# Activin and Its Receptors during Gastrulation and the Later Phases of Mesoderm Development in the Chick Embryo

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We have cloned chick homologues of the type-II activin receptor, which we have designated cActR-IIA and -IIB. Binding assays show that the two receptors are indistinguishable in their ability to bind activin-A, with comparable $k_d$s. Injection of mRNAs encoding these receptors into *Xenopus* embryos causes axial duplications. Expression of both receptors can first be detected in the primitive streak by *in situ* hybridization. This suggests that these genes may be activated in response to mesoderm induction. In agreement with this, we find that treatment of preprimitive streak chick embryos with activin-A leads to rapid induction of the expression of cActR-IIB. At later stages, cActR-IIA transcripts become localized mainly in the notochord and myotome and cActR-IIB in the dorsal neural tube, proximal-anterior part of the limb bud, sensory placodes, and specific regions of the fore- and midbrain. To test the response of early chick embryonic tissues to activin, we designed a new *in vitro* assay for differentiation. We find that explants of area opaca epiblast or posterior primitive streak from various stages can respond to activin treatment by differentiating into a variety of mesodermal cell types in a dose-dependent manner. These results suggest that the importance of activin-related signaling pathways is not confined to pregastrulation stages and that these receptors may be involved in mediating the effects of inducing signals during later stages of development of the mesoderm, limbs, and nervous system.   © 1995 Academic Press, Inc.

## INTRODUCTION

In *Xenopus,* considerable advances have been made in understanding the molecular bases of mesodermal induction. Among the most exciting findings is the identification of the mesoderm-inducing factor XTC-MIF as activin, a member of the TGF-$\beta$ superfamily of peptide growth factors (Smith *et al.,* 1990). Mesoderm induction in amphibians is thought to take place during the blastula stage (Nieuwkoop, 1969; Gurdon, 1987; Smith *et al.,* 1990; Green and Smith, 1991; Slack, 1991; Green *et al.,* 1992). Consistent with this,

isolated frog animal caps lose their ability to respond to activin treatment after about stage $9\frac{1}{2}$–10, during the early stages of gastrulation (Green and Smith, 1991; Lamb *et al.,* 1993; Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou *et al.,* 1994; Lemaire and Gurdon, 1994).

In the chick, there is also some evidence that activin-like molecules might play a role in the early stages of formation of the mesoderm. Mitrani and his colleagues discovered that activin can replace the hypoblast in isolated central fragments of blastoderms by allowing them to form an embryonic axis. Moreover, activin mRNA appears to be transcribed in the embryo from early stages of development (Mitrani *et al.,* 1990; Mitrani and Shimoni, 1990; Ziv *et al.,* 1992). Activin-B transcripts seem to appear first, followed by activin-A after formation of the primitive streak (Mitrani *et al.,* 1990). Cooke and

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Wong (1991) also found that avian cells can respond to activin treatment by enhanced spreading on fibronectin substrates and by varying their pattern of incorporation into different sites of an untreated host blastoderm and that embryos flooded with activin develop an abnormal axis. Finally, localized application of activin (Ziv *et al.,* 1992) or of activin together with Wnt-1 (Cooke *et al.,* 1994) can induce the formation of a supernumerary primitive streak.

Despite this information in avian embryos, the only evidence that activin can induce mesoderm is based on the ability of the central part of the embryo to form a complete embryonic axis despite the absence of the hypoblast, and there is no direct assay for mesodermal differentiation comparable to the animal cap assay in amphibians. A further complication when extrapolating results from amphibians to the amniote embryo is that in amniotes, mesoderm formation occurs over a much more protracted period than appears to be the case in amphibians. For example, when single cells in the superficial layer of Hensen's node at the full primitive streak stage (stage 4 and later) are marked by intracellular injection of a lineage tracer, their descendants are sometimes found in both ectodermal (floor plate, neural tube) and mesodermal (notochord, somites) derivatives (Selleck and Stern, 1991). Therefore, mesoderm induction does not end at the beginning of gastrulation, at least in avian embryos (Stern, 1992; Stern *et al.,* 1992).

To resolve some of these issues, we have isolated full-length cDNAs of chick homologues of two activin receptors, which we designate as cActR-IIA and cActR-IIB, from a Hensen's node cDNA library. We find that both receptors start to be transcribed in the primitive streak and continue to be expressed at later stages of development. These chick receptors can induce axial duplications and gastrulation defects when injected into *Xenopus* embryos, and both receptors bind iodinated activin-A. We have developed a new assay for mesodermal differentiation in the chick embryo, in which small explants of extraembryonic, area opaca epiblast are placed in culture and their differentiation assessed using cell-type-specific markers for different mesodermal tissues and for the organizer. The results suggest that the epiblast of the chick embryo maintains responsiveness to activin treatment into the stages of gastrulation and that there is a hierarchy of responsiveness among different regions of the embryo, the primitive streak itself being the most sensitive. Finally, we show that transcription of cActR-IIB itself is induced by activin treatment, which provides a molecular basis for the hierarchy of activin responsiveness seen in explants. We conclude that in the chick, the role of activin-like molecules in mesoderm formation may extend into gastrulation and even to later stages of development.

## MATERIALS AND METHODS

### *Construction and Screening of cDNA Libraries*

Hensen's node tissue was dissected from stage 3–4 (Hamburger and Hamilton, 1951) chick embryos (White Leghorns

from McIntyre Poultry, CA) and RNA was extracted by the lithium chloride method (Auffray and Rougeon, 1980). After purification of poly(A) RNA, a cDNA library was constructed in Lambda ZAP, which contained inserts up to 2.5 kb (see Kakizuka *et al.,* 1993). A stage 17–18 chick embryonic library was similarly constructed. Approximately $3 \times 10^5$ plaques per library were screened with a *Eco*RV–*Hinc*II fragment of the mouse activin receptor cDNA (mActR-II; Mathews and Vale, 1991) at low stringency. Hybridization was performed at 60°C in 1 *M* NaCl, 50 m*M* Tris, 1% SDS, 10% dextran sulfate, 100 $\mu$g/ml denatured salmon sperm DNA, 200 $\mu$g/ml yeast RNA, and 6 ng/ml $^{32}$P-labeled random primed probe (approx. $2 \times 10^8$ cpm/$\mu$g). The filters were washed in 6× SSC and 0.1% SDS at 55°C. Positive clones were characterized; sequence compilation and comparison were performed with the GCG computer program packages (Devereux *et al.,* 1984).

### *COS Cell Transfection and Binding Studies*

The complete coding regions of cActR-IIA and -IIB were subcloned into pCMX-PL2, a modified eukaryotic expression vector (Umesono *et al.,* 1991). COS-7 cells were transiently transfected with either cActR-IIA or -IIB expression plasmids and competition binding assays with human recombinant activin A (supplied by Genentech, Inc.) were performed as described by Mathews and Vale (1991).

### *Xenopus mRNA Injection Assays*

The complete coding regions of cActR-IIA and -IIB cDNAs were subcloned into pSP64T (Krieg and Melton, 1984), and capped, polyadenylated mRNAs were generated with SP6 polymerase. Two doses (0.5 and 2 ng) of cActR-IIA, cActR-IIB, or $\beta$-galactosidase mRNA were injected bilaterally into the marginal zone of 2- to 4-cell stage embryos. The embryos were incubated in 0.1× MMR to stage 28 (Nieuwkoop and Faber, 1967).

### *Whole-Mount in Situ Hybridization*

Whole-mount *in situ* hybridization was performed according to Harland (1991), with several modifications (Izpisúa-Belmonte *et al.,* 1993). Chick embryos were staged according to Hamburger and Hamilton (1951) for postprimitive streak stages and to Eyal-Giladi and Kochav (1976) for prestreak stages and fixed in MEMFA, treated for 30 min in 10 $\mu$g·ml$^{-1}$ proteinase-K (Sigma), and postfixed in 4% paraformaldehyde. Antisense and sense RNA probes were generated from linearized pBluescript plasmids containing the coding regions of cActR-IIA and -IIB in the presence of digoxigenin-labeled UTP with either T3 or T7 RNA polymerase and detected with anti-digoxigenin alkaline phosphatase antibody (Boehringer-Mannheim). Hybridization was performed at 70°C overnight. High stringency washes consisted of two 30-min incubations at 37°C in 2× SSC and two incubations of 30 min at 62°C in 0.2× SSC, both in the

presence of 0.1% Chaps. The color reaction was performed with 4-nitro blue tetrazolium chloride (Boehringer-Mannheim) and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma). After the color reaction, embryos were washed in PBS and postfixed in buffered formol (4%) saline (pH 7.0).

### Activin Treatment of Embryos

To investigate whether activin-A treatment can induce expression of the receptors, whole chick embryos at stage XII (Eyal-Giladi and Kochav, 1976) were treated with activin and then subjected to *in situ* hybridization. Fertile hens' eggs were incubated for 3–5 hr to obtain embryos at stage XII–XIII. These were explanted in Tyrode's solution at room temperature, attached to small squares of vitelline membrane. The embryos, still on their membrane, were then transferred to glass scintillation vials (6–12 embryos per vial) containing 2 ml Tyrode's solution with 1:100 antibiotic/antimycotic solution (penicillin, streptomycin, amphotericin-B; Sigma) and incubated for 8 hr at 38°C in the presence (experimental) or absence (control) of human recombinant activin-A at a final concentration of 35 or 70 *Xenopus* units · ml$^{-1}$.

Under these conditions, the primitive streak does not develop, and the embryos remain flat enough for fixation and whole-mount *in situ* hybridization. After incubation, the squares of vitelline membrane with the embryos attached were washed twice in a large volume of PBS and placed into MEMFA. Soon after this, the embryos were detached from their vitelline membrane and processed for whole-mount *in situ* hybridization as described above, except that the proteinase-K step was reduced to 20 min. Four separate experiments were conducted, each with four vials which were used, respectively, as control and experimental for probing with cActRIIA and control and experimental for cActRIIB.

### In Vitro Assay for Differentiation

To investigate whether activin can induce the differentiation of various mesodermal cell types from chick epiblast, small explants (about 150 × 150 μm) were cut out from four different regions of chick embryos at two different stages of development: (a) the inner lateral margin; or (b) the inner posterior margin (just outside and excluding Koller's sickle) of the area opaca of chick embryos at stage XI–XIII; (c) the posterior end of the primitive streak; or (d) the inner lateral margin of the area opaca of stage 3 embryos. All sets of experiments included positive controls (explants of the anterior half of the primitive streak).

Collagen was prepared by dissolving, overnight at 4°C, 8–10 sets of tendons obtained from the terminal sections of the tails (approximately 11 cm long) of two young rats into 50 ml sterile distilled water containing 7 μl glacial acetic acid. The volume was brought up to 110 ml with sterile distilled water and the solution centrifuged at 35,500$g$ for 30 min at 4°C. The supernatant was collected, its optical density (280 nm) was adjusted to 0.15 with sterile distilled water, and it was stored at 4°C for up to 4 weeks.

Cultures of the explants described above were set up in 24-well plates. The medium consisted of 7 parts of collagen supernatant as described above, 1 part of 10× medium-199, 1 part sodium bicarbonate solution (1.1 mg · ml$^{-1}$ in water), 1 part of fetal calf serum, and 1:100 antibiotic/antimycotic solution (Sigma), kept on ice. First, a drop of human recombinant activin-A (a generous gift of Dr. J. C. Smith, NIMR, London) was placed in the center of each well so as to give a final concentration in the range of 0–80 *Xenopus* units · ml$^{-1}$ (1 *Xenopus* unit in this preparation corresponds to approximately 0.2 ng activin A; J. C. Smith, personal communication) after adding 400 μl of the collagen-medium mixture. This mixture was then added and one to two explants quickly transferred into it. The solution usually gels at room temperature in 1–5 min, as the pH increases. After setting up the explants in the collagen gels, the plates were transferred to an incubator at 37°C supplied with 5% CO$_2$. Cultures were grown for 5–7 days and examined every day by phase-contrast microscopy. After this they were fixed for 24 hr in absolute methanol in the presence of 0.3% H$_2$O$_2$ prior to immunocytochemistry or in MEMFA for *in situ* hybridization to determine the cell types that differentiated.

### Immunohistochemistry to Assess Cell Phenotype

A variety of monoclonal antibodies was used: Not-1 (Yamada *et al.,* 1991; Selleck and Stern, 1992) for notochord, 3A10 (Yamada *et al.,* 1991; see also Storey *et al.,* 1992) specific for a phosphorylated neurofilament-associated antigen, a mixture of three monoclonal antibodies against neurofilament proteins (Boehringer-Mannheim), Sigma or Calbiochem monoclonal antibodies against skeletal muscle myosin, 13F4 (Rong *et al.,* 1992) for skeletal muscle, and Sigma monoclonal antibody against smooth muscle actin.

To stain cultured explants, the methanol-fixed wells were first rehydrated to PBS containing 0.25% Triton X-100 and 0.02% thimerosal (PBT) and then blocked with 1% BSA and 1% heat-inactivated goat serum in PBT overnight. Monoclonal antibody was then added to a suitable dilution (1:4 for Not-1, 1:1 for 3A10, 1:50 for neurofilament antibodies, 1:100 for Sigma and 1:500 for Calbiochem antibodies against muscle myosin, 1:3 for 13F4, 1:500 for Sigma anti-smooth muscle actin) and the explants incubated at 4°C for 1–3 days. They were then washed four to six times (1–2 hr per wash) in PBT and then incubated in appropriate peroxidase-coupled secondary antibody (1:100 goat anti-mouse IgM, Sigma, for 13F4; 1:5000 goat anti-mouse IgG, Jackson Immunoresearch, for the remaining antibodies) for 24 hr at 4°C. After four to six further washes in PBT they were rinsed twice in 0.1 $M$ Tris (pH 7.4) and placed into 1 ml diaminobenzidine (500 μg · ml$^{-1}$) in Tris for 3 hr in the dark at 4°C. Ten microliters of hydrogen peroxide (100 V, which had been diluted 1:100 in Tris) were then added to each well and incubated for 5–10 min. The wells were then washed repeatedly with tap water and then 70% ethanol, in which

they were stored. The stained explants were photographed using TMAX-100 (Kodak) through an inverted microscope with bright-field or phase-contrast optics or using Fuji 64T through a dissecting microscope with dark-field optics.

### In Situ Hybridization to Assess Cell Phenotype in Explants

To determine the expression of the homeobox gene *goosecoid,* a marker for the organizer (Izpisúa-Belmonte *et al.,* 1993), collagen gels containing explants cultured overnight in the presence or absence of activin were fixed in MEMFA overnight, dehydrated in methanol, removed from the wells, and placed in glass scintillation vials. *In situ* hybridization with digoxigenin-labeled RNA probes was then performed exactly as described above except that the antibody incubation and postantibody washes were performed in saline containing 1% Tween 20.

## RESULTS

### Identification of Chick Activin Receptor cDNAs in Embryonic cDNA Libraries

Hensen's node and stage 17–18 cDNA libraries were screened with a mouse ActR-II cDNA fragment (Mathews and Vale, 1991). Two classes of cDNAs were isolated. One class strongly resembled the mActR-II; the longest open reading frame contained a coding region of 1542 bp, with a long (1.2 kb) 5′ untranslated region. The 3′ untranslated region did not contain a poly(A) stretch but contained a potential polyadenylation signal at nucleotide 3371. Comparison of the nucleotide sequence with that of the mouse activin receptor cDNA (Mathews and Vale, 1991) showed 82% nucleotide identity in the coding region and a similarly high degree of homology (84%) in the 3′ untranslated region, including the conservation of a potential polyadenylation signal. However, the two cDNAs diverge upstream of the proposed initiator methionine codon (Mathews and Vale, personal communication). The predicted protein product consists of 513 amino acids (Fig. 1) which align perfectly with those encoded by mActR-II (Mathews and Vale, 1991), with an overall amino acid conservation between the chick and mouse proteins of 92%. This degree of homology allows us to conclude that the protein encoded by these chick cDNAs, which we designated cActR-IIA (see below), is structurally almost identical to mActR-II.

The second class of cDNAs generated weaker hybridization signals with the mouse probe and its restriction map differed from the cActR-IIA clones. Sequence analysis of a full-length clone revealed 69% nucleotide identity to cActR-IIA and mActR-II within their coding regions. The longest open reading frame encodes a predicted 512-amino-acid long protein (Fig. 1), with up to 500 bp 5′ untranslated region and a 163-bp 3′ untranslated region, which lacks a poly(A) tail. The homology of this second group of cDNAs

(referred to as cActR-IIB) to both the mouse ActR-II and cActR-IIA (Fig. 1) was calculated to be 70% at the amino acid sequence level, and it appears that both chick cDNAs code for related transmembrane receptor kinase proteins, with domain structures analogous to the mouse ActR-II (Mathews and Vale, 1991).

By comparing the chick receptors with the mActR-II, we predict the transmembrane domain to extend from amino acid 135 to 160 in cActR-IIA and from amino acid 134 to 159 in cActR-IIB. Significant amino acid conservation in the extracellular domains of the two cActR-II proteins is limited to a region of less than 90 amino acids, which probably corresponds to the ligand-binding domain. In the proposed kinase domain (Mathews and Vale, 1991), we noted the conservation of a phosphorylation consensus site (Pearson and Kemp, 1991), in subdomain XI of this class of serine/threonine kinases (the TGF-$\beta$ type-II receptor and *Caenorhabditis elegans* daf-1 proteins also belong to this class; Lin *et al.,* 1992; Georgi *et al.,* 1990).

### Affinity of the Chick Activin Receptor Proteins for Activin-A

To test whether both types of chick ActR-IIs bind activin, we conducted a radioligand-binding assay for activin-A (Mathews and Vale, 1991) with each of the chick ActR proteins expressed in mammalian cells. COS cells transiently transfected with the cActR-II expression plasmids exhibit a specific binding activity for iodinated activin-A, which can be competitively inhibited by an excess amount of unlabeled ligand (Fig. 2). Relative binding affinities of the two cActR-II proteins, as revealed by displacement curves in the presence of increasing amounts of unlabeled activin-A, are similar (Fig. 2). This indicates that the two subtypes of chick receptors are comparable in their activin-A-binding capacities.

### Expression of the Chick Activin Receptors in Xenopus Embryos Generates an Ectopic Axis

To test the possibility that chick ActR-II proteins, when expressed in *Xenopus* embryos, may affect axial patterning (Thomsen *et al.,* 1990; Asashima *et al.,* 1991a,b), the two chick ActR-II proteins in *Xenopus* embryos were compared by injecting approximately 0.5–2 ng of *in vitro* transcribed mRNA into 2- to 4-cell stage *Xenopus* embryos at the boundary between the animal and vegetal poles. Compared to control embryos (injected with $\beta$-galactosidase mRNA), the injection of synthetic mRNAs for either of the chick cActR-IIs resulted in an increased incidence of spina bifida, ectopic protrusions, and secondary axes (as observed after injection of *Xenopus* ActR mRNA; Kondo *et al.,* 1991; Mathews *et al.,* 1992; Hemmati-Brivanlou *et al.,* 1992). These results show that both chick receptors are functional *in vivo,* causing the formation of ectopic axial structures in amphibian embryos.

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                     1
cActR-IIA   MGAATKLGSPVFLISCSSGAILGRSETQECIYYNANWEKDKTNRSGIEPCYGDKDKRRHCFATWKNISGSIE
            | ||| ||| |||||||| ||| || |            ||  | | | | |    | | | ||
cActR-IIB   MSASW-LTLAVLCATLGAGPGHGEAETRECIYYNANWELEKTNQSGVERCEGEKDKRLHCYASWRNNSGSIE
                     1

IIA   IVKQGCWLDDINCYDRNDCIEKKDSPEVFFCCCEGNMCNERFFYFPEMEVTQPTSNPVTPKPPLFNTLLYSLVPIMG
      | |       |      || ||||||| |      |    ||| |||||||||| || | | | |   | || 
IIB   LVKKGCWLDDFNCYDRQECVATEENPQVFFCCCEGNYCNEKFTHLPEVTGPEVIYEPPPPTPSLLNILVYSLLPIAV

IIA   IAVIVLFSFWMYRHHKLAYPPVLVPTQDPGPPPPSPLMGLKPLQLLEIKARGRFGCVWKAQLLNEYVAVKIFPIQDK
      || || ||  |||||| |||  |      |||||| |||||||||||||||||||||||  ||  |||||||||||
IIB   LSVAILLAFWMYRHRKPPYGHVDI-NEDPGPPPPSPLVGLKPLQLLEIKARGRFGCVWKAQLMNDYVAVKIFPIQDK

IIA   QSWQNEYEIYSLPGMKHDNILQFIGAEKRGTSIDVDLWLITAFHEKGSLTDFLKANVVSWNELCHIAQTMARGLAYL
      ||| |  |||  |||||| || |||| |||  |||||||||||  ||||| ||  ||||||||| |  ||||||  |
IIB   QSWQSEREIFNTPGMKHENLLQFIAAEKRGTNLETELWLITAFHDKGSLTDYLKGNIISWNELCHVAETMARGLSYL

IIA   HEDIPGLK-DGHKPAISHRDIKSKNVLLKNNLTACIADFGLALKFEAGKSAGDTHGQVGTRRYMAPEVLEGAINFQR
      | || ||  ||||||| ||| |||||||| |||  ||||||| ||  || ||  ||||||||||||||||||||||||
IIB   HEDVPWCKGEGHKPAIAHRDFKSKNVLLKNDLTAVLADFGLAVRFEPGKPPGDTHGQVGTRRYMAPEVLEGAINFQR

IIA   DAFLRIDMYAMGLVLWELASRCTASDGPVDEYMLPFEEEIGQHPSLEDMQEVVVHKKKRPVLRECWQKHSGMAMLCE
      ||||||||||||||||||| |||  ||||||||||||||||||||||| ||||||||| ||| | | |     | | |
IIB   DAFLRIDMYAMGLVLWELVSRCRAVDGPVDEYMLPFEEEIGQHPSLEDLQEVVVHKKMRPVFKDHWLKHPGLAQLCV

                                                            513
IIA   TIEECWDHDAEARLSAGCVEERIIQMQKLTNIITTEDIVTVVTMVTNVDFPPKESSL
      ||||||||||||||||||||||| | || || |||| || | | |    |||||| |
IIB   TIEECWDHDAEARLSAGCVEERIAQIRKSVNGTTSDCLVSIVTSVTNVDLPPKESSI
                                                            512
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**FIG. 1.** Alignment of the predicted amino acid sequence of the chick activin receptors IIA and IIB. The vertical lines show amino acids that are not conserved. The complete nucleotide sequences, including the 3′ and 5′ untranslated regions, has been submitted to GenBank (accession numbers: cActRIIA: U31222; cActRIIB: U31223).

## In Situ Hybridization Studies of ActR gene Expression during Development

We used whole-mount *in situ* hybridization to study the expression of cActR-IIA and -IIB during chick embryonic development.

*cActR-IIA.* Transcripts of cActR-IIA are first detected at stage $3^+$ (Hamburger and Hamilton, 1951) and are strongest in the primitive streak (Fig. 3A); at lower levels, they are also seen throughout the mesoderm that has emerged from the primitive streak. Within the primitive streak, expression is stronger in the right primitive ridge than in the left. By stage 5, expression is still found in the right side of Hensen's node but is much weaker in the rest of the primitive streak. Outside the streak, mesodermal expression becomes confined to the precardiac regions and, at lower level, to the forming head process/notochord (Fig. 3B).

At stages 10–11, the expression of cActR-IIA is strongest in the notochord, and at a lower level in the dorsal part of the somites (Figs. 3C and 3D). By stage 16, cActR-IIA is expressed in the dorsal part of the younger (most caudal) somites, as seen at earlier stages, but in older somites transcripts become concentrated in the myotome (Figs. 3E–3H). This pattern persists throughout later stages of development (Figs. 3F–3H). At stage 22, in addition to the pattern described for the somites, diffuse expression is seen in the limb buds (Fig. 3F). In sections, this appears to be associated with the forming dorsal and ventral muscle masses (not shown), which are made up of cells of dermomyotomal origin.

*cActR-IIB.* cActR-IIB mRNA starts to be expressed slightly earlier than the other receptor, from stage 3, when it is found exclusively in the primitive streak (Fig. 3I). By stage $3^+$–4 it is stronger in the anterior two-thirds of the primitive streak (including Hensen's node) and weaker posteriorly (Fig. 3J). Unlike cActR-IIA, this receptor mRNA is expressed symmetrically with respect to the mediolateral axis at these stages.

From about stage 9, cActR-IIB mRNA is strongly expressed in the whole of the neural tube (Fig. 3K). Expression gradually becomes restricted to the dorsal part of the neural tube (Fig. 3L), a landmark feature of this receptor mRNA that can be seen as late as stage 28 (the latest stage examined; Fig. 3P). Between stages 12 and 22, cActR-II shows restricted expression in segmented structures: rhombomeres 2 and 4 of the hindbrain, the caudal halves of the youngest (most posterior) somites, both halves of the somites in the middle of the trunk, and the rostral halves of the oldest (most anterior) somites (not shown). At stage 22, cActR-IIB is expressed at the boundaries between all rhombomeres of
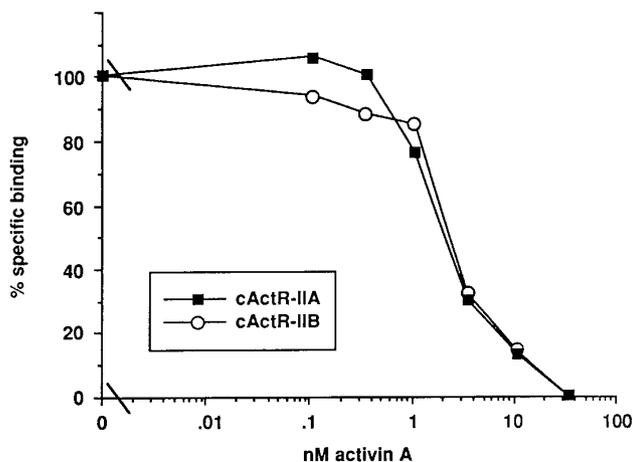
**FIG. 2.** Radioreceptor assay of chick ActR-II binding to activin-A. Profiles of $^{125}$I-activin-A binding to COS cells expressing either cActR-IIA (solid square) or cActR-IIB (open circle) proteins are plotted. Binding was performed on cell monolayers and was competed with unlabeled activin-A. Data are shown as percentage specific binding, where 100% specific binding was set as 2.6% of input cpm in the absence of competitor which was retained on the cells expressing cActR-IIB. Nonspecific binding (0.6% of input cpm) was determined in the presence of 500-fold molar excess of unlabeled activin-A. An activin-A-binding profile of cActR-IIA was plotted over that for cActR-IIB, taking its maximum binding activity as 80% of that of cActR-IIB in the absence of the competitor.

the hindbrain and in the anterior-proximal part of the limb buds (Figs. 3N–3P). In addition, transcripts are found in the telencephalic neuroepithelium, in the second diencephalic neuromere (D2) including the epiphysis, and in the sensory placodes (nasal, otic, and optic) and their derivatives, as well as in the optic nerve. At this stage, the distal, ventrolateral (limb-facing) edge of the dermomyotome expresses mRNA encoding this receptor (Fig. 3L).

### *Activin Treatment Induces Expression of the Receptor cActRIIB*

Since activin treatment of stage XIII embryos has been reported to induce the formation of a primitive streak and subsequent development of an embryonic axis in central discs of chick epiblasts (Mitrani *et al.,* 1990; Mitrani and Shimoni, 1990), and since the cActR-IIA and -IIB receptors only appear to be expressed at relatively late stages of development, it seemed likely that activin might induce the expression of these receptors. We therefore treated whole embryos at stages XII–XIII with activin-A, under conditions (submerged in saline) that do not allow the formation of a primitive streak during the 8- to 9-hr incubation. After this treatment, embryos were subjected to *in situ* hybridization to examine the expression of the IIA and IIB receptors as described above.

The results are illustrated in Fig. 4A. Treatment with 70 units $\cdot$ ml$^{-1}$ activin-A induces the expression of cActR-IIB

**FIG. 3.** Expression of cActR-IIA (A–H) and cActR-IIB (I–P) visualized by whole-mount *in situ* hybridization with digoxigenin-labeled probes. (A) Expression of cActR-IIA is first seen in the primitive streak (PS) and lateral plate mesoderm at stage 3$^+$, particularly concentrated on the right primitive ridge. (B) By stage 5, expression is concentrated in Hensen's node (HN) and in two lateral regions of the mesoderm corresponding to the future heart-forming area. The emerging head process and the primitive streak express at a much lower level. (C) At stage 11, the most prominent site of expression is the notochord (arrow). (D) This is seen in transverse section (arrow), which also reveals fainter dorsal expression in the somite and lateral plate mesoderm. (E) At stage 15–16, the expression in the somites has intensified. (F and G) By stage 22, it becomes clear that somitic expression is exclusively within the myotome. (H) In transverse section, myotomal expression is apparent (green arrows), as is a lower level of transcripts forming two concentric rings in the gut mesenchyme. (I) cActR-IIB is first expressed at stage 3, in the entire primitive streak (PS). (J) By stage 4, the posterior part of the primitive streak (PS) has lost its expression, but the anterior two-thirds express strongly. (K) At stage 11–12, the most prominent site of expression is the whole of the neural tube (NT). (L) In transverse section, the neural tube shows strongest expression in its dorsal part (arrows); the dorsal root ganglia display a much lower level of expression. The ventrolateral edge of the dermomyotome displays localized cActR-IIB expression. (M) At stage 17, expression is seen along the dorsal part of the neural tube (white arrow) and in the somites. (N–P) Stage 22. At this stage, in addition to dorsal expression in the neural tube (dNT in N; NT in P), the anterior-proximal expression of the receptor in the limb bud (arrow in N, O, P) is most conspicuous. N and O are lateral views. P is a dorsal view.

**FIG. 4.** (A) Treatment of early embryos with activin-A enhances the expression of cActR-IIB mRNA. The embryo on the upper left of the panel has been cultured in the absence of activin (Cont.); the three on the right (Exp.) in the presence of 70 *Xenopus* units/ml activin-A. *In situ* hybridization reveals strong induction of cActR-IIB mRNA throughout the treated embryos except in a most peripheral ring of area opaca. (B and C) Explants of area opaca epiblast cultured in the absence (B) or presence (C) of activin-A overnight. In the absence of factor, the explant rounds up into a sphere. Explants exposed to activin-A develop a protrusion (arrow). (D–F) Effects of activin treatment on differentiation of smooth muscle *in vitro*. (D) Explant of area opaca epiblast cultured in the absence of activin, stained with antibody against smooth muscle actin. No immunoreactivity is apparent. (E) Positive control: explant of primitive streak cultured in the absence of factors; smooth muscle develops. (F) Explant of area opaca epiblast cultured in the presence of 40 *Xenopus* units/ml activin-A: smooth muscle develops. (G–I) Effects of activin treatment on expression of the homeobox gene *goosecoid,* a marker for the organizer, revealed by *in situ* hybridization. (G) Control explant cultured in the absence of activin: no *goosecoid* mRNA expression is apparent. (H) Positive control: explant of Hensen's node cultured in the absence of activin: *goosecoid* mRNA expression can be seen. (I) Explant of area opaca epiblast cultured in the presence of 80 *Xenopus* units/ml activin-A. Strong *goosecoid* expression is seen.
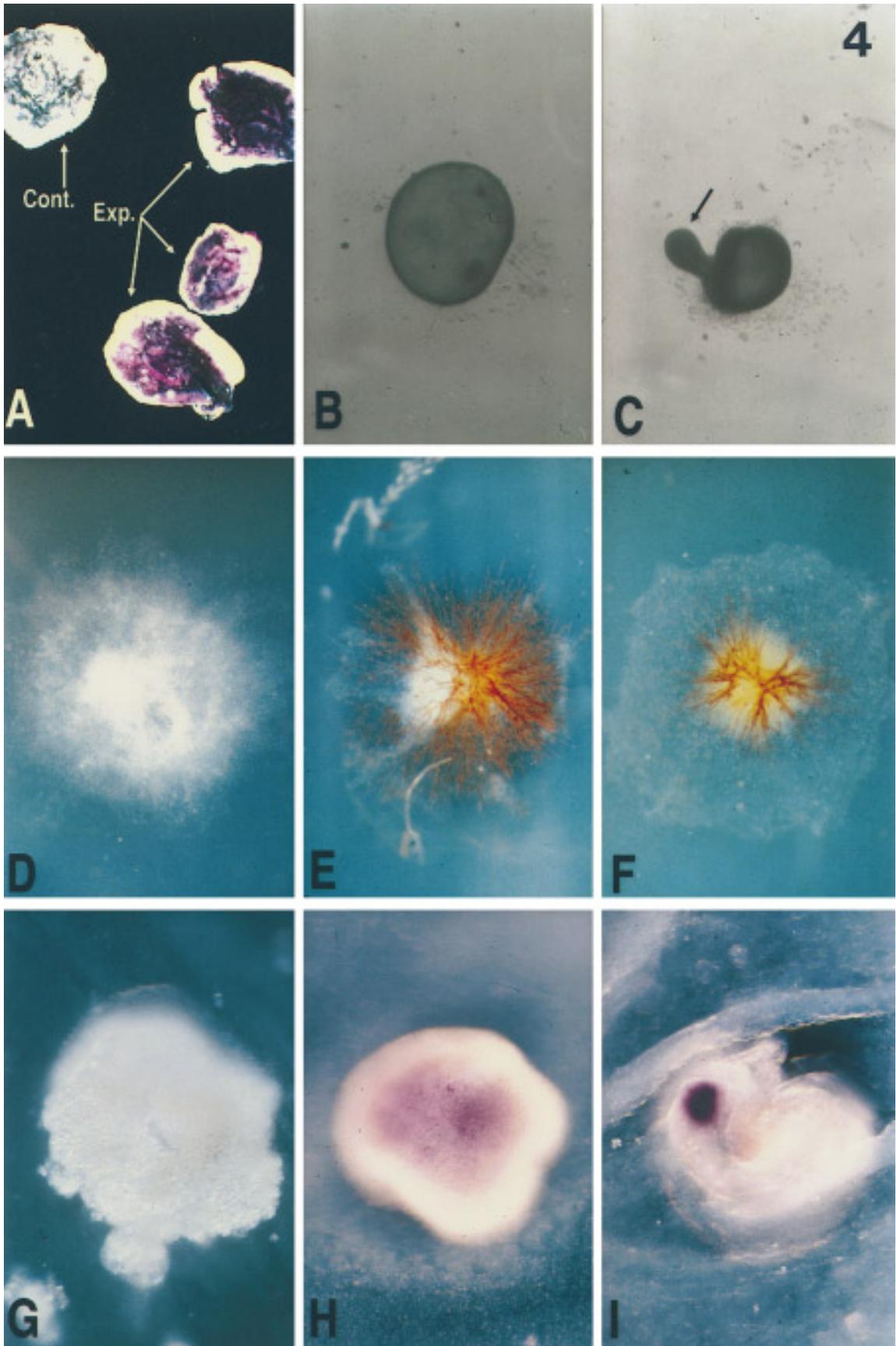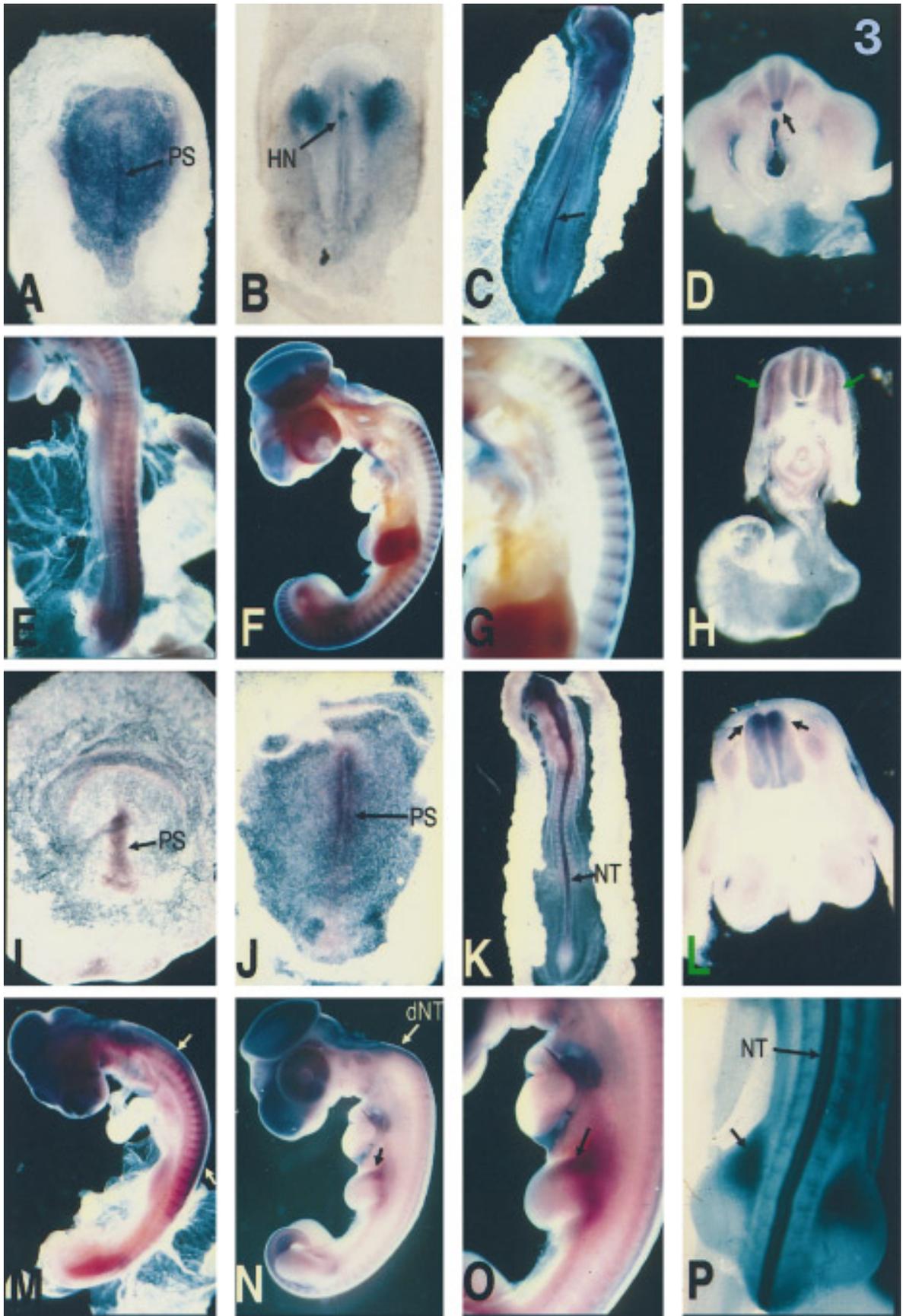
throughout the epiblast of the treated embryos, except in the most peripheral rim of the area opaca. Control embryos incubated for the same length of time in the absence of activin did not exhibit detectable levels of cActR-IIB expression but did express moderately high levels of cActR-IIA, as did treated ones. One of the four experiments (33 embryos in all) was conducted in medium 199 containing 10% heat-inactivated (for 30 min at 55°C) fetal calf serum, instead of Tyrode's saline. In this experiment, the untreated control embryos expressed the genes for both receptor subtypes. These results are in agreement with recent reports (Dalkin *et al.,* 1994; Trudeau *et al.,* 1994) that the levels of expression of the activin type-II receptor is regulated by its ligand.

### In Vitro Assay for Mesodermal Differentiation in Chick

We investigated whether different regions of the chick embryo at different developmental stages can respond to activin treatment by differentiating into mesoderm, as does the amphibian animal cap. In amphibians, the higher the dose of activin, the more dorsal/axial the mesoderm that forms (Green *et al.,* 1992), and the competence to respond to this ends in the late blastula stage, at about stage $9\frac{1}{2}$ (Nieuwkoop and Faber, 1967). We therefore treated anterior/lateral margins or posterior margins of ''mid-blastula'' stage (stage XII) or the lateral margin or posterior primitive streak of ''mid-gastrula'' stage (stage 3) chick embryos with different concentrations of activin-A. The results obtained are shown in Figs. 4B–4I and 5.

Like the amphibian animal cap, the chick epiblast responds to activin in a dose-dependent way. After a few hours' culture, untreated explants roll up into a sphere which swells with fluid, while treated explants develop a proboscis-like protrusion (Figs. 4B and 4C). After more prolonged culture, all explants spread in the collagen matrix. Those treated with activin differentiate into cells with a variety of morphologies. The type of mesoderm that develops, assessed with a number of molecular markers (see Materials and Methods for details), broadly follows the concentration of activin applied. Smooth muscle develops at the lowest concentrations (Figs. 4D–4F and 5), then skeletal/cardiac muscle, and finally notochord and organizer (*goosecoid*-positive; Figs. 4G–4I and 5) at the highest concentrations. In contrast with published results from amphibians, however, the competence of the chick epiblast to respond to activin by differentiating into all of these types of mesoderm does not end at the start of gastrulation. Perhaps surprisingly, explants from later stage embryos seem to be more sensitive to activin, with more dorsal/axial cell types differentiating at lower doses than in younger explants (Fig. 5).

## DISCUSSION

### Multiple Activin Receptors in Vertebrate Development and their Phylogenetic Conservation

Our results show that at least two types of functional type-II activin receptors exist in the chick embryo, that these are expressed from the primitive streak stage, and that the two receptor types appear to be very similar in their ligand-binding affinity for activin-A. However, the divergence of their ligand-binding domains suggests that they may differ significantly in their affinities for other forms of activin or other related members of this family of molecules. Analysis of these presumed ligand-binding domains reveals that amino acid conservation is limited to a region of less than 90 amino acids, which may constitute a minimal activin-binding domain. Further detailed studies with other members of the TGF-$\beta$ family, which might be potential ligands, as well as with the type-I receptor, should shed more light on the specificity of these two receptor proteins.

In addition to their similarity in binding specificity for activin-A, the two receptors resemble each other in that overexpression of either in early *Xenopus* embryos produces supernumerary axial structures and muscle, as found when *Xenopus* activin receptor mRNAs are injected (Kondo *et al.,* 1991; Mathews *et al.,* 1992; Hemmati-Brivanlou *et al.,* 1992). This indicates that the avian receptors can activate downstream activin-related signaling pathways in *Xenopus* embryos. This may find a parallel in the finding that the ''vegetalizing factor'' identified in chick embryos by its mesoderm-inducing activity in *Xenopus* has been shown to correspond to chick activin-A (Tiedemann *et al.,* 1992). It therefore appears that the signaling pathway for activin-related molecules in embryonic induction is highly conserved across species.

### Functional Roles of the Activin Receptors during the Early Steps of Mesodermal Induction

In *Xenopus,* activin responsiveness is lost by animal caps at the time gastrulation begins (stage $9\frac{1}{2}$–10) (Green and Smith, 1991; Lamb *et al.,* 1993; Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou *et al.,* 1994; Lemaire and Gurdon, 1994). Similarly, in the chick, Eyal-Giladi, Mitrani, and their colleagues (Eyal-Giladi, 1984; Khaner *et al.,* 1985; Eyal-Giladi and Khaner, 1989; Khaner and Eyal-Giladi, 1989; Mitrani and Shimoni, 1990; Mitrani *et al.,* 1990; Eyal-Giladi *et al.,* 1992; Ziv *et al.,* 1992) have argued that mesoderm induction occurs at a stage equivalent to the ''late blastula'' of amphibians, just before the appearance of the primitive streak (about stage XIII).

However, our *in situ* hybridization experiments reveal that both activin receptor mRNAs are expressed during development at stages other than when mesoderm induction is believed to take place. At the level of sensitivity of this technique, neither can be detected before the primitive streak stage. Both receptors continue to be expressed at later stages, in the somites and neural tissues among other sites. Therefore, if activin is involved in mesoderm induction and if this induction indeed occurs just before the appearance of the primitive streak (as seems likely), then receptors other than cActR-IIA and -IIB (or other isoforms of the type-IIB receptor; see Mathews, 1994) must be involved.
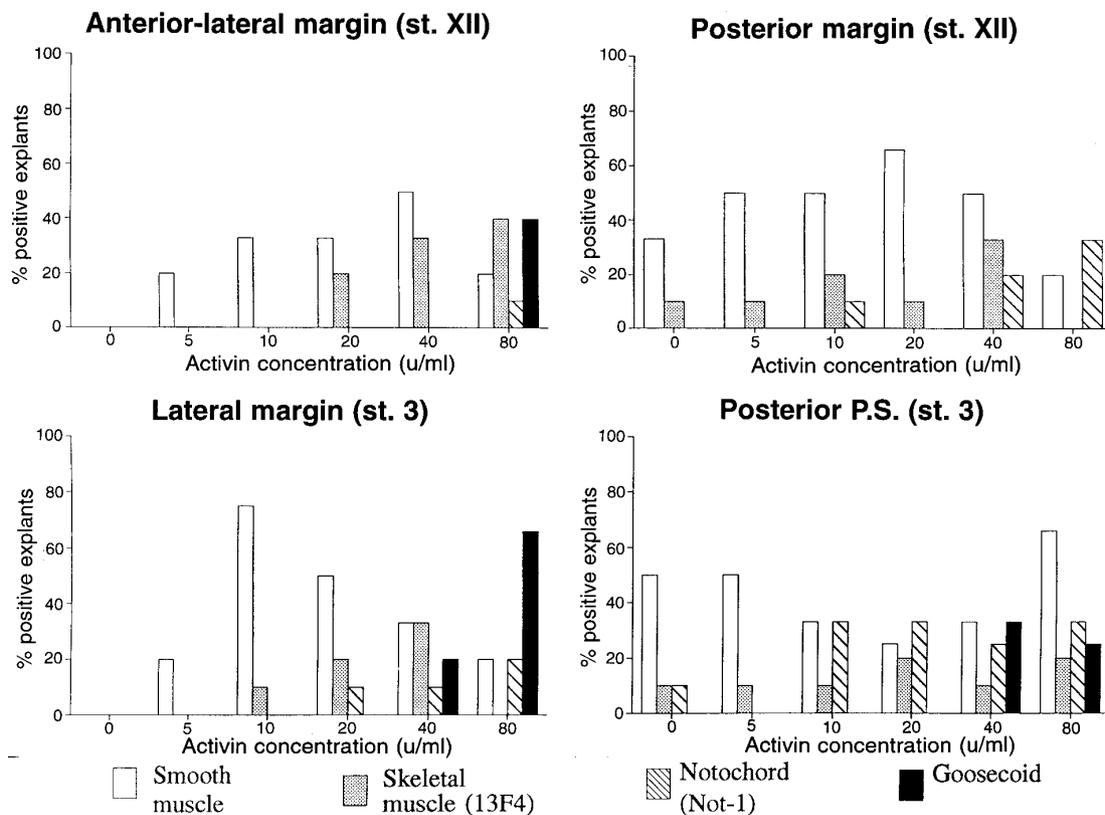
**FIG. 5.** Dose–response relationship between activin-A concentration and four tissue types that differentiate *in vitro,* in explants obtained from four regions of the early embryo.

In *Xenopus,* Hemmati-Brivanlou *et al.* (1992) have isolated an activin receptor (XAR1) that appears to be unrelated in its pattern of expression to those reported in the present paper, despite its high degree of structural similarly to cActR-IIB. This is expressed ubiquitously and the mRNA is of maternal origin, suggesting that it could mediate the earlier steps of mesodermal induction *in vivo.* In support of this, the phenotype of dominant negative XAR1 mutants indicates that this receptor may be involved in normal mesodermal induction (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou *et al.,* 1994). By contrast, null mutants in the mouse homologue of the cActR-IIA gene, produced by homologous recombination, do not display obvious defects in mesoderm induction (Matzuk *et al.,* 1995b).

It seems possible, by analogy with results in other species (see Mathews, 1994), that the chick should possess other activin receptors, expressed at earlier stages of development than the cActR-IIs reported here. This would account for our finding that preprimitive streak stage embryos can respond to activin treatment. Such treatment in fact leads to ubiquitous induction of expression of cActR-IIB, which therefore can itself be considered as an early marker for the mesoderm of the primitive streak. But what, then, is the function of these later receptors, which only appear to be expressed in *response* to activin treatment?

One possibility is that their *in vivo* ligands are peptide growth factors other than activin, perhaps other members of the TGFβ family, including Vg-1 or BMPs, as has recently been reported in other systems (Bhushan *et al.,* 1994; Koenig *et al.,* 1994; Schulte-Merker *et al.,* 1994; Kingsley, 1994; Kessler and Melton, unpublished results). This receives strong support from the finding that null mutants for either the βA or the βB subunits, or the double-mutant, do not display defects in mesodermal induction (Matzuk *et al.,* 1995a). Another possibility, however, is that the functions of these receptors continues during development, beyond the early primitive streak stage, as suggested by the later phenotypic consequences of targeted mutations in activin, activin receptor, and follistatin genes (Matzuk *et al.,* 1995a,b,c).

### A New in Vitro Assay for Mesodermal Differentiation in the Chick Embryo

In the experiments of Mitrani and his colleagues (Mitrani and Shimoni, 1990; Mitrani *et al.,* 1990; Ziv *et al.,* 1992), mesoderm induction in the chick embryo is assessed according to whether embryos deprived of hypoblast and marginal zone form a complete embryonic axis. There is, as yet,

no direct assay for mesodermal differentiation independent of other morphogenetic events, as is possible with small *Xenopus* animal caps (see reviews by Green and Smith, 1991; and Stern, 1992). For this reason, and to test the possibility suggested above that activin responsiveness may continue beyond the early stages of primitive streak formation, we have designed a new *in vitro* assay for mesodermal differentiation in small isolated explants of epiblast. With this assay, we were able to investigate the stage and position dependence of the responses of the epiblast to activin treatment.

### Activin Responsiveness of Mesodermal Differentiation Persists through Later Stages of Development

At early stages of development (stage XII), explants of anterior/lateral area opaca epiblast generate smooth muscle, skeletal muscle, notochord and organizer (goosecoid-expressing cells) in response to activin treatment, in a concentration-dependent manner. The relationship between activin concentration and the mesodermal cell types that differentiate is closely reminiscent of the results obtained by Green *et al.* (1992). However, at later stages of development, the area opaca epiblast retains its ability to respond to activin in a concentration-dependent manner, unlike findings made in amphibian animal caps, which lose their responsiveness to activin as gastrulation begins. Our experiments reveal that there is a hierarchy of sensitivity of explants to activin: early anterior/lateral margin is least sensitive, followed by the early posterior margin, the later stage lateral margin, and finally the posterior primitive streak, which is most sensitive. Thus, not only does the epiblast of the area opaca retain its responsiveness to activin at later stages, but also these stages show an *enhanced* responsiveness to the factor. It will be interesting to investigate whether equivalent regions of the frog gastrula, such as the lips of the blastopore, show similar behavior.

The finding that the area opaca epiblast can be induced by activin to differentiate into various mesodermal cell types appears at first to contrast with the findings of Khaner *et al.* (1985). They showed that this region is ''not competent to develop any sort of axial structures'' at stages X–XIII, based on grafting experiments. The present results show that activin can indeed induce mesoderm including the most axial types, notochord and organizer, from area opaca epiblast. These two results suggest either that the marginal zone used in the experiments of Khaner *et al.* (1985) is not a source of activin or that the area opaca is competent to form mesodermal tissues but is not able to organize them into a coherent axis. The experiments of Storey *et al.* (1992) argue that the latter may not be the case, even at later stages of development, since a graft of Hensen's node is able to induce a complete axis, which also expresses a full range of regional markers (*engrailed, Hox-c6,* and diencephalic and hindbrain markers).

### Activin, Hensen's Node, and the Origins of the Axial Mesoderm

The preceding argument brings us back to the original question: how do mesendodermal progenitor cells acquire their fates during gastrulation in Hensen's node? The expression of both of these receptors in the primitive streak and Hensen's node makes them obvious candidates to mediate the effects of activin seen in culture on explants of the primitive streak.

In the experiments of Selleck and Stern (1991), single cells in the epiblast of Hensen's node at the full primitive streak stage (stage 4) were seen to contribute progeny to both the neural tube (an ectodermal derivative) and to the notochord (axial mesoderm). Therefore, at this relatively late stage in development, some cells derived from uncommitted precursors are still acquiring mesodermal phenotypes. It seems possible that activin-related signals could play a role in this process, an idea supported both by the patterns of expression of the cActR-IIs and by the persistent sensitivity of explants to activin at these late stages.

These results lead to a simple model: that a chick homologue of the amphibian XAR1 and/or type-I receptor might mediate the earlier steps of mesoderm induction and that this early response could result initially in the activation of transcription of the cActR-IIs in the primitive streak. In turn, all of these receptors would then cooperate to account for the full range of activin responsiveness. Activin itself could compete with other ligands for the same receptors, and different ligands might have different affinities for these various receptors. Thus, in addition to concentration thresholds (Green *et al.,* 1992), more subtle timing and sequential activation of transcription of specific receptors could be responsible for the complex patterns of mesoderm formation and differentiation seen at earlier and later stages of development.

### Activin and goosecoid

Among the responses of early embryonic tissues to activin is the activation of transcription of the homeobox gene *goosecoid* (Cho and De Robertis, 1990; Gaunt *et al.,* 1993; Green *et al.,* 1992; and present results). This gene is normally expressed in the dorsal lip of the blastopore of the amphibian embryo (Blumberg *et al.,* 1991) and in Hensen's node of the chick embryo (Izpisúa-Belmonte *et al.,* 1993). Its expression pattern is consistent with the localization of transcripts for the activin receptors studied here, and Hensen's node might be expected to be the most sensitive tissue, with other regions of the primitive streak following it in the hierarchy.

### Activin Signaling Pathways in Limb Pattern Formation and Neural Development

Interestingly, we find that cActR-IIB, like *goosecoid,* is expressed particularly strongly in the proximal-anterior part

of the limb bud (see also Gaunt *et al.,* 1992; Ohuchi *et al.,* 1992; Nohno *et al.,* 1993). This suggests that cActR-II receptors could underlie the activation of *goosecoid* both in Hensen's node and in the limb mesenchyme. This conclusion is particularly interesting because it has been shown that a graft of Hensen's node into the anterior margin of the limb bud mimics a graft of a polarizing region or of a local source of retinoids by producing extra digits (Hornbruch and Wolpert, 1986). In addition, activin treatment of limb bud mesenchyme can induce chondrogenesis (Chen *et al.,* 1993; Jiang *et al.,* 1993). It is also interesting that ectopic application of TGF-$\beta$1, which acts through receptors structurally related to the ActR-IIs (Lin *et al.,* 1992), also causes alterations in the skeletal elements in the developing limb in a stage- and position-specific manner (Hayamizu *et al.,* 1991). It therefore seems likely that activin-related signaling is somehow involved in the events leading to limb pattern formation.

The two receptors are also expressed in the nervous system, which could be related to reports that activin is a nerve cell survival molecule for P19 cells (Schubert *et al.,* 1990) and that it can modulate neuronal phenotype (Hashimoto *et al.,* 1990; Fann and Patterson, 1994). While the present results support these suggestions, the precise role of activin-related signaling pathways in the development of the vertebrate CNS remains to be elucidated.

In summary, therefore, the expression of activin receptors at later stages of development correlates with an extended period of responsiveness to this factor, initially of the epiblast and later of other tissues. This conclusion allows us to predict that the neural tube and somites should also be responsive to activin, although at present it seems difficult to design an appropriate assay to demonstrate this directly.

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*Note added in proof.* Since acceptance of this paper, Pituello *et al.* have reported that activin-A regulates dorsoventral patterning of the neural tube *in vitro* (Pituello, F., Yamada, G., and Gruss, P. (1995). Activin-A inhibits Pax-G expression and perturbs cell differentiation in the developing spinal cord *in vitro. Proc. Natl. Acad. Sci. USA* **92,** 6952–6956).

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