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Expression pattern of the activin receptor type IIA gene during differentiation of chick neural tissues, muscle and skin

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To elucidate target cells of activins during embryogenesis we isolated cDNAs of chick activin receptor type II (*cActR-II*) and studied expression patterns of the *cActR-II* gene by in situ hybridization. Transcripts of *cActR-II* were observed in neuroectoderm developing to spinal cord, brain and eyes, in surface ectoderm differentiating to epidermis, and in myotomes differentiating to muscles. The expression patterns of *cActR-II* suggest that activin and its receptor are involved in differentiation of chick neural tissues, muscle and skin after inducing the dorsal mesoderm.

Activin receptor type IIA gene; Hybridization, in situ; Chick embryogenesis; Neural tissue; Muscle; Skin

1. INTRODUCTION

Activin, a member of TGF-β family, was originally recognized as a factor that causes the release of follicle-stimulating hormone from anterior pituitary cells [1,2]. Three types of activins are known; homodimers of two βA chains (activin A), heterodimers of a βA and βB chain (activin AB) and activin B (homodimer βB βB). Activin A was shown to be identical to an erythroid differentiation factor [3,4]. Furthermore, activins were found to be dorsal mesoderm-inducing factors not only in *Xenopus* [5–7] but also in chick embryo [8]. However, their role during development has not yet been established. Recently, two distinct types of activin receptor cDNA were found: Mathews and Vale cloned a mouse activin receptor type II (*mActR-II*) cDNA and found that it had a serine/threonine kinase domain [9]. A variant type of *mActR-II*, designated *cActR-IIB*, was found by Attisano et al. [10]. However, nothing has been reported about cell types expressing the *ActR-II* gene in vivo. Thus, to elucidate spatial and temporal expression patterns of the *ActR-II* gene during embryogenesis, we cloned cDNAs of a chick *ActR-II* homologue and performed in situ hybridization on sections of chick embryos in stage, from 5 to 36 stages (Hamburger-Hamilton [11]).

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2. MATERIALS AND METHODS

2.1. *Isolation of chick activin receptor cDNAs*

Chick embryos (White Leg-Horn) were commercially purchased from Fukuda Co. (Okayama, Japan). One hundred 4-day embryos were treated to obtain mRNAs and constructed a cDNA library with a λgt10 vector. The library (ca. 10^8 recombinant phages) was screened with a mouse probe obtained by the polymerase chain reaction method. Three of five clones isolated (designated CAR3, CAR7, CAR13) were subcloned into pGEM7 vector and sequenced by the dideoxy chain-termination method using double-stranded plasmids as templates [12,13]. The riboprobes for in situ hybridization were synthesized with pCAR13 in which CAR13 was subcloned into pGEM7 vector.

2.2. *In situ hybridization*

Chick embryos from stage 5 to 36 were fixed with 4% paraformaldehyde, dehydrated with ethanol and then embedded in paraffin as described previously [14,15]. Both antisense and sense riboprobes were labeled with [α-^{35}S]UTP (Amersham Co.). The specific activity of both probes was ca. 5×10^8 dpm/g. The slides were analyzed on an Olympus microscope using bright- and dark-field optics.

3. RESULTS AND DISCUSSION

3.1. *Cloning of chick activin receptor cDNAs*

We isolated three independent clones, CAR3, CAR7 and CAR13, from a cDNA library, the scheme and the restriction maps of which are shown in Fig. 1. The CAR7 contained 2.1 kb, coding for a protein with 457 amino acids. The CAR13 contained *Cla*I, *Pst*I and *Xho*I sites corresponding to those in the CAR7 clone. Since both clones were incomplete at the 5' end, the lacking sequence (nucleotides 1–57 in Fig. 2) was complemented with the corresponding sequence of the CAR3 clone. Since both CAR3 and CAR7 had the long 5' non-coding

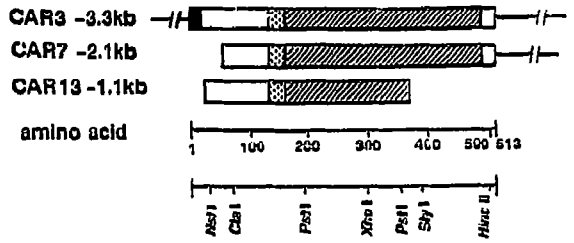

Fig. 1. Schematic representation and restriction map of three isolated cDNAs, CAR3, CAR7 and CAR13. Amino acid numbering is from the first ATG codon of CAR3. Boxes represent coding sequences; black is the signal peptide, white is the extracellular ligand-binding domain, dotted is the transmembrane domain, and hatched is the intracellular kinase domain.

region we used CAR13 to synthesize riboprobes to avoid non-specific hybridization.

The translated amino acid sequence of the CAR cDNA is shown in Fig. 2. The open reading frame extends from the initial methionine for 513 amino acids in the cDNA. The CAR amino acid sequence is compared with the sequences of mouse and frog activin receptors. The sequence of the CAR is highly homologous to the sequences of *mActR-II* [9] and *Xenopus* activin receptor (*xActR-II*) [16], as shown in Fig. 2. The overall amino acid sequence homology is 92% with *mActR-II*, 91% with *xActR-II*, and 67% with *mActR-IIB*. Thus, the cloned cDNA was concluded to be an avian homologue of *mActR-II*, and was designated *cActR-IIA*.

3.2. *Expression pattern of the* cActR-IIA *gene in chick embryogenesis*

We performed in situ hybridization on parasagittal and transverse sections of chick embryos at various stages from 5 to 36. Several typical results are shown in Figs. 3 and 4. The dense accumulation of the silver grains was observed as white under dark-field view. Since no significant accumulation of the grains was observed when the corresponding sense probe was used (data not shown), the accumulation of the grains observed with the antisense probe indicates the presence of the transcripts of *cActR-IIA*.

3.2.1. Expression pattern in differentiation of neuroepithelium

Since activin is known as a putative inducer of dorsal mesoderm [5–8] a restricted expression of the *cActR-IIA* gene was expected in early stages of the embryos. At stage 5–6 (19–25 h of incubation), however, the *cActR-IIA* transcripts were observed ubiquitously over the embryo (data not shown). During the late phase of gastrulation at stage 6–7 the notochordal process migrates to the cephalic region with inducing neural plate from the ectoderm. This is known as the first event in the formation of the central nervous system, and regarded as the beginning of neurulation. No specific expression pattern of *cActR-IIA* was observed in these stages, although transcripts were detected in any cells (data not shown). At stage 8–10 (4–10 somites, 26–38 h), when neural folds meet in the cephalic portion but still do not fuse in the caudal portion, a high level of hybridization signal was detected in the neural groove (Fig. 3A), notochord (Fig. 3A), mesoderm (Fig. 3A), ectoderm (Fig. 3B) and somites (Fig. 3B), but not in endoderm (Fig. 3A and B). At these stages regionalization of the neural tube occurs as a result of changes in the shape of the tube, and then each of the lateral walls of the forebrain bulge laterally and form the primary optic vesicle. A significant level of hybridization was detected in the embryonic brain region, although no specific expression pattern was observed in neural tissues such as optic vesicles (data not shown).

With differentiation of the spinal cord (stage 11–20) the expression of *cActR-IIA* became relatively weak (Fig. 3C). At stage 23 the gene remained to be expressed in the floor plate and in the basal plate of the neural tube where the motoneurons developed later on (Fig. 3D). On the other hand, the expression in the notochord also became weak during subsequent maturation (Fig. 3C) and almost disappeared at stage 23 (Fig. 3D). The change in the *cActR-IIA* expression pattern of the notochord appears to be correlated to the formation of the neural tube. The transcripts were also detected in the prospective spinal ganglia derived from neural crest cells (Fig. 3D).

Intense expression of *cActR-IIA* was detected in the ventral region of the mesencephalon and that of the diencephalon at stage 23 (Fig. 4A). A high level of hybridization was observed in the optic chiasma located in the ventral region of the diencephalon where the optic stalk enters. In the ventral region of the mesencephalon, the *cActR-IIA* transcripts were observed in both the germinal and mantle layer, whereas the hybridization signal was higher in the mantle layer than in the germinal one in the ventral region of the diencephalon. A significant signal was also observed in the neural retina, pigmented epithelium (PE), optic stalk, lens epithelial cells and corneal epithelium at stage 23 (Fig. 4A). Although eye pigments appear around this time hybridization signals were distinguishable from the pigments by microscopic observation at high magnification. Activins thus appeared to be involved in differentiation of neuroepithelia to the spinal cord, brain and eye.

3.2.2. Expression pattern in differentiation of myotomes to muscle

Somites are derived from the paraxial mesoderm which extends along both sides of the neural plate and notochord. The first somites are visible around stage 8. The *cActR-IIA* transcripts were detected in somites at stage 8–10 (Fig. 3A and B). Somites change their form in response to signals from the neural tube and notochord. The cells located in the ventromedial region of

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cActR-IIA    1 MGAATKLALAVFLISCSS-GAILGRSETQECIYYNANWEKDKTIAVGIEP
xActR-II       ........F.........A.S.......K.............NSL.T.I
mActR-IIA      ....A...F.....-.....LFF.....R.R.NQT.V..
mActR-IIB      ..T.PWA...LLWG.LCA..SGR.EA..R.........LER.NQS.L.R

cActR-IIA   51 CYGDKDKRRHCFATWKNISGSIEIVKQGCWLDDINCYDRNDCIEKKDSPE
xActR-II       ....N...K...........................NKSK.T......D
mActR-IIA      ....................................T.........
mActR-IIB      .E.EQ...I..Y.S.R.S..T..L..K......F.....WE.VATEEN.Q

cActR-IIA  101 VFFCCCEGNMCNERFSYFPEMEVTQPTSNPVTPKPPLFNTLLYSLVPIMG
xActR-II       .........Y...K.YHS.............T..............V
mActR-IIA      .Y..........K......N..............YY.I.......L.L
mActR-IIB      .Y.......F.....THL..PGGPEV.YE.PPTA.T.LTV.A...LT.G.

cActR-IIA  151 IAVIVLFSFWMYRHHKLAYPPVLVPTQDPGPPPPSPLMGLKPLQLLEIKA
xActR-II       V.....................................L.........V..
mActR-IIA      ..G..ICA..V.....M.....................L.........V..
mActR-IIB      LSL...LA......R.PP.GH.DIHE-..........V...........

cActR-IIA  201 RGRFGCVWKAQLLNEYVAVKIFPIQDKQSWQNEYEIYSLPGMKHDNILQF
xActR-II       ..............T.......V...L..............E...Y.
mActR-IIA      ....................................V........E.....
mActR-IIB      ............M.DF.......L.......S.R..F.T.....E.L...

cActR-IIA  251 IGAEKRGTSIDVDLWLITAFHEKGSLTDFLKANVVSWNELCHIAQTMARG
xActR-II       ........NL.T...............Y............L..E.....
mActR-IIA      .........V...............S................E.....
mActR-IIB      .A.....SNLE.E........D......Y..G.IIT......V.E..S..

cActR-IIA  301 LAYLHEDIPGLK-DGHKPAISHRDIKSKNVLLKNNLTACIADFGLALKFE
xActR-II       .SH.........-.......VA.................A.........
mActR-IIA      ............-.................................AI.........
mActR-IIB      .S.....V.WCRGE....S.A...F........SD...VL...A..VR..

cActR-IIA  351 AGKSAGDTHGQVGTRRYMAPEVLEGAINFQRDAFLRIDMYAMGLVLWELA
xActR-II       ..........................................P........
mActR-IIA      ..........................L.....................
mActR-IIB      P..PP.....................................V

cActR-IIA  401 SRCTASDGPVDEYMLPFEEEIGQHPSLEDMQEVVVHKKKRPVLRECWQKH
xActR-II       ....................V...................I......Q.
mActR-IIA      .....A.....................................DY....
mActR-IIB      ...K.A.....................EL........M..TIKDH.I..

cActR-IIA  451 SGMAMLCETIEECWDHDAEARLSAGCVEERIIQMQKLTNIITTEDIVTVV
xActR-II       A.....................................................
mActR-IIA      A.........................G...T...R.............
mActR-IIB      P.L.Q..V.....................VSLIRRSV.GT.SDC..SI.

cActR-IIA  501 TMVTNVDFPPKESSL
xActR-II       ...............
mActR-IIA      ...............
mActR-IIB      .S..M..LL.....I
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Fig. 2. Comparison of the amino acid sequences of *cActR-IIA*, *mActR-IIA* [9], *xActR-II* [16] and *mActR-IIB* [10]. Dots in *mActR-IIA*, *xActR-II* and *mActR-IIB* indicate residues that are identical to the corresponding residues of *cActR-IIA*

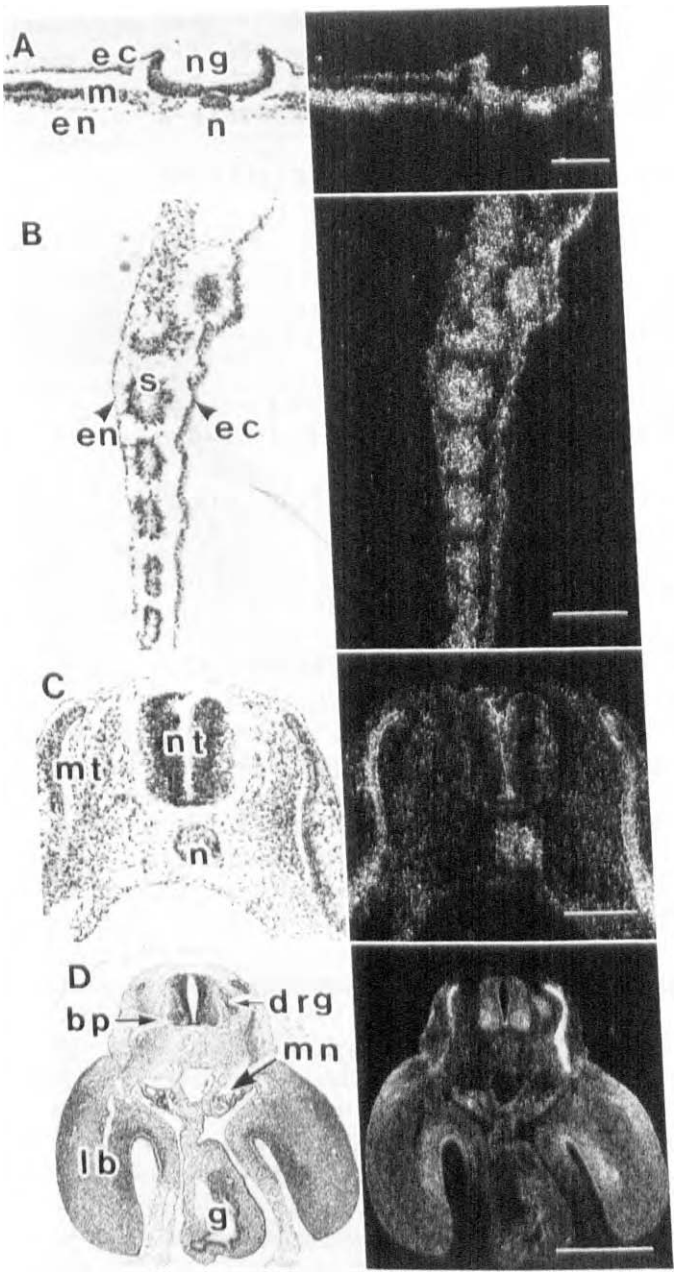

Fig. 3. Expression of the *cActR-IIA* gene in stage 8–23 chick embryos. Bright- and dark-field views are shown in the left and right panels, respectively. (A) A transverse section at stage 8. (B) A parasagittal section at stage 10. (C) A transverse section at stage 20. (D) A transverse section containing limb buds at stage 23. The white scale bars represent 100 μm in A, B and C, and 500 μm in D. ec, ectoderm; m, mesoderm; en, endoderm; ng, neural groove; n, notochord; s, somite; nt, neural tube; mt, myotome, bp, basal plate; drg, dorsal root ganglion; mn, mesonephros; lb, limb bud; g, gut.

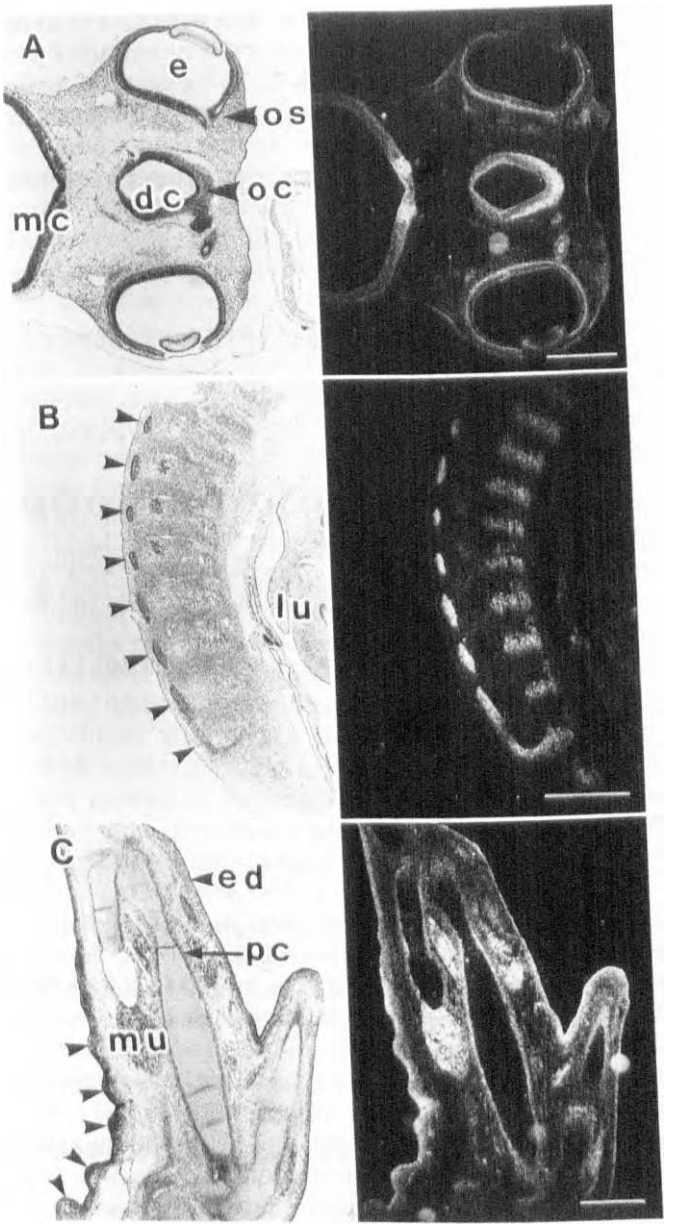

Fig. 4. Expression of the *cActR-IIA* gene in stage 23–36 chick embryos. Bright- and dark-field views are shown in the left and right panels, respectively. (A) A transverse section containing brain and eyes at stage 23. (B) A parasagittal section at stage 23. (C) A parasagittal section of stage 36 wing. The white scale bars represent 500 μm in A and C, and 200 μm in B. Arrowheads indicate myotomes in B and feather buds in C. mc, mesencephalon; dc, diencephalon; e, eye; os, optic stalk; oc, optic chiasma; lu, lung; ed, epidermis; pc, perichondrium; mu, muscle.

somites begin rapid proliferation and become sclerotomes, which migrate to the ventral region of the notochord and neural tube to form the axial skeleton. The dorsolateral walls of somites are divided into two layers: the outer layer is the dermatome (prospective dermis) and the inner layer is the myotome (prospective skeletal muscle). Since a high level of transcripts was detected in the inner layer of somites at stage 20–21 (Fig. 3C) and stage 23 (Figs. 3D and 4B), myotomes were concluded to express *cActR-IIA*. This conclusion is consistent with the fact that the expression was localized to the epithelium, perichondrium and muscles of wings at stage 36 (10 days), but not to the dermis (Fig. 4C). Since expression of *cActR-IIA* was restricted to

myotomes, activins may determine the fate of multipotential mesenchymal cells of paraxial mesoderm to presumptive myoblasts and to muscle.

By stage 18 limb buds become quite conspicuous and begin to exhibit nipple-shaped apices. The *cActR-IIA* transcripts were observed in mesenchymal and ectodermal cells of limb buds at stage 20–21 but without any significant pattern (data not shown). In the stage 23 limb bud (Fig. 3D) the transcripts were detected in proximoventral and proximodorsal regions. The transcripts were also observed in other mesoderm-derived tissues including splanchnopleura, heart and mesonephros.

3.2.3. Expression pattern in differentiation of surface ectoderm

The *cActR-IIA* expression was observed significantly in surface ectoderm at stage 8–10 (Fig. 3A and B). We observed the transcripts in epidermis as well as epithelia of gut and trachea at later stages, which are all differentiated from the surface ectoderm.

Our results showed that *cActR-IIA* transcripts are expressed in cells belonging to three distinct cell lineages; (i) from neuroectoderm to brain, eye and spinal cord, (ii) from surface ectoderm to epidermis and epithelia of gut and trachea, and (iii) from a part of the mesoderm to the notochord and muscles, etc. It is interesting to note that activin mimics the vegetal pole signal in frog embryos by eliciting mesodermal differentiation when added to animal cap explants (see [17] for a review). Furthermore, it was demonstrated that activin can induce neural-inducing cells, epidermis and muscle from frog animal cap explants, depending upon its concentration: treatment of frog animal cap with high doses of activin induces neural-inducing cells that, though not themselves neural, can induce neural differentiation in ectodermal cells; treatment with medium and low doses induces muscle and epidermis, respectively (see [17] for a review). Thus it is likely that chick cells expressing *cActR-IIA* differentiate to the three distinct types of cells in response to the activin level. Thus activin may be a possible candidate for a morphogen in embryogenesis. To elucidate roles of activin, however, further investigation is needed on an activin-binding protein of follistatin and other types of activin receptor, such as *ActR-IIB*, which has higher affinity to activin A than *ActR-IIA* [10].

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