/PAGE under reducing conditions, followed by autoradiography. For equilibrium binding assays, cells were incubated with ^{125}I-activin A for 3 hr at 4°C, washed, and solubilized in 1% (vol/vol) Triton X-100 to release bound activin, which was quantitated by γ counting.

Antibody Production and Immunocytochemistry. Recombinant Atr-II protein (amino acids 231–516) produced in *Escherichia coli* using the T7 pRSet expression vectors (Invitrogen) was used to immunize rabbits. Collected sera was passed over a column of staphylococcal protein A to obtain a purified immunoglobulin fraction. For immunocytochemistry, embryos from a *y,w* stock were collected and fixed as described (23). Atr-II protein was localized by using a horseradish peroxidase-coupled goat anti-rabbit secondary antibody and diaminobenzidine detection (24). In immunoblot assays using this antibody and receptor-transfected COS-1 cell lysates, the signal obtained with ActR-II or ActR-IIB was only ≈10% as intense as the signal with Atr-II.

RESULTS

Cloning and Cytological Location of the *Drosophila Atr-II* Gene. Probing of a *Drosophila* genomic library with separate portions of the mouse ActR-II activin receptor cDNA (12) yielded one strong positive plaque per ≈10,000 plaques screened. Purification, restriction mapping, and hybridization analysis of 10 of these phage indicated that they all were derived from a single locus, which we now refer to as *Atr-II* (for activin receptor type II). The cytological location of *Atr-II* determined by *in situ* hybridization to polytene chromosomes was found to be on the right arm of the third chromosome within the 88D subdivision. This location was confirmed by additional hybridization experiments employing several deficiency-containing chromosomes. The locus mapped just distal to *Df(3R) red1* (88B1–88D2-3) but within *Df(3R) ry[506–85C]* (*87D1*–88E5-6) (25). Analysis of both wild-type and deficiency chromosomes suggests that the locus was within the D interval just distal to the D2-3 break associated with *Df red1* (data not shown).

***Atr-II* Encodes a Putative Transmembrane Serine/Threonine Kinase Receptor.** One Atr-II cDNA isolate, obtained from a 0- to 4-hr embryonic cDNA library, was sequenced and analyzed. Following a consensus translation start site (26) the cDNA sequence contains one large open reading frame of 1548 bp. The predicted amino acid sequence contains two hydrophobic regions at amino acids 1–29 and 148–172 that correspond to a putative signal sequence and a transmembrane region, respectively. The cysteine-rich extracellular domain contains three potential N-linked glycosylation sites. Most of the putative cytoplasmic region consists of a protein kinase domain with sequences characteristic of serine/threonine kinases (27). A mature protein of 488 amino acids is predicted from the Atr-II sequence (Fig. 1).

Comparison of the Atr-II amino acid sequence with other proteins revealed the closest degree of similarity to the mammalian and *Xenopus* activin receptors (12–14). The kinase domain of Atr-II is 61% and 60% identical to mouse ActR-II and ActR-IIB2, respectively (Fig. 1), and 40% identical to the kinase domains of TβR-II (25) and daf-1 (18). Outside of the kinase domain, the similarity among these receptors is very limited. However, the spacing of 10 cysteine residues in the extracellular domains of the vertebrate activin receptors is well conserved in Atr-II. This is in contrast to the poor conservation of extracellular cysteines when the activin receptors are compared with daf-1 or TβR-II. An exception to these general observations is the "cysteine box," a cluster of cysteine residues located near the transmembrane domain, which is a feature retained by all these receptors. These results suggested that the Atr-II cDNA could represent a *Drosophila* activin receptor.

Atr-II Is an Activin Receptor. We investigated the ability of Atr-II to bind factors representing the activin, TGF-β, and BMP families, by transiently expressing Atr-II in monkey COS-1 cells. Cells expressing Atr-II bound markedly higher levels of ^{125}I-activin A than did cells expressing pCMV5 alone or TβR-II (Fig. 2*A*). No significant increases over controls were observed when binding of either ^{125}I-BMP-2 or ^{125}I-TGF-β1 was assayed in Atr-II transfectants (Fig. 2*A*). Analysis by SDS/PAGE of extracts from Atr-II transfectants crosslinked to bound ^{125}I-activin A revealed a major labeled species of 80 kDa and a minor species of 92 kDa (Fig. 2*B*), similar in size to the labeled species obtained in ActR-II and ActR-IIB1 transfectants (Fig. 2*B*). The larger species may represent receptors linked to internally crosslinked activin dimers (13).

Atr-II bound activin A with high affinity ($K_d \approx 400$ pM; Fig. 2*C*). Inhibin A (an α–β_A heterodimer) (2) competed less potently for this binding than did activin A (a β_A–β_A homodimer) (Fig. 2*D*). These binding parameters are comparable to those observed for mouse activin receptors expressed in COS-1 cells (refs. 12 and 13; Fig. 2*D*). Affinity crosslinking studies in mammalian cells have identified two types of activin receptors of 53 kDa and 70–80 kDa (12, 13). They are designated receptor types I and II, respectively, based on the similarity of their size to the TGF-β receptors I and II. Given the high degree of sequence similarity, as well as similarity in affinity crosslinking profiles and binding properties, between Atr-II and the two mammalian activin type II receptors, we conclude that Atr-II is a *Drosophila* activin type II receptor.

Temporal Expression Pattern and Localization of Atr-II Transcripts. A single Atr-II transcript, of ≈2.8 kb, was detected at all developmental stages by Northern analysis

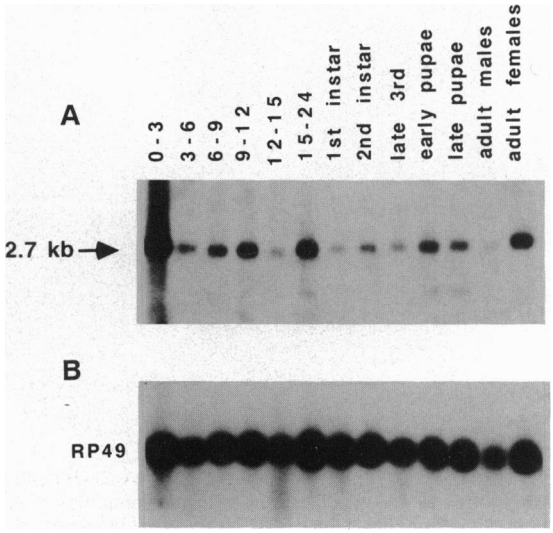

FIG. 3. Developmental profile of the Atr-II receptor mRNA. Poly(A)$^+$ RNA isolated from embryos (0–3 hr, 3–6 hr, etc.), larvae, pupae, and adults of the indicated stages was electrophoresed in a 2.2 M formaldehyde/1% agarose gel, blotted to a nylon membrane, and hybridized with a full-length Atr-II cDNA (*A*) or with a probe for the gene that encodes ribosomal protein 49 (RP49) (*B*).

(Fig. 3*A*). After normalization of the hybridization signal with a ribosomal protein 49 probe (Fig. 3*B*), it is apparent that Atr-II gene expression is maintained throughout development, although the levels fluctuate somewhat. The relatively high levels of this message in adult females and in 0- to 3-hr embryos suggest that in addition to constitutive zygotic expression, the Atr-II transcript is supplied maternally to the embryo.

The spatial distribution of the Atr-II message and protein was examined in oocytes, embryos, and imaginal tissue by *in situ* hybridization with an antisense Atr-II RNA probe or by immunostaining (embryos only) with Atr-II antibody (Fig. 4). High levels of Atr-II transcripts were observed in both nurse cells and the developing oocyte (Fig. 4*A*). The maternal transcript persisted at high levels in the embryo at all stages prior to cellularization (Fig. 4*B*). During cellularization, there was a sharp decrease in message levels yielding an asymmetric transcript distribution within the zygote, with the highest levels remaining at the posterior end underneath the developing pole cells (Fig. 4*C*). At the start of gastrulation, high Atr-II expression was observed in the invaginating mesoderm, although significant transcript levels were also present in other cells (Fig. 4*D*). This enriched expression in the mesoderm might represent specific activation of zygotic expression, whereas the lower level of staining seen in other cells might represent residual maternal transcript or low-level zygotic expression.

By the time germ-band extension was completed during stage 11 (Fig. 4*E*), expression of Atr-II was high along the entire length of the germ band in the mesoderm, as well as in the developing gut. Fore-, mid-, and hindgut continued to show the highest levels of Atr-II transcript accumulation during stages 13 and 14 (Fig. 4*F*). After dorsal closure was completed during stage 16, a particularly high level of Atr-II expression was observed in the hindgut and the anterior end of the midgut (Fig. 4 *G* and *H*). In third-instar larvae, all imaginal-disc cells showed prominent Atr-II expression (data not shown). Embryos hybridized with a sense Atr-II RNA probe of similar specific activity gave little or no signal (Fig. 4*I*). The pattern of immunochemical staining obtained in whole embryos with a polyclonal antibody raised against bacterially expressed Atr-II kinase domain mirrored the RNA expression pattern (Fig. 4 *J–L*). Staining with preimmune serum was used as a control (data not shown). Although our antibody discriminates against mammalian activin receptors, we cannot discard the possibility of crossreaction with a closer Atr-II isoform.

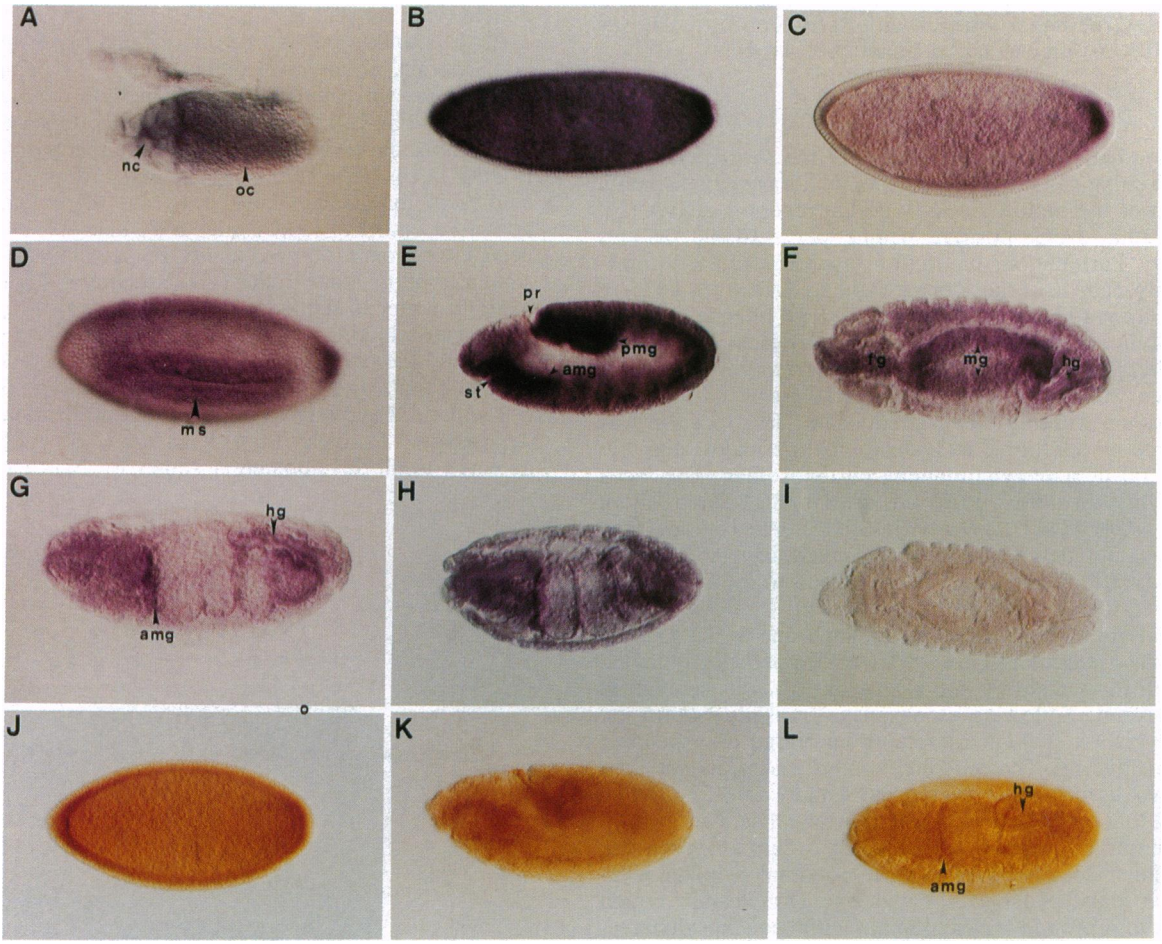

FIG. 4. Spatial distribution of the Atr-II mRNA (*A–H*) and protein (*J–L*) during embryogenesis. Ovaries and embryos were hybridized to digoxigenin-labeled antisense RNA probes corresponding to full-length Atr-II (*A–H*) or to the Atr-II ectodomain (data not shown), with similar results. (*A*) Stage 11 ovary showing Atr-II message in nurse cells and the developing oocyte. (*B*) Lateral view of a syncytial blastoderm embryo showing high levels of maternal product. (*C*) Slightly older embryo undergoing cellularization showing loss of maternal message and a graded posterior localization. (*D*) Ventral view of early gastrulation showing accumulation in the invaginating cells of the mesoderm. (*E*) Stage 11 embryo showing continued enriched expression in the mesoderm and pronounced accumulation of message in the developing endoderm. (*F–H*) Stage 14, 15, and 16 embryos, respectively, displaying continued high-level expression in the fore-, mid-, and hindgut. (*I*) Control stage 14 embryo hybridized with a digoxigenin-labeled sense RNA probe. (*J–L*) Atr-II antibody staining of a syncytial blastoderm embryo (*J*), a stage 11 embryo (*K*), and a stage 15 embryo (*L*). nc, Nurse cells; oc, oocyte; st, stomadium; pr, proctodeum; ms, mesoderm; fg, foregut; mg, midgut; amg, anterior midgut; pmg, posterior midgut; hg, hindgut. (×200.)

DISCUSSION

In this report, we describe the cloning, sequence, ligand binding, and embryonic expression pattern of Atr-II, a receptor serine/threonine kinase identified in *Drosophila*. Like all previously characterized activin receptors, Atr-II contains a short, cysteine-rich extracellular domain followed by a transmembrane region and an intracellular region containing a predicted protein-serine/threonine kinase domain. A feature that distinguishes Atr-II from other identified activin receptors is the very low level of sequence conservation (≈20%) in the extracellular domain. The two mouse activin receptor isoforms, ActR-II and ActR-IIB, show 50% identity when compared with each other in this region. Atr-II does show good conservation of the spacing between 10 extracellular cysteine residues, although it contains one extra pair of cysteines when compared with ActR-II and -IIB. The Atr-II sequence is equidistant from those of ActR-II and -IIB. These three receptors might have evolved from a single ancestor or, alternatively, Atr-II might represent the *Drosophila* homologue of a third activin receptor isoform not yet identified in vertebrates.

The three activin species identified in vertebrates are homo- or heterodimers of β_A and β_B chains, whereas inhibins A and B are heterodimers of a β_A or β_B chain with an α chain, and their biological effects are often opposite to those of the activins (2). The affinity of Atr-II for human activin A is within the range of affinities (K_D = 100–400 pM) observed with the vertebrate activin receptors (12–14). Further, these receptors all show significantly lower affinity for inhibin A than for activin A. Although previous searches for *Drosophila* members of the TGF-β superfamily have uncovered only dpp and 60A, two factors closely related to human BMPs (5–7), we conclude that Atr-II is a receptor for an activin-like ligand on the basis of its sequence similarity to activin receptors from vertebrates and its ability to bind activin A with high affinity and specificity.

It is noteworthy that 16 of the 22 extracellular amino acids conserved between Atr-II and ActR-II or -IIB are cysteines or an adjacent residue. Thus, the ability of these receptors to discriminate between various members of the TGF-β superfamily as ligands may be primarily determined by the particular pattern of extracellular disulfide bonds characteristic of each receptor subgroup. Supporting this view, the activin receptors diverge substantially in both the number and the spacing of extracellular cysteine residues compared with daf-1, the TGF-β type II receptor, and another group of serine/threonine kinase receptors that we have recently identified from both humans and *Drosophila* (unpublished data). Additional details concerning binding specificity determinants will have to await mutational and biophysical analyses of receptor–ligand complexes.

A determination of the precise developmental roles for a *Drosophila* activin signaling pathway will require detailed mutational analysis of both the Atr-II gene and its ligand, as well as the identification of other receptor components that may be involved in signaling as is the case for the TGF-β receptor (28). However, the maternal contribution of Atr-II into the oocytes is noteworthy because it could reflect a role for an activin-like factor during early axial patterning in *Drosophila*, as has been suggested for activin in *Xenopus* development (8–10). During later stages of development, the Atr-II gene shows particularly enriched expression in the developing mesoderm and endoderm. Most striking is the strong accumulation of both mRNA and protein in the developing gut. The strong accumulation of Atr-II in the developing mesoderm, foregut, and hindgut may reflect a role of this receptor in specifying these tissues. Eventual analysis of mutant phenotypes should allow us to assess these various possibilities. In addition, it may be possible to devise genetic screens employing Atr-II mutations to identify downstream components of TGF-β-type signaling pathways.

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