

## Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins

A. Feijen, M. J. Goumans and A. J. M. van den Eijnden-van Raaij\*

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

\*Author for correspondence

### SUMMARY

Using *in situ* hybridization we have studied the localization of the messenger RNAs encoding the inhibin/activin subunits ( $\alpha$ ,  $\beta$ A,  $\beta$ B), the activin-binding protein follistatin and activin receptors (IIA, IIB) in mouse embryos during postimplantation development.

From 6.5- to 9.5-days post coitum (p.c.) activin  $\beta$ A and  $\beta$ B subunit expression was restricted to the decidua, while activin receptor type IIB messages were exclusively detected in the embryo. Expression of activin receptor type IIA was apparent in the embryo as early as 9.5 days p.c. In contrast, follistatin transcripts were present in both the decidua and the embryo at the early postimplantation stages. In particular, the primitive streak region, specific rhombomeres in the developing hindbrain, somites, paraxial mesoderm and parietal endoderm cells attached to the Reichert's membrane showed strong expression of follistatin.

In 10.5- and 12.5-day embryos expression of the  $\beta$ A subunit message was abundant in mesenchymal tissue, in particular in the developing face, the body wall, the heart, precartilaginous condensations in the limb and in the mesenchyme of structures that show both epithelial and mesenchymal components, including tissues of the embryonic digestive, respiratory and genital tracts. The distribution of  $\beta$ B transcripts was quite different from that observed for  $\beta$ A.  $\beta$ B is strongly expressed in selected regions of the brain, in particular the fore- and hindbrain, and in the spinal cord. Specific hybridization signals were also present in the epithelium of the stomach and oesophagus. Common sites of  $\beta$ A and  $\beta$ B expression are blood vessels, interver-

tebral disc anlagen, mesenchymal condensations in the flank region and the gonad primordium. The latter organ is the only site in the embryo where the  $\alpha$  subunit is expressed, and thus where inhibin activity may be present.

During the period of organogenesis the sites of expression of activin receptors type IIA and IIB messenger RNA (mRNA) generally coincide with or are adjacent to the sites of  $\beta$  subunit expression. Differences in the expression patterns of the receptor RNAs are the whisker follicles, where type IIA is expressed, and the metanephros and the forebrain where type IIB transcripts are present.

Taken together, the present data suggest that follistatin, but not one of the known activin forms (A,B,AB) is involved in early postimplantation development. During organogenesis a role is suggested for (1) activin A in the formation of the inner ear, tongue and lung and during morphogenesis of craniofacial structures, (2) activin B in the development of the stomach and the central nervous system as well as in the development of the eyelids, and (3) activin AB in limb and shoulder development. However, in or adjacent to some of these developing structures follistatin is also produced. The different forms of follistatin have different capacities to regulate activin activity so that both the particular form of follistatin and its distribution in relation to that of activins will determine whether activins are indeed involved in the development or morphogenesis of specific organs.

Key words: activins, activin-binding proteins, murine embryogenesis, *in situ* hybridization

### INTRODUCTION

Activins are now thought to be important regulators of early developmental processes in vertebrates. Originally, however, these factors and their relatives, the inhibins, were isolated as gonadal proteins from follicular fluid and purified on the basis of their capacity to regulate the release of follicle-stimulating hormone (FSH) from the pituitary (for a review see Ying, 1988

and Ling et al., 1988). Activins and inhibins are members of the transforming growth factor (TGF) $\beta$  superfamily of structurally related, but functionally diverse proteins. The activins are dimeric proteins, composed of disulphide-linked  $\beta$ A or  $\beta$ B subunits. At least three different forms of activin are known to occur *in vivo*; the homodimeric activin A ( $\beta$ A $\beta$ A) and activin B ( $\beta$ B $\beta$ B) forms and the heterodimeric activin AB ( $\beta$ A $\beta$ B; Ying, 1988; Ling et al., 1988; Mason et al., 1989; Nakamura

et al., 1992a; Fukui et al., 1993). There are two forms of inhibin (A and B) consisting of a  $\beta$ A or  $\beta$ B subunit linked to a distantly related inhibin-specific  $\alpha$  subunit.

Activins bind to at least two prominent classes of cell surface proteins, type I receptors ( $50\text{--}55 \times 10^{-3} M_r$ ) and type II receptors ( $70\text{--}75 \times 10^{-3} M_r$ ; Hino et al., 1989; Centrella et al., 1991; Mathews et al., 1991). Cloning of the activin type IIA receptor (Act R-IIA; Mathews and Vale, 1991; Kondo et al., 1991; Matzuk and Bradley, 1992 a,b; Donaldson et al., 1992; Shinozaki et al., 1992) and the activin type IIB receptors (Act R-IIB; Attisano et al., 1992; Mathews et al., 1992; Legerski et al., 1992; Nishimatsu et al., 1992) has shown that these receptors have intracellular kinase domains with predicted serine/threonine specificity. Recently a *Drosophila* type II activin receptor, Atr-II, has been identified which might represent a third activin type II receptor isoform not yet identified in vertebrates (Childs et al., 1993).

Recently two human activin type I receptors, Act R-I (Attisano et al., 1993) and Act R-IB (Carcamo et al., 1994) were cloned and shown to be essential for activin signalling. Act R-I and Act R-IB are identical to the activin receptor-like kinases 2 (ALK-2) and 4 (ALK-4), respectively (ten Dijke et al., 1993, 1994). Also the sequences of the rat homologues of Act R-I and Act R-IB (Tsuchida et al., 1993; R-1, He et al., 1993) and the mouse Act R-I homologue (Ebner et al., 1993a) have been reported. The latter murine receptor, Tsk-7L, was originally isolated as a TGF $\beta$  type I receptor, but was later shown to be promiscuous in that it binds both activin and TGF $\beta$  (Ebner et al., 1993b). Signalling, however, occurs only for activin (Attisano et al., 1993). In addition to these receptors a *Drosophila* activin type I receptor (Atr-I; Wrana et al., 1994), and another shared TGF $\beta$ /activin type I receptor from human (TSR-I, Attisano et al., 1993) or rat (R-3, He et al., 1993) have been identified. Whether these receptors also have signalling capacity is still unknown.

Apart from cell surface receptors, there are other activin-binding proteins known as follistatins: these are glycosylated polypeptides originally isolated from porcine follicular fluid, that, like the inhibins, inhibit FSH secretion (Ueno et al., 1987; Shimasaki et al., 1988a,b; for a review see Michel et al., 1993). At least six isoforms of follistatin have been identified that are generated by truncations in the C terminus and/or the presence of Asn-linked carbohydrate chains. The isoforms can be divided into three groups, representing follistatins of 315 amino acids (FS-315), 303 (FS-303) and 288 amino acids (FS-288). FS-303 is probably the major component in the follistatin preparation from porcine follicular fluid (Inouye et al., 1991; Sugino et al., 1993). This protein has a specific and high affinity for activin and neutralizes its effects in a variety of systems (Nakamura et al., 1990; Kogawa et al., 1991; Xiao and Findlay, 1991; Asashima et al., 1991; Hashimoto et al., 1992; Shiozaki et al., 1992). Although follistatin binds to both activin and inhibin through the common  $\beta$  subunit (Shimonaka et al., 1991), binding does not affect the biological activity of inhibin (Ying et al., 1987).

Activins are involved in many biological processes outside the pituitary-gonadal axis (for a review see Ueno et al., 1990; Vale et al., 1990). In particular, the different forms of activin have the capacity to act as mesoderm-inducing factors in amphibian development (Albano et al., 1990; Asashima et al., 1990; Smith et al., 1990; Thomsen et al., 1990; van den

Eijnden-van Raaij et al., 1990; Nakamura et al., 1992a). The sharp thresholds and multiple stable responses to activin A indicated that this factor has the properties of a classical morphogen (Green and Smith, 1990). Recently it was found that activin A also induces axial structures, including notochord and somites in the chick epiblast (Mitrani and Shimoni, 1990; Mitrani et al., 1990) and newt presumptive ectoderm (Moriya and Asashima, 1992). Exposure of animal cap cells of zebrafish to activin A induces the zebrafish *T* gene (*z-f-T*), which is a marker for mesodermal tissue including notochord (Schulte-Merker et al., 1992). These results suggest that activin may be one of the natural inducers of mesoderm in a wide range of species. This hypothesis was strengthened by the observation that injection of a truncated activin type IIB receptor into *Xenopus* embryos completely prevents mesoderm induction and dorsal body axis formation (Hemmati-Brivanlou and Melton, 1992, 1994). In addition, it was shown that inhibition of activin receptor signalling promotes neuralization in *Xenopus*, indicating a role for activin in the control of neural induction (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou et al., 1994).

Little is known about the function of activins and activin-binding proteins during murine embryogenesis. Using the P19 embryonal carcinoma (EC) cell line as a model for studying mesodermal and neural differentiation in the mouse, we have recently shown that activin A itself has no mesoderm-inducing activity in this system. In fact, activin A completely inhibits differentiation of P19 EC cells into derivatives of any of the three germ layers (van den Eijnden-van Raaij et al., 1991; Hashimoto et al., 1990). In addition, depending on the culture conditions, activin A acts as a potent mitogen for P19 EC cells or promotes their survival in monolayer (Schubert and Kimura, 1991; Schubert et al., 1990). These results together with the identification of activin-binding sites on P19 EC cells (Kondo et al., 1989; Nakamura et al., 1992b) suggest that activins may have a regulatory function in differentiation during early murine development.

Expression studies in differentiating EC and embryonic stem (ES) cells, and in the mouse embryo itself showed that the expression of inhibin subunits and follistatin, but not of activin type IIA receptor is developmentally regulated (van den Eijnden-van Raaij et al., 1992; Albano et al., 1993; Lu et al., 1993). For the  $\beta$ A and  $\beta$ B subunits, this was confirmed at the protein level with subunit-specific antibodies (Paulusma et al., 1994). The detection of the inhibin subunits in early postimplantation mouse embryos by reverse transcription-polymerase chain reaction (RT-PCR) analysis contrasts with the results of in situ hybridization studies by Manova et al. (1992). These authors showed that in 5.5- to 9.5-day mouse embryos inhibin  $\beta$ A is expressed exclusively in maternal decidual tissue while  $\beta$ B and  $\alpha$  transcripts are not present in either the embryo proper or the decidua. The mRNA level of the inhibin subunits is probably below the detection limit of in situ hybridization. We have recently shown in some preliminary in situ hybridization studies of 12.5-day mouse embryos that there are characteristic and distinct expression patterns for the three inhibin subunits and for activin-binding proteins, which suggested that different forms of activin have different functions during murine development (Mummery and van den Eijnden-van Raaij, 1993). In the present paper we describe in detail the expression of the inhibin subunits, follistatin and activin

receptors type IIA and IIB in histological sections of mouse embryos from 6.0- to 12.5-days post coitum (p.c.). The results are discussed in terms of the putative functions of the different forms of activin in mammalian embryonic processes in vivo.

## MATERIALS AND METHODS

### Embryos

Mouse embryos collected between 6.0- and 12.5-days post coitum (p.c.) were obtained from F1 crosses between C57BL6 females and CBA males (midday on the date of finding a vaginal plug was designated 0.5 day). 6.0- to 8.5-day embryos were left in the decidua and 9.5- to 12.5-day embryos were dissected free from their membranes.

### Cell culture, RNA isolation and northern blotting

Culture of P19 embryonal carcinoma (EC) cells, embryonic stem cells (ES-5) and the P19-derived mesodermal cell line MES-1 was as described previously (Mummery et al., 1985, 1986, 1990a,b). Isolation of polyadenylated RNA and northern blotting were carried out as described by van den Eijnden-van Raaij et al. (1992).

### Probe synthesis

Constructs for in situ hybridization were generated by cloning the following fragments into the pBluescript SK II<sup>-</sup> vector (Stratagene) in anti-sense orientation with respect to the T7 promoter:

βA, a 276 nucleotide fragment, spanning amino acid residues 309-399 (Albano et al., 1993); βB, a 350 nucleotide fragment, spanning amino acid residues 196-312 (Esch et al., 1987); α, a 249 nucleotide fragment, spanning amino acid residues 194-277 (Albano et al., 1993); follistatin, a 324 nucleotide fragment, spanning amino acid residues 171-279 (Shimasaki et al., 1988a); activin type IIA receptor, a 239 nucleotide *SspI-KpnI* fragment (Mathews and Vale, 1991) spanning amino acid residues 61-140; activin type IIB receptor, a 235 nucleotide fragment, spanning amino acid residues 75-153 (Attisano et al., 1992).

Except for the activin type IIA receptor fragment, all fragments were obtained by amplification of reverse transcriptase products of total RNA from mouse cells or tissue expressing the particular gene, using the polymerase chain reaction (PCR). In brief, first strand cDNA was synthesized using RNA isolated from P19 (Act R-IIB, follistatin), ES-5 (βB), MES-1 (βA) cells and mouse ovary (α) as template and oligo (dT) as primer. The cDNA was amplified in a PCR reaction using specific primers, containing unique cloning sites (*Bam*HI site for 5' primer; *Eco*RI site for 3' primer).

All constructs were verified by sequencing qiagen purified DNA preparations and tested on northern blots for specificity. RNA probes were generated by transcription of the T3 or T7 RNA polymerase promoter in the presence of α<sup>35</sup>S-UTP (Amersham).

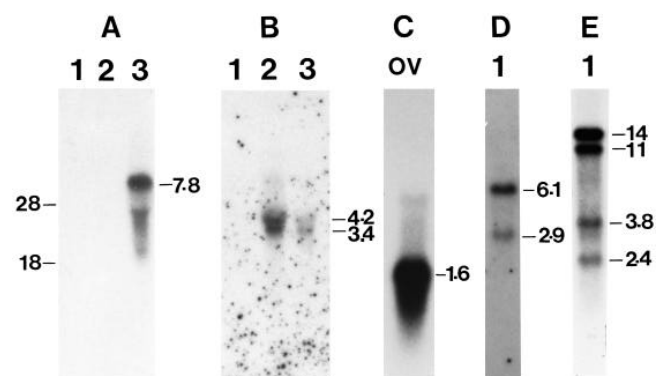
### In situ hybridization

In situ hybridization analysis was performed as described by Wilkinson et al. (1990) with slight modification. Embryos were fixed in 4% paraformaldehyde, dehydrated through alcohol and embedded in paraffin wax. Sections of 6-8 μm were cut and floated on to TESPA (3-aminopropyltriethoxysilane)-coated slides. The slides were pre-treated with 20 μg/ml proteinase K and 0.25% (v/v) acetic anhydride to reduce background and hybridized overnight at 55°C in a moist chamber. After hybridization, coverslips were removed in 5× SSC, 25 mM DTT at 50°C. The slides were then washed at high stringency for 30 minutes at 65°C in a 50% formamide, 2× SSC, 100 mM DTT solution and treated with 20 μg/ml RNase A at 37°C for 30 minutes to remove any non-specifically bound probe. The high-stringency washing was repeated. The sections were washed in 2× SSC and 0.1× SSC for 15 minutes at room temperature and dehydrated in ethanol containing 300 mM ammonium acetate and air dried. Autoradiogra-

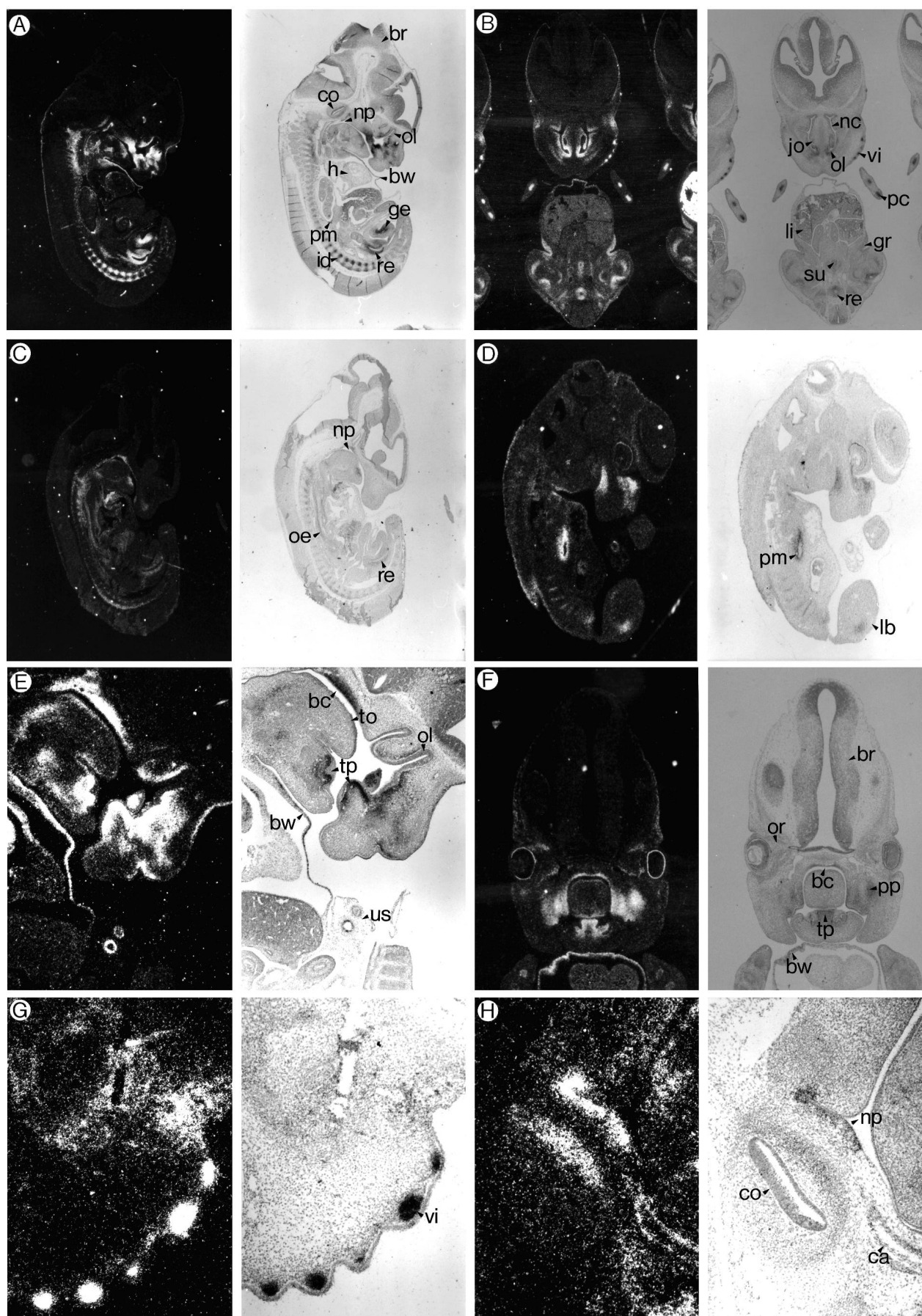
phy was performed by using Ilford G5 photo emulsion 1:1 diluted with 2% glycerol/water. The sections were air dried and exposed for 2 weeks in a light-safe box containing silica gel at 4°C. Slides were developed in Kodak D19, fixed in Kodak UNIFIX and counterstained with haematoxylin.

## RESULTS

Recent data on the expression of inhibin subunits, follistatin and type II activin receptors in differentiating murine EC and ES cells, and in the mouse embryo, pointed to a potential role for these factors during murine development (van den Eijnden-van Raaij et al., 1992; Albano et al., 1993, 1994; Mummery and van den Eijnden-van Raaij, 1993). In order to define more precisely the tissues and cell types expressing these genes we carried out an in situ hybridization study in 6.0- to 12.5-day mouse embryos, using <sup>35</sup>S-labelled single-stranded antisense riboprobes. The specificity of the probes for the inhibin subunits and the activin receptors IIA and IIB was checked by using the cDNAs from which they were generated on northern blots of mouse cells expressing the different genes. As shown in Fig. 1 each probe detected only the predicted transcript sizes for the respective gene in the cell or tissue type expected (see also van den Eijnden-van Raaij et al., 1992); there is therefore no cross-reactivity between the different probes. This, together with the high stringency conditions used for in situ hybridization should result in the specific detection of the messenger RNAs (mRNAs) of interest. Indeed each gene shows a unique transcript distribution during murine embryogenesis. A comparison of the mRNA localization of the inhibin/activin subunits, activin receptors and follistatin in a 12.5-day mouse embryo is shown in Table 1. These expression patterns are quite similar to those observed in rat embryos (Roberts et al., 1991; Roberts and Barth, 1994). Control hybridizations using the corresponding sense RNA probes gave no specific signal above background (not shown).



**Fig. 1.** Northern blot analysis of the inhibin α, βA and βB subunit probes and the activin type IIA and IIB receptor probes used for this study. (A) βA subunit, (B) βB subunit, (C) α subunit, (D) activin type IIA receptor, (E) activin type IIB receptor. 1, 2 and 3 refer to P19 EC, ES-5 and MES-1 cells, respectively. Ov refers to murine ovary. Each probe showed a different specificity. Transcript sizes (in kilobases) are indicated on the right, positions of ribosomal 18S and 28S subunits are shown on the left. Equal loading was confirmed using a glyceraldehyde phosphate dehydrogenase cDNA as probe (not shown).



**Fig. 2.** Inhibin/activin  $\beta$ A subunit mRNA expression in sagittal (A,C,D,E,G,H) and frontal sections (B,F) of 12.5- (A,B,E-H) and 10.5- (C,D) day p.c. mouse embryos. Dark-field micrographs (left) and corresponding bright-field images (right). bc, buccal cavity; br, brain; bw, body wall; ca, carotid artery; co, cochlea; ge, genital eminence; gr, groin; h, heart; id, intervertebral disc primordium; jo, Jacobson's organ; lb, limb bud; li, liver; nc, nasal cavity; np, nasopharynx; oe, oesophagus; ol, olfactory epithelium; or, orbita; pc, precartilaginous condensations; pm, pleuroperitoneal membrane; pp, palatine process; re, rectum; su, septum urogenitale; to, tongue; tp, tooth primordium; us, umbilical stalk; vi, vibrissa.

### Localization of $\beta$ A subunit mRNA

A general observation from the *in situ* hybridization studies was that expression of the  $\beta$ A subunit mRNA was absent in early postimplantation mouse embryos (6.0 to 9.5 days p.c.), but was present in a specific subset of decidual cells, confirming the expression pattern described earlier (Manova et al., 1992; Albano et al., 1994).

In 10.5- and 12.5-day mouse embryos  $\beta$ A subunit mRNA was abundant in the mesenchymal cells of structures that show both epithelial and mesenchymal components (Figs 2, 3). In particular the condensed mesenchyme in the head, surrounding the olfactory epithelium (Fig. 2A,B,E) and Jacobson's organ (Fig. 2B), and the mesenchyme adjacent to the epithelia of the cochlea (Fig. 2A,H) were positive. A strong hybridization signal was also present in the mesenchyme along the epithelium lining the nasal cavity (Fig. 2F), the nasopharynx (Fig. 2A,C,H) and lining the roof of the buccal cavity (Fig. 2E,F), as well as in the mesenchyme of the palatine processes (Fig. 2F). In addition, the mesenchyme of the tooth primordia in the upper and lower jaws (Fig. 2E,F), the mesenchyme in the developing vibrissae (Fig. 2G), the condensed mesenchyme in the orbita around the developing eye (Fig. 2F) and mesenchymal structures in the tongue (Fig. 2A,E) express the  $\beta$ A message.  $\beta$ A subunit mRNA is also localized on the dorsal side of the genital eminence and the septum urogenitale (Figs 2A, 3A), and is present in tissues of the embryonic digestive and respiratory tract.  $\beta$ A transcripts are seen in the mesenchyme in the hilus of the lung (Fig. 3C) and in the mesenchymal tissue underlying the epithelial layer of the oesophagus, rectum and trachea (Fig. 3A,B,D). In the alveoli, stomach, liver, metanephros and intestine no specific hybridization signal was observed (Figs 2, 3). Also the central nervous system, including brain and spinal cord was completely negative (Fig. 2).

The data further show that  $\beta$ A subunit mRNA is present in the mesenchyme of the anterior body wall (Fig. 2A,E,F), in the pleuroperitoneal membrane (Figs 2A,D, 3G,H) and in the wall of large blood vessels, including the aorta (Fig. 3B,D), the truncus arteriosus and truncus pulmonalis (Fig. 3G), the carotid artery (Fig. 2H) and blood vessels in the umbilical stalk (Fig. 2E). In the heart, expression is seen in the atrioventricular bulbar cushion tissue, while the walls of the heart ventricles are negative (Fig. 3D). High levels of  $\beta$ A subunit transcripts were visible in the intervertebral disc anlagen (Figs 2A, 3B,D) and mesenchymal condensations in the cervical region (Fig. 3E), shoulder girdle (Fig. 3E) and groin region (Fig. 2B). In the limbs,  $\beta$ A expression was restricted to precartilaginous condensations (Fig. 3F). A striking observation was that the gonad primordium was either positive (Fig. 3G) or negative (not

**Table 1.** Distribution of inhibin/activin subunits, follistatin and activin type IIA and IIB receptor in 12.5-day p.c. mouse embryos determined by *in situ* hybridization

Tissue	$\beta$ A	$\beta$ B	$\alpha$	Foll	R-IIA	R-IIB
Olfactory	<sup>1</sup>	—	—	<sup>1</sup>	<sup>2</sup>	<sup>2</sup>
Cochlea	<sup>1</sup>	—	—	<sup>2</sup>	<sup>2</sup>	<sup>2</sup>
Vibrissa	<sup>1</sup>	—	—	<sup>1</sup>	<sup>2</sup>	—
Eye <sup>3</sup>	+	+	—	+	+	+
Tooth primordium	<sup>1</sup>	—	—	—	—	—
Tongue mesenchyme	+	—	—	+	+	+
Genital eminence	+	—	—	—	—	—
Lung	+	—	—	—	+/-	+/-
Oesophagus	<sup>1</sup>	<sup>2</sup>	—	—	—	—
Rectum	<sup>1</sup>	—	—	—	—	—
Trachea	<sup>1</sup>	—	—	—	—	—
Stomach	—	<sup>2</sup>	—	<sup>1</sup>	+/-	+/-
Liver	—	—	—	—	—	—
Metanephros	—	—	—	—	—	+
Intestine	—	—	—	<sup>1</sup>	+	+
Brain	—	+	—	+	+	+
Spinal cord	—	+	—	+	+	+
Body wall	+	—	—	+	—	—
Blood vessels	+	+/-	—	+	—	—
Heart	+	—	—	+	—	—
Intervert. discs	+	+	—	+	—	—
Gonad primordium <sup>5</sup>	+/-	+	+	+	—	+/-
Pleuroperit. membr.	+	+	—	+	—	—
Fascia	—	—	—	+	+	+
Limb/shoulder	<sup>4</sup>	+	—	+	+	+

<sup>1</sup>Restricted to mesenchyme.

<sup>2</sup>Restricted to epithelium.

<sup>3</sup>Expression in specific regions (see text).

<sup>4</sup>Restricted to precartilaginous condensations.

<sup>5</sup>+/- means either + or -, possibly by sex difference.

shown) for  $\beta$ A transcripts. In the latter case the mesonephros was positive. This variation in  $\beta$ A expression in the developing gonad might be due to sex differences between embryos and will be further investigated.

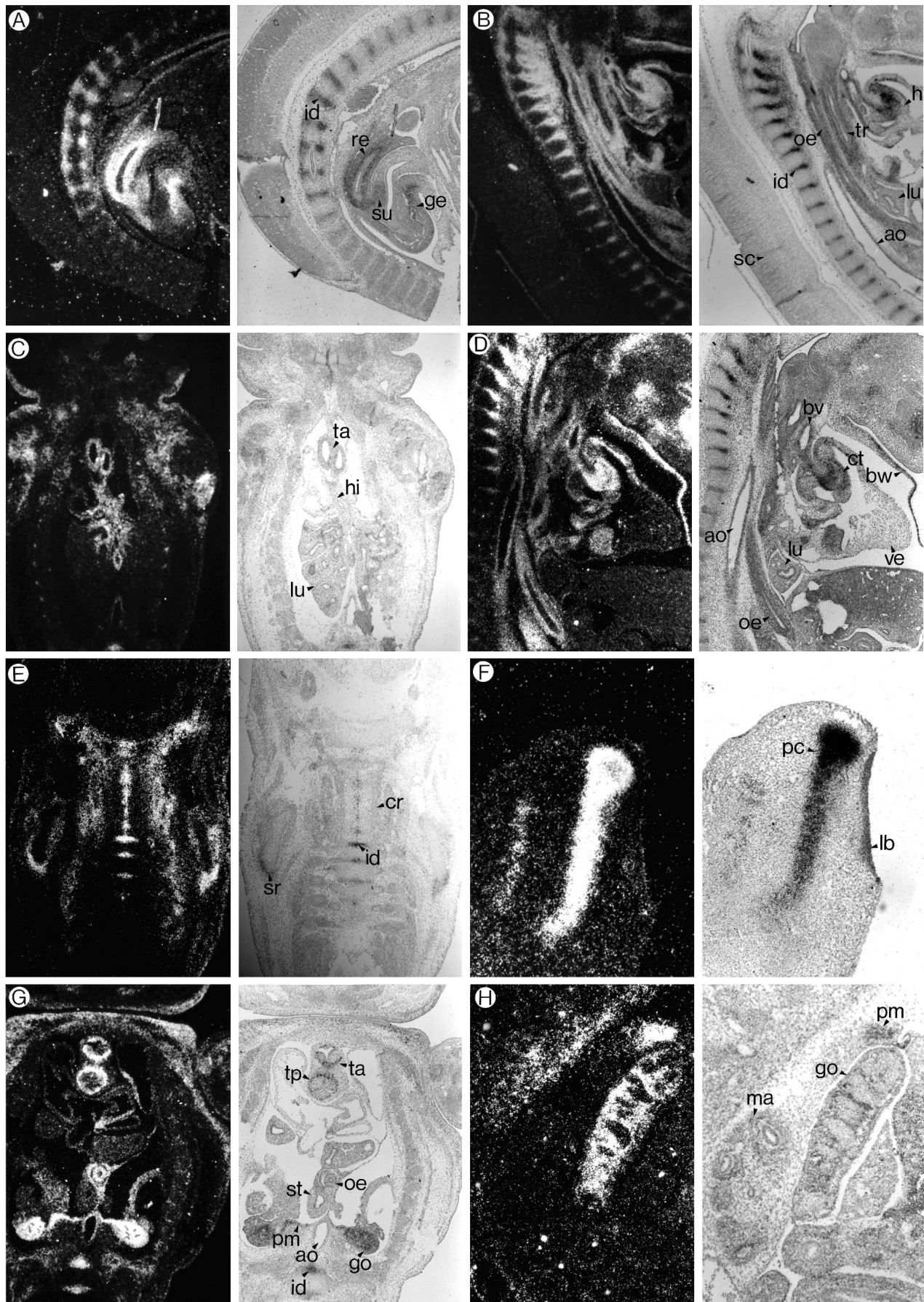
### Localization of $\beta$ B subunit mRNA

The distribution of  $\beta$ B transcripts is quite different from that of  $\beta$ A. Consistent with the results of Albano et al. (1994) activin  $\beta$ B subunits are absent in the embryo, but are expressed in a broad zone of decidual cells underlying the uterine lumen at 6.0 days p.c. Decidual cells located at the position of the embryo are negative for  $\beta$ B transcripts, which is in contrast with the expression pattern of the  $\beta$ A subunits (Manova et al., 1992). During further development  $\beta$ B expression in the decidua is slightly decreased.

In 10.5- and 12.5-day mouse embryos  $\beta$ B is strongly expressed in certain parts of the brain. In particular the neopalial cortex (telencephalon, anterior of the choroid plexus), the intraventricular portion of the cerebellum and the floor of the rhombencephalon and diencephalon are strongly positive (Figs 4, 5A). The choroid plexus, and the mesencephalon are negative. High expression of the  $\beta$ B subunit was also observed around the lumen of the spinal cord (Fig. 5B). It is striking that only the rapidly dividing cells of the ependymal layer of the neural tube and the brain regions contain the  $\beta$ B message, while the mantle layer and marginal layer are negative.

Fig. 5C shows that the epithelial lining of the oesophagus is strongly positive for the  $\beta$ B subunit mRNA while the mesenchymal tissue is negative. This is in contrast to the distribu-





**Fig. 3.** Inhibin/activin  $\beta$ A subunit mRNA expression in sagittal (A,B,D,F,H) and frontal (C,E,G) sections through the thoracic and abdominal region (A-D) and through the shoulder/cervical region (E), limb (F) and gonad primordium (G,H) of a 12.5 day p.c. mouse embryo. Dark-field micrographs (left) and corresponding bright-field images (right). ao, aorta; bv, blood vessel; bw, body wall; cr, cervical region; ct, cushion tissue; ge, genital eminence; go, gonad primordium; h, heart; hi, hilus of the lung; id, intervertebral disc primordium; lb, limb bud; lu, lung; ma, metanephros; oe, oesophagus; pc, precartilaginous condensation; pm, pleuroperitoneal membrane; re, rectum; sc, spinal cord; sr, shoulder region; su, septum urogenitale; st, stomach; ta, truncus arteriosus; tp, truncus pulmonalis; tr, trachea; ve, ventricle.

tion pattern of  $\beta$ A transcripts. Another difference is that  $\beta$ B mRNA is expressed in mesenchymal condensations of the tarsus in the developing eye (Fig. 5H). Furthermore, very specific hybridization signals were present in the stomach, in particular in the epithelial layer lining the lumen of the glandular part of the stomach (Fig. 5E), and the epithelium of the pylorus (Figs 4A, 5F).

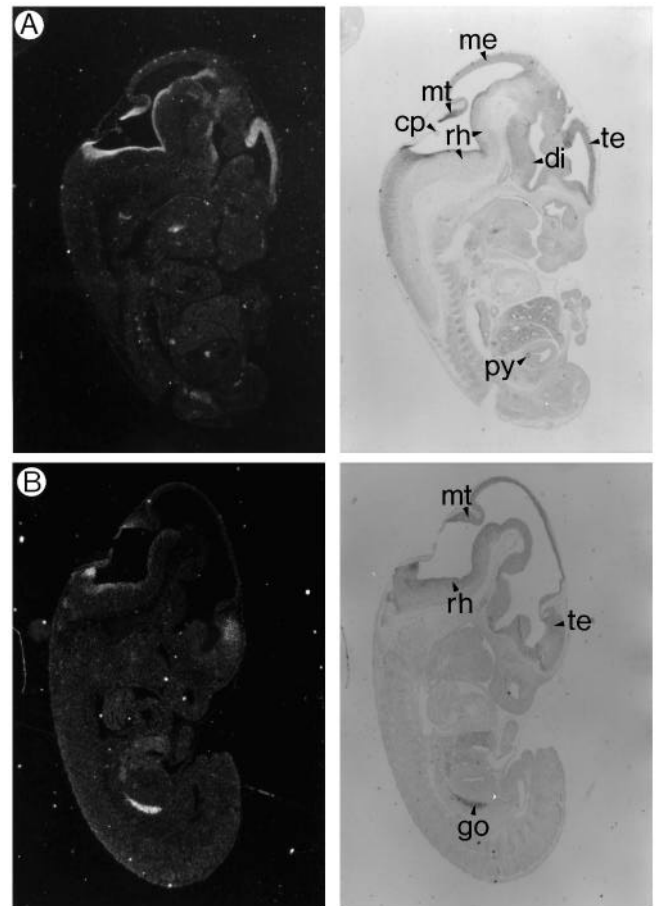
A common site of expression for the  $\beta$ B and  $\beta$ A subunits is the mesenchyme in the wall of large blood vessels in the heart region, including the truncus arteriosus (not shown). In addition, the pleuroperitoneal membrane is positive for both  $\beta$ A and  $\beta$ B mRNA (Fig. 5F), as well as the mesenchymal condensations in the flank region (not shown). In the intervertebral disc anlagen  $\beta$ A and  $\beta$ B (Fig. 5D) appeared to be expressed in the mantle part and nuclear part, respectively. A strong signal for  $\beta$ B was observed in the gonad primordium, whereas both the metanephros and mesonephros were devoid of any signal (Figs 4B, 5F,G).

#### Localization of a subunit mRNA

In contrast to the  $\beta$ A and  $\beta$ B subunit mRNAs the  $\alpha$  message was absent in the decidua and the embryo at early postimplantation stages and was found in only one developing organ in 10.5- and 12.5-day mouse embryos, namely the gonad primordium (Fig. 6). However, the cellular localization of this message could not be clearly determined. Comparison of the expression patterns of the three inhibin subunits in the developing gonads of male and female mice is under investigation.

#### Localization of follistatin mRNA

The expression pattern of follistatin showed little overlap with those of inhibin  $\beta$ A and  $\beta$ B subunits. In early postimplantation embryos follistatin is expressed both in the embryo and the decidua. Fig. 7C shows that, except for a narrow zone of decidual cells around the embryo, the whole decidua is strongly positive for follistatin mRNA. Striking sites of follistatin expression in the 7.5-day embryo are the primitive streak region and the parietal endoderm cells in the Reichert's membrane surrounding the embryo (Fig. 7C). Although similar results were obtained by Albano et al. (1994), the levels of follistatin expression in the present study are dramatically higher. Furthermore, as shown in Fig. 7A,B parietal endoderm of 6.0 and 6.5-day embryos contains high levels of follistatin transcripts. In these embryos also an asymmetric expression pattern in the egg cylinder was observed. In 8.5-day embryos a strong hybridization signal was still present in the parietal endoderm, and in somites, paraxial mesoderm and developing hindbrain, while the developing forebrain, spinal cord and

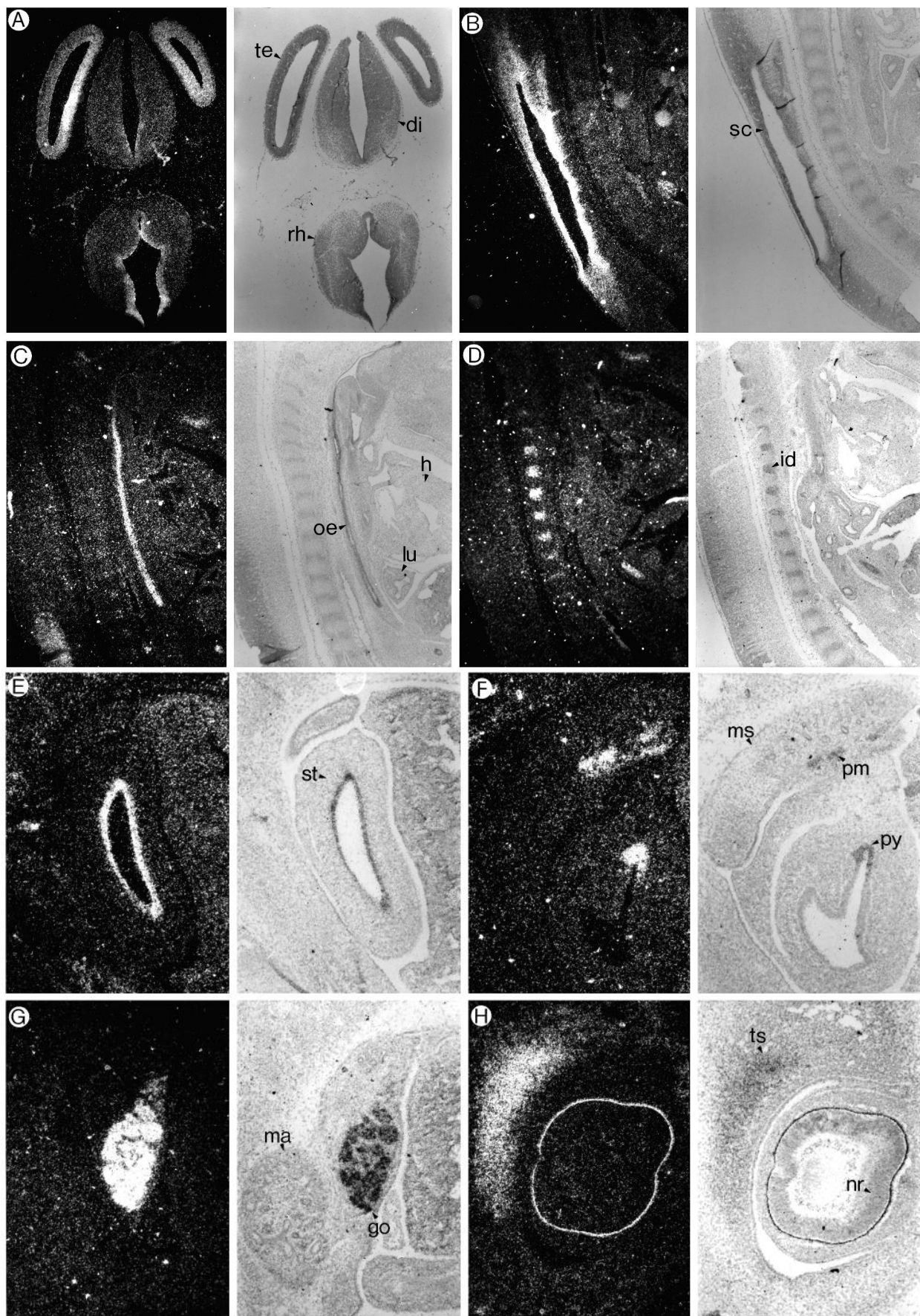


**Fig. 4.** Inhibin/activin  $\beta$ B subunit mRNA expression in sagittal sections of 12.5- (A) and 10.5- (B) day p.c. mouse embryos. Dark-field micrographs (left) and corresponding bright-field images (right). cp, choroid plexus; di, diencephalon; go, gonad primordium; me, mesencephalon; mt, metencephalon; py, pylorus; rh, rhombencephalon; te, telencephalon.

neural plate were negative (Fig. 7D-F). As shown in Fig. 7E follistatin was expressed in specific rhombomeres of the developing hindbrain, in particular rhombomeres 2, 4 and 6 (Albano et al., 1994). In 9.5-day embryos follistatin transcripts appear in the mesencephalon and diencephalon, while the telencephalon remains negative (not shown).

Fig. 8 shows that in 10.5- and 12.5-day embryos follistatin is expressed in mesoderm- and ectoderm-derived tissues. In the developing nervous system the most predominant hybridization signal is seen in regions where  $\beta$ B mRNA is absent or very weakly expressed, namely in the ependymal layer of the diencephalon (Figs 8, 9C) and the roof of the mesencephalon (Figs 8, 9A). It is striking that a small region in the developing midbrain is negative. A weak signal is also visible in the floor of the rhombencephalon (Figs 8, 9C), the roof of the metencephalon (Fig. 9A) and around the lumen of the spinal cord in the tail region (not shown) where  $\beta$ B is also expressed.

In the snout, follistatin mRNA is present in the roof of the nasal cavity, in particular in the mesenchyme surrounding the olfactory epithelium as well as in mesenchymal structures in the tongue (Fig. 9B). Although  $\beta$ A is also expressed in these tissues, the messages do not appear to be colocalized. Follis-





**Fig. 5.** Inhibin/activin  $\beta$ B subunit mRNA expression in the brain (A), spinal cord (B), oesophagus (C), intervertebral disc primordium (D), stomach (E,F), gonad primordium (G) and eye (H) of a 12.5-day mouse embryo. (B-H) sagittal sections and (A) transverse section. Dark-field micrographs (left) and corresponding bright-field images (right). di, diencephalon; go, gonad primordium; h, heart; id, intervertebral disc primordium; lu, lung; nr, neural retina; ma, metanephros; ms, mesonephros; oe, oesophagus; pm, pleuroperitoneal membrane; py, pylorus; rh, rhombencephalon; sc, spinal cord; st, stomach; te, telencephalon; ts, tarsus. Note: Serial sections of the brain (A) were also hybridized with the follistatin antisense probe (see Fig. 9C).

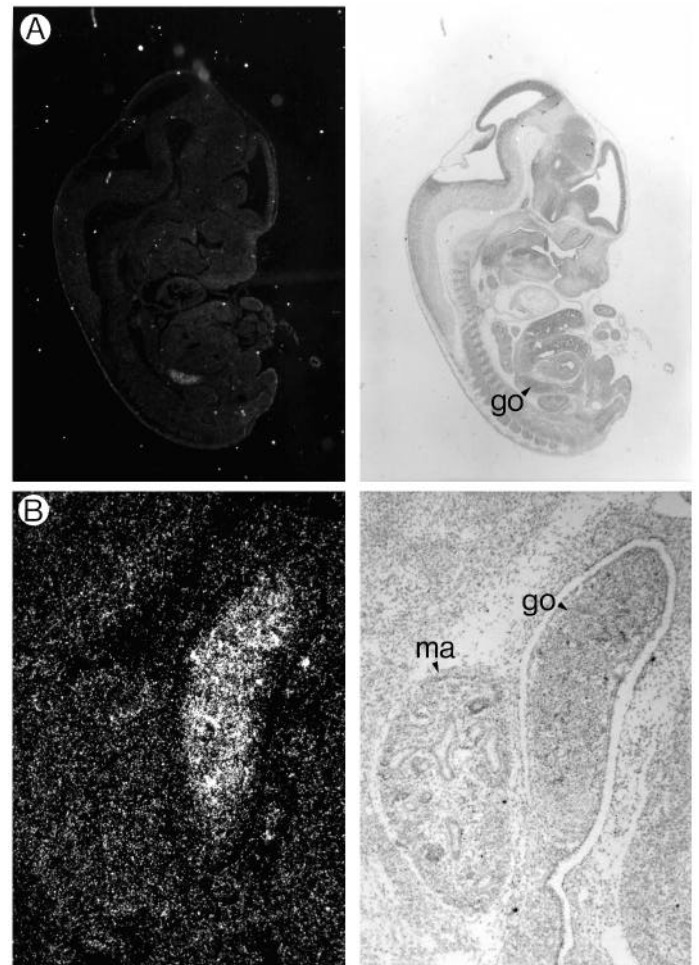
tatin transcripts appear to be restricted to the slowly growing regions of the developing conchae (Fig. 9B), whereas  $\beta$ A subunits are expressed in the actively dividing mesenchymal cells. Another difference with  $\beta$ A subunit expression is the presence of follistatin mRNA in the sensory epithelia of the cochlea and its absence in the surrounding mesenchyme (Fig. 9D). Furthermore, while  $\beta$ B is expressed in the stomach epithelium, follistatin is strongly expressed in the underlying mesenchymal layer, but not in the muscle tissue (Fig. 9E). In the body wall follistatin is expressed in the epidermis (Fig. 9B,H), while  $\beta$ A subunit mRNA is present in the underlying mesenchymal tissue. These results indicate that in certain epithelial/mesenchymal structures there is a reciprocal expression pattern for inhibin  $\beta$  subunit and follistatin mRNA.

Striking sites of follistatin expression are the sinoauricular septum of the heart (Fig. 9H), the mesenchyme of the intestine (Fig. 9F) and the fascia in the flanks of the embryo (Fig. 9E). Other specific expression sites are the mesenchymal condensations in the limbs (Fig. 9E) and in the developing pelvis (Fig. 9E), as well as the sclera of the developing eye (Fig. 9G) and the peripheral blood vessels (Fig. 8A). The intervertebral disc anlagen (Fig. 9F), the pleuroperitoneal membrane (Fig. 9F), the developing vibrissae (not shown) and the gonads (Fig. 9H) form common sites of follistatin,  $\beta$ A and/or  $\beta$ B subunit expression.

### Localization of activin receptors

The distribution of the type IIA and IIB receptors in postimplantation mouse embryos has been investigated in order to determine possible target cells for activins. In 6.0- to 8.5-day embryos, type IIB activin receptor was strongly expressed in embryonic and extraembryonic tissue, while no transcripts for the activin type IIA receptor could be observed. Type IIA receptor could be detected in the mouse embryo as early as 9.5 days p.c. (not shown). Both receptors were absent in the decidua. These results confirm the hypothesis of Blum et al. (1992) that activin receptors are present in early postimplantation embryos.

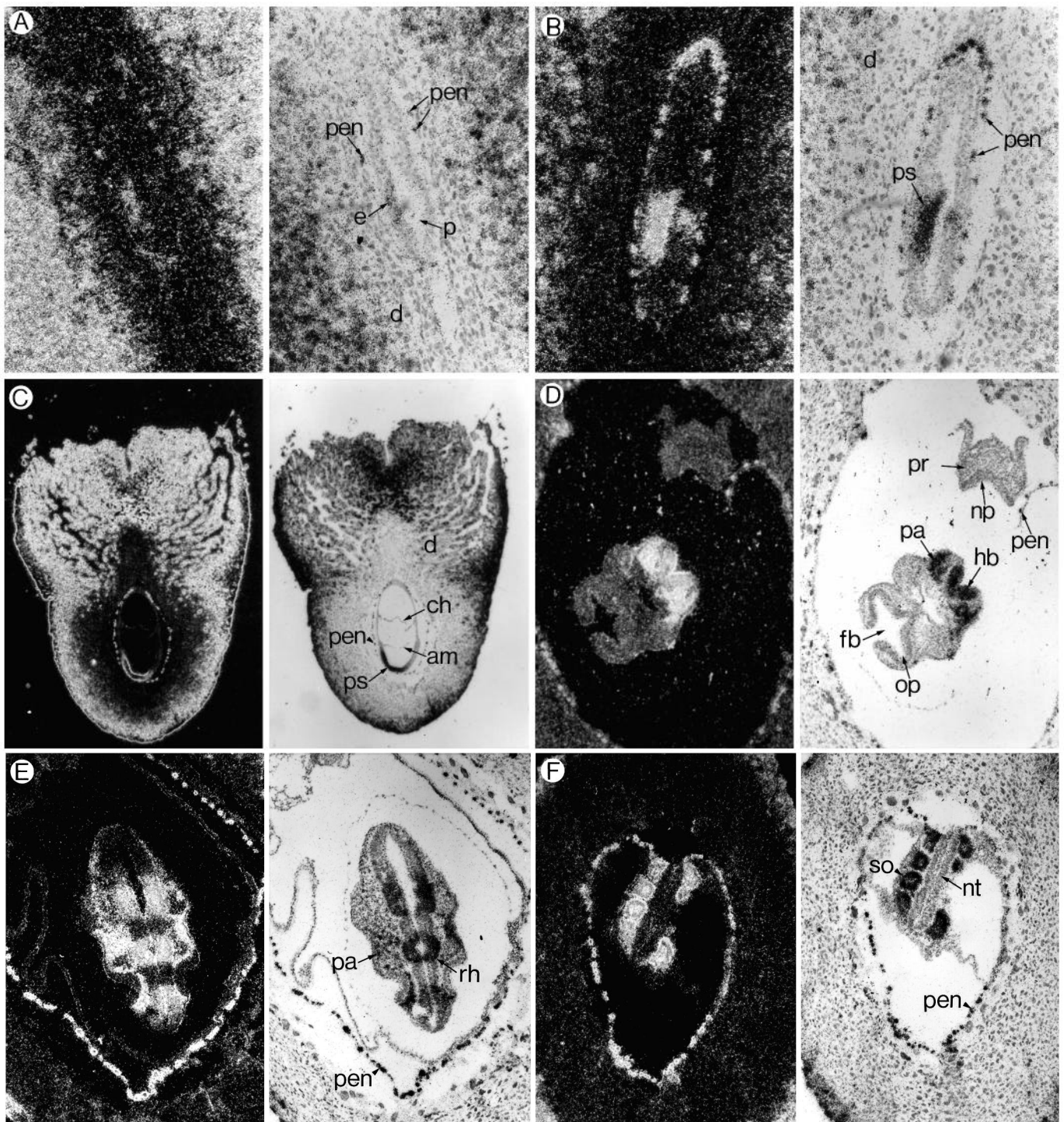
In 12.5-day mouse embryos there are many sites where activin receptor type IIA and IIB are coexpressed. As shown in Fig. 10 both receptors are predominantly present in the central nervous system, including the brain, ganglia and spinal cord. Furthermore, the mRNA for the type II receptors is colocalized in several mesenchymal and epithelial cell types outside the central nervous system. There are, however, a number of striking differences between the expression patterns of the two receptors. While activin receptor type IIA is selectively expressed in the mantle layer of the brain and spinal cord (Fig. 10B,C), activin receptor type IIB mRNA is also present



**Fig. 6.** Inhibin  $\alpha$  subunit mRNA expression in the gonad primordium of a 12.5-day p.c. mouse embryo. Dark-field micrographs (left) and corresponding bright-field images (right). go, gonad primordium; ma, metanephros.

in the ependymal layer (Fig. 10E-G). Furthermore, type IIB activin receptor is expressed in the telencephalon and diencephalon (Fig. 10E,F), while type IIA receptor is expressed only in the mantle layer of the corpus striatum and the diencephalon (Fig. 10B). Another difference is the expression pattern in the developing eye. A specific hybridization signal for the activin type IIB receptor was seen in the neural layer of the retina (Fig. 10H), where the type IIA receptor is absent. The latter receptor was present in the fascia of the developing ocular muscles (Fig. 10A). In addition, developing vibrissae only express type IIA (Fig. 10D), but not type IIB activin receptor. As shown in Fig. 10E,G the peripheral structures in the metanephros are positive for the type IIB receptor, while no signal was observed for type IIA. Furthermore, activin type IIB receptor transcripts were absent in some embryos and present in others in the developing gonads, while type IIA receptor expression was never observed in the gonad primordium (not shown). Possible sex-related differences in the activin type II receptor expression patterns cannot be excluded. The mesonephros was negative for both receptor types.

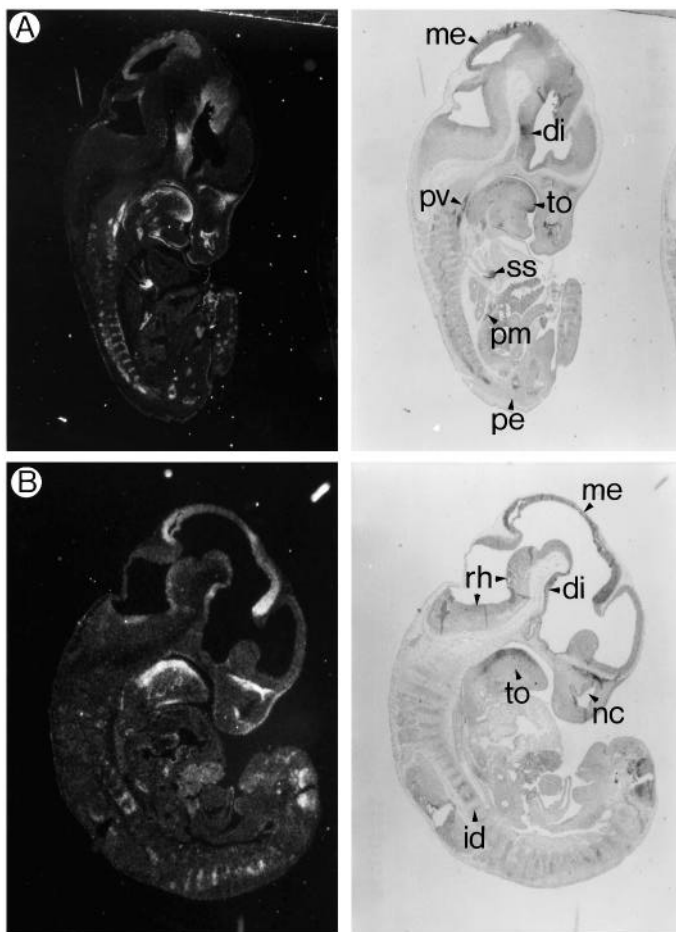
Colocalization of the receptor type IIA and IIB messages



**Fig. 7.** Expression of follistatin mRNA in 6.0-days p.c. (A), 6.5-days p.c. (B), 7.5-days p.c. (C) and 8.5-days p.c. (D-F) mouse embryos. (A-C) sagittal sections; (E) frontal section; (D,F) transverse sections. Dark-field micrographs (left) and corresponding bright-field images (right). am, amnion; ch, chorion; d, decidua; e, embryonic ectoderm; fb, developing forebrain; hb, developing hindbrain; np, neural plate; nt, neural tube; op, optic pit; p, proamniotic cavity; pa, paraxial mesoderm; pen, parietal endoderm; pr, presomitic mesoderm; ps, primitive streak; rh, rhombomere; so, somite. Note: The dark region in the center of the signal in the primitive streak region (C; dark-field micrograph) is the result of the high follistatin expression, thus blocking light transmission through the silver grains. In the bright-field image clear expression is visible.

was observed in the epidermis of the developing eye (Fig. 10D,H), the epithelium of the cochlea (Fig. 10A,F) and the

epithelium of the semicircular canals (Fig. 10F). The two receptors are also coexpressed in the mesenchyme and devel-



**Fig. 8.** Follistatin mRNA expression in sagittal sections of 12.5- (A) and 10.5- (B) day p.c. mouse embryos. Dark-field micrographs (left) and corresponding bright-field images (right). di, diencephalon; id, intervertebral disc primordium; me, mesencephalon; nc, nasal cavity; pe, pelvis; pm, pleuroperitoneal membrane; pv, peripheral blood vessels; rh, rhombencephalon; ss, sinoauricular septum; to, tongue.

opening muscles of the tongue (Fig. 10C). A weak signal for both receptors was seen in the epithelium and mesenchymal layer of the stomach (Fig. 10A,E).

Preliminary experiments have shown a striking colocalization of the type II and one of the activin type I (Act R-I/ALK-2) receptors in the mesenchymal condensations of the flank and back of the embryo, in particular in the limb and shoulder region (not shown). In the central nervous system the type I activin receptor appeared to be present in the floor of the mesencephalon and the floor of the rhombencephalon and diencephalon. Also mesenchymal condensations in the tail region and in the snout expressed this receptor. A more detailed inventory of the specific expression sites of different activin type I receptors during murine development is currently in progress.

## DISCUSSION

In this paper we have described the first comparative analysis of the localization of inhibin/activin  $\alpha$ -,  $\beta$ A- and  $\beta$ B-subunit,

follistatin and activin type II receptor mRNA in postimplantation mouse embryos. The data presented in this study suggest a role for activins in a variety of developmental processes during the period of organogenesis.

In early postimplantation embryos (6.0- to 9.5-days p.c.) activin  $\beta$  subunits are exclusively expressed in specific regions of the decidua, not in the embryo. Manova et al. (1992) have suggested that activin, produced by the decidua, might reach the embryo and function during mesoderm formation and gastrulation. However, because of the spectacular expression levels of follistatin in the decidua and also in the parietal endoderm surrounding the embryo (this study) it is very unlikely that the embryo is exposed to biologically active activin. We therefore suggest that at least the known forms of activin are not involved in early postimplantation development. However, recently it has been shown that different follistatin forms have different activin-neutralizing capacity, which might be due to differences in affinity for cell surface proteoglycans (Nakamura et al., 1991; Sugino et al., 1993). For this reason the exact protein form present in the embryo and decidua needs to be established. In addition, as at least type IIB activin receptors are present in the embryo, the occurrence of new members of the activin family cannot be excluded at these stages. Knowledge of the appearance of signalling type I activin receptors will also help to establish whether activins are functional in early postimplantation development.

A striking observation was the high level of follistatin expression in the primitive streak region of 6.5- and 7.5-day embryos. Although follistatin effects are always coupled to activin neutralization, it was shown recently that follistatin itself can act as a paracrine modulator upon thecal cells to directly stimulate the production of progesterone, independently of activin (Shukovski et al., 1993). These results suggest the presence of a signalling pathway for follistatin in certain cells. We are now studying the involvement of follistatin in mesoderm induction in the mouse embryo. In 8.5-day embryos follistatin is expressed in specific rhombomeres, paraxial mesoderm and somites, indicating a role for this protein in the development of the hindbrain, and in somite development.

In 10.5- and 12.5-day embryos  $\beta$ A subunit transcripts are expressed predominantly in mesenchymal structures, including those surrounding the epithelia of several internal organs. It is striking that other members of the TGF $\beta$  superfamily, in particular TGF $\beta$ 2 and TGF $\beta$ 3 are either expressed in the same region or in tissues adjacent to the site of  $\beta$ A expression in the mouse embryo. Like the  $\beta$ A subunit, TGF $\beta$ 3 is expressed in the tracheal mesenchyme and in the intervertebral disc anlagen (Schmid et al., 1991; Millan et al., 1991). A coordinated expression of  $\beta$ A and TGF $\beta$ 2 was found in the nasal and mandibular mesenchyme that forms the soft tissue components of the face (Schmid et al., 1991). In addition, Pelton et al. (1989) reported the presence of TGF $\beta$ 2 RNA in mesenchymal cells of the embryonic oesophagus where  $\beta$ A is also expressed. In several structures where TGF $\beta$ 2 is expressed in the epithelial component, including cochlear epithelium and olfactory epithelium, the  $\beta$ A subunit is present in the adjacent mesenchymal tissue (Schmid et al., 1991; Millan et al., 1991). These results together with the recent finding that TGF $\beta$  influences the expression of activin  $\beta$  subunits in mesodermal cell lines (van der Kruijssen et al., 1993) suggest that the activin  $\beta$

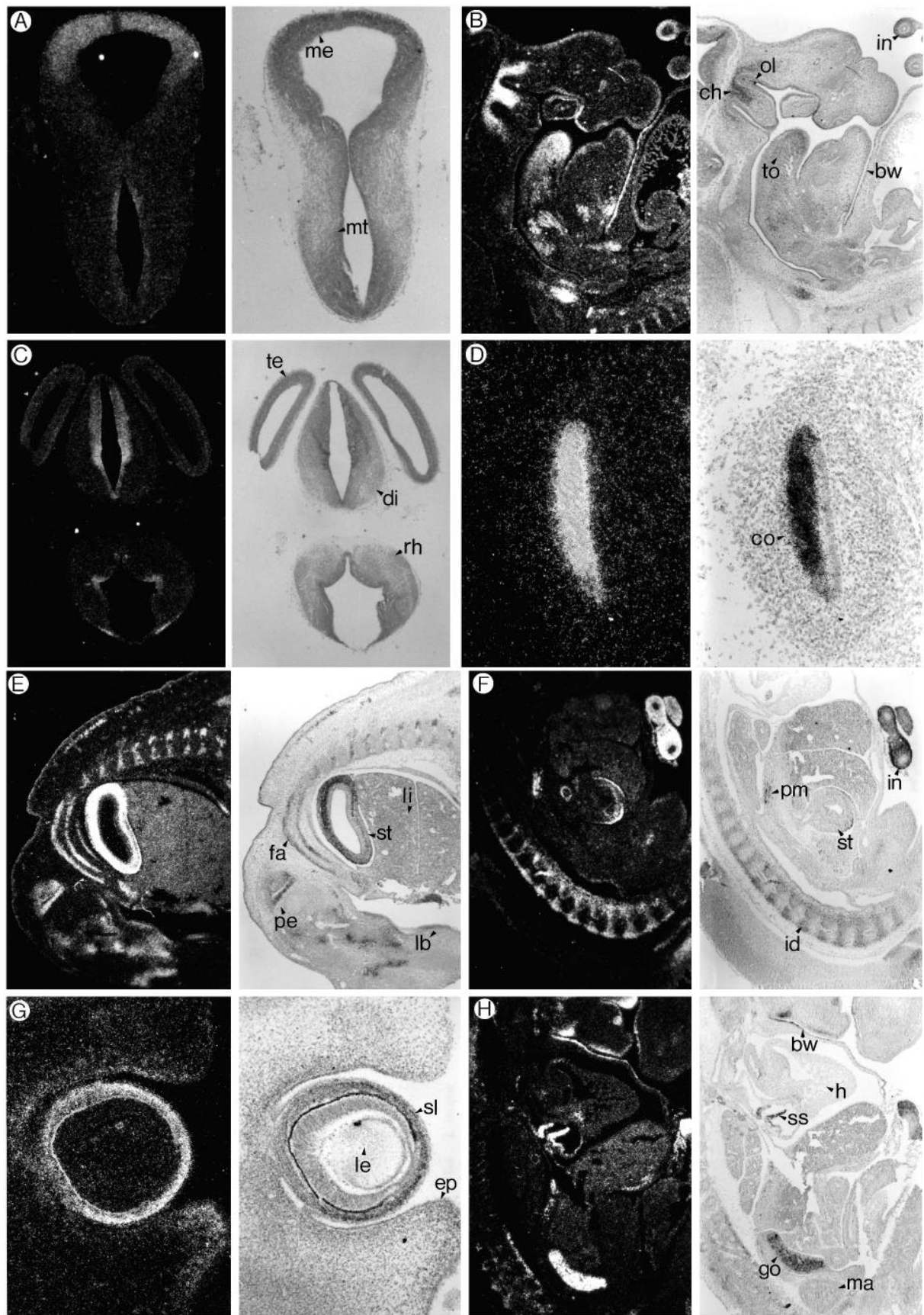


Fig. 9. For legend see p. 3634



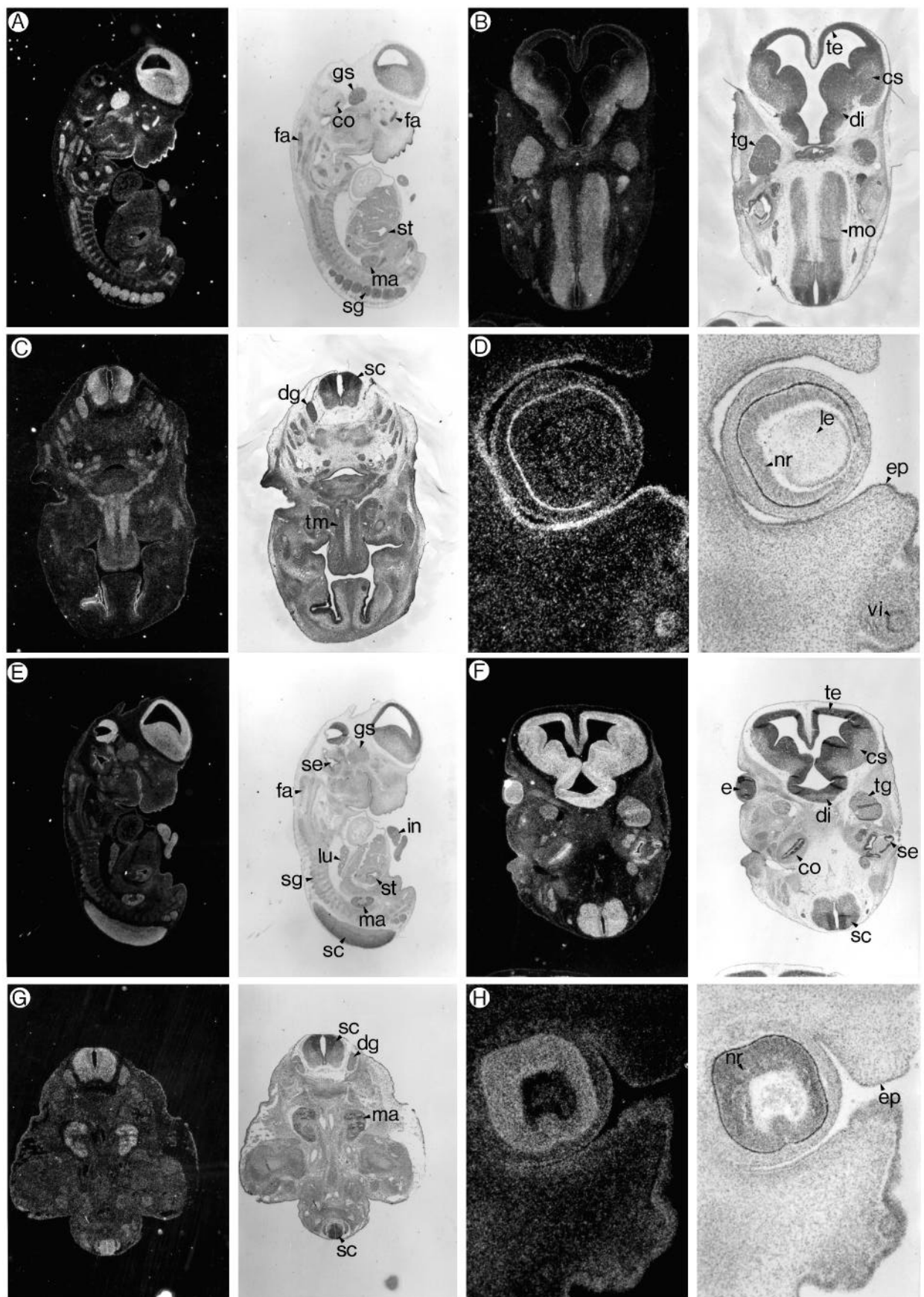


Fig. 10. For legend see p. 3634



**Fig. 9.** Follistatin mRNA expression in sagittal (B,D,E-H) and transverse (A,C) sections through the brain (A,C), snout (B), cochlea (D), abdominal region (E,F), eye (G) and thoracic region (H) of a 12.5-day p.c. mouse embryo. Dark-field micrographs (left) and corresponding bright-field images (right). bw, body wall; ch, developing conchae; co, cochlea; di, diencephalon; ep, epidermis; fa, fascia; go, gonad primordium; h, heart; id, intervertebral disc primordium; in, intestine; lb, limb bud; le, lens; li, liver; ma, metanephros; me, mesencephalon; mt, metencephalon; ol, olfactory epithelium; pe, pelvis; pm, pleuroperitoneal membrane; rh, rhombencephalon; sl, sclera; ss, sinoauricular septum; st, stomach; te, telencephalon; to, tongue.

**Fig. 10.** Activin receptor type IIA (A-D) and type IIB (E-H) mRNA expression in sagittal (A,D,E,H) and transverse (B,C,F,G) sections of a 12.5-day p.c. mouse embryo. Dark-field micrographs (left) and corresponding bright-field images (right). co, cochlea; cs, corpus striatum; dg, dorsal root ganglion; di, diencephalon; e, eye; ep, epidermis; fa, fascia; gs, ganglion statoacousticum; le, lens; lu, lung; ma, metanephros; mo, medulla oblongata; nr, neural retina; sc, spinal cord; se, semicircular canal; sg, spinal ganglion; st, stomach; te, telencephalon; tg, trigeminal ganglion; tm, muscle of the tongue; vi, vibrissa. Note: The sagittal sections are serial sections from the same embryo.

subunit expression levels may be regulated by TGF $\beta$ s in a tissue-specific way during murine development.

In contrast to the expression pattern of the  $\beta$ A subunit, high levels of  $\beta$ B transcripts were present in the central nervous system, in particular in the forebrain, hindbrain and the ependymal layer around the lumen of the spinal cord. The absence of  $\alpha$ - and  $\beta$ A subunit mRNA suggests that activin B, and not activin A/AB or inhibins, might regulate neuronal cell growth and/or differentiation. Although activin A has been shown to promote the survival of some types of nerve cells (Schubert et al., 1991) it is not yet known whether activin B has a similar effect. In the rat embryo,  $\beta$ B subunits are also expressed in the developing forebrain from 14 days p.c. (Roberts et al., 1991). At later developmental stages, however,  $\beta$ A subunit mRNA was also observed in the brain, in particular in the striatum, and later in the cerebral cortex. Whether  $\beta$ A subunits are also expressed at equivalent stages in the mouse embryo remains to be established.

Because of the coexpression or adjacent expression of  $\beta$ A and  $\beta$ B subunits in the oesophagus, the pleuroperitoneal membrane, the intervertebral disc anlagen, the mesenchyme in the wall of the blood vessels and the mesenchymal condensations in the flank, these are potential sites where all three forms of activin (A,B,AB) might be present. The developing gonads are the only site of both activin and inhibin expression with both  $\alpha$  and  $\beta$  subunits colocalized in these organs. A recent study by Matzuk et al. (1992c) has shown that expression of inhibin  $\alpha$  in the embryonic gonad is not essential for normal sexual differentiation and development. It was shown, however, that inhibin-deficient mice develop gonadal tumours, demonstrating that inhibin acts as a tumour-suppressor protein in the gonads.

The sites of expression of activin receptors type IIA and IIB mRNA generally coincide with or are adjacent to the sites of  $\beta$  subunit expression. Although the exact protein distribution of the  $\beta$ A and  $\beta$ B subunits will reveal which activin form is present in the developing organs and differentiating structures of the mouse embryo, one could suggest a role for activin A in the formation of the inner ear, tongue and lung and during

morphogenesis of craniofacial structures. Activin AB could have a function in limb and shoulder development. Activin B on the other hand may be involved in the development of the stomach and the central nervous system as well as in the development of the eyelids. Indeed, it has been shown recently by Vassalli et al. (1994) that activin  $\beta$ B subunit gene disruption leads to defects in eyelid outgrowth and closure during mouse development.

A striking difference in the expression pattern of the type IIA and IIB activin receptors is observed in the roof of the telencephalon, where receptor IIB is strongly expressed and receptor IIA is completely absent. Coexpression of the  $\beta$ B subunit in the forebrain strongly suggests that activin B might be involved in forebrain development, thereby exerting its function via the activin type IIB receptor. Developing whisker follicles only express type IIA, not type IIB receptor. Coexpression of the  $\beta$ A subunit mRNA in these structures suggests that activin A might play a role in their differentiation to mature follicles via the type IIA activin receptor. Some tissues, including spinal ganglia and metanephros (for type IIB) display a high hybridization signal of the type II activin receptor mRNA, whereas they exhibit undetectable levels of  $\beta$  subunit mRNA. This might indicate that yet unknown ligands exist for these receptors that are expressed in these regions. On the other hand, the absence of type II activin receptor mRNA in tissues expressing  $\beta$  subunits, suggests the existence of related receptors that mediate activin function. Comparison of the type II activin receptor expression patterns with that recently described for the type II TGF $\beta$  receptor during a similar period of development (Lawler et al., 1994) has revealed that there is no overlapping hybridization signal. As TGF $\beta$ 1 is the major ligand for the type II TGF $\beta$  receptor these results suggest that TGF $\beta$ 1 and activins have completely different functions during murine embryogenesis.

In addition to a type II activin receptor several type I receptors that are also putative transmembrane serine/threonine kinases, were shown to be essential for activin signalling (Attisano et al., 1993; Carcamo et al., 1994). Preliminary studies on the expression of one of the type I receptors (Act R-I/ALK-2) have indicated that there are only a few sites in common with those of the type II receptors. These include the floor of the mesencephalon, rhombencephalon and diencephalon in the brain and the mesenchymal condensations in the limb and shoulder. For this reason the expression patterns of other signalling type I receptors, including ALK-4, will be studied for possible colocalization with the type II activin receptors.

Although specific functions could be suggested for the different activin forms on the basis of the colocalization of activin subunits and type II activin receptors, there are several tissues in the developing mouse embryo in which the activin-neutralizing protein follistatin is colocalized or expressed in an adjacent cell layer. These tissues include various parts of the developing brain, mesenchymal structures in the snout, the cochlea, stomach, whisker follicles, and the gonads. Therefore, until the distribution of activin and follistatin proteins and their ratios is known it will be difficult to establish whether activins are involved in the maturation of some or all of these tissues or not. Furthermore, it is important to know which form of the follistatin protein is present. In addition to the sites of common expression with activins and their receptors, follistatin mRNA

was also detected at very specific sites, including the mesencephalon, intestine, fascia and the sinoauricular septum of the heart. This might indicate that other (unknown) activins are expressed at these sites, and that their activity is regulated by follistatin. Another possibility is that follistatin itself, independent of activins, might function as a regulator of tissue development.

In conclusion, the present results indicate that follistatin, and not the known activin forms (A,B,AB) are involved in early postimplantation development. However, based on their expression patterns, different types of activin might have different functions during the period of organogenesis. Further studies will be needed to determine the protein localization of activins and activin-binding proteins in the developing mouse embryo. In addition, the precise combination of the different activin receptors through which the different ligands might exert their function remains to be established.

We are grateful to Dr W. Vale for the activin receptor type IIA probe, to P. Lanser and E. Freund for excellent technical assistance, to Dr K.A. Lawson for helpful discussions, to Dr O. Destrée for confirmation of anatomical details and, together with Dr C. Mummery, for critical reading of the manuscript.

## REFERENCES

- Albano, R. M., Godsave, S. F., Huylebroeck, D., van Nimmen, K., Isaacs, H. V., Slack J. M. W. and Smith, J. C. (1990). A mesoderm-inducing factor produced by WEHI-3 murine myelomonocytic leukemia cells is activin A. *Development* **110**, 435-443.
- Albano, R. M., Groome, N. and Smith, J. C. (1993). Activins are expressed in preimplantation mouse embryos and in ES and EC cells and are regulated on their differentiation. *Development* **117**, 711-723.
- Albano, R. M., Arkell, R., Beddington, R. S. P. and Smith, J. C. (1994). Expression of inhibin subunits and follistatin during postimplantation mouse development: decidual expression of activin and expression of follistatin in primitive streak, somites and hindbrain. *Development* **120**, 803-813.
- Asashima, M., Nakano, H., Shimada, K., Kinoshita, K., Ishii, K., Shibai, H. and Ueno, N. (1990). Mesoderm induction in early amphibian embryos by activin A (erythroid differentiation factor). *Roux's Arch. Dev. Biol.* **198**, 330-335.
- Asashima, M., Nakano, H., Uchiyama, H., Sugino, H., Nakamura, T., Eto, Y., Ejima, D., Davids, M., Plessow, S., Cichocka, I. and Kinoshita, K. (1991). Follistatin inhibits the mesoderm-inducing activity of activin A and the vegetalizing factor from chicken embryo. *Roux's Arch. Dev. Biol.* **200**, 4-7.
- Attisano, L., Carcamo, J., Ventura, F., Weis, F. M. B., Massagué, J. and Wrana, J. L. (1993). Identification of human activin and TGF- $\beta$  type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* **75**, 671-680.
- Attisano, L., Wrana, J. L., Cheifetz, S. and Massagué, J. (1992). Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. *Cell* **68**, 97-108.
- Blum, M., Gaunt, S. J., Cho, K. W. Y., Steinbeisser, H., Blumberg, B., Bittner, D. and DeRobertis, E. M. (1992). Gastrulation in the mouse: the role of the homeobox gene goosecoid. *Cell* **69**, 1097-1106.
- Carcamo, J., Weis, F. M. B., Ventura, F., Wieser, R., Wrana, J., Attisano, L. and Massagué, J. (1994). Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor  $\beta$  and activin. *Mol. Cell. Biol.* **14**, 3810-3821.
- Centrella, M., McCarthy, T. L. and Canalis, E. (1991). Activin-A binding and biochemical effects in osteoblast-enriched cultures from fetal-rat parietal bone. *Mol. Cell. Biol.* **11**, 250-259.
- Childs, S. R., Wrana, J. L., Arora, K., Attisano, L., O'Connor, M. B., and Massagué, J. (1993). Identification of a Drosophila activin receptor. *Proc. Natl. Acad. Sci. USA* **90**, 9475-9479.
- Donaldson, C. J., Mathews, L. S. and Vale, W. W. (1992). Molecular cloning and binding properties of the human type II activin receptor. *Biochem. Biophys. Res. Commun.* **184**, 310-316.
- Ebner, R., Chen, R. H., Shum, L., Lawler, S., Zioncheck, T. F., Lee, A., Lopez, A. R. and Derynck, R. (1993a). Cloning of a type I TGF- $\beta$  receptor and its effect on TGF- $\beta$  binding to the type II receptor. *Science* **260**, 1344-1348.
- Ebner, R., Chen, R. H., Lawler, S., Zioncheck, T. and Derynck, R. (1993b). Determination of type I receptor specificity by the type II receptors for TGF- $\beta$  or activin. *Science* **262**, 900-902.
- Esch, F. S., Shimasaki, S., Cooksey, K., Mercado, M., Mason, A. J., Ying, S. Y., Ueno, N. and Ling, N. (1987). Complementary deoxyribonucleic acid (cDNA) cloning and DNA sequence analysis of rat ovarian inhibins. *Mol. Endocrinol.* **1**, 388-396.
- Fukui, A., Nakamura, T., Sugino, K., Takio, K., Uchiyama, H., Asashima, M. and Sugino, H. (1993). Isolation and characterization of *Xenopus* follistatin and activins. *Dev. Biol.* **159**, 131-139.
- Green, J. B. A. and Smith, J. C. (1990). Graded changes in dose of a *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. *Nature* **347**, 391-394.
- Hashimoto, M., Kondo, S., Sakurai, T., Etoh, Y., Shibai, H. and Muramatsu, M. (1990). Activin/EDF as an inhibitor of neural differentiation. *Biochem. Biophys. Res. Commun.* **173**, 193-200.
- Hashimoto, M., Shoda, A., Inoue, S., Yamada, R., Kondo, T., Sakurai, T., Ueno, N. and Muramatsu, M. (1992). Functional regulation of osteoblastic cells by the interaction of activin A with follistatin. *J. Biol. Chem.* **267**, 4999-5004.
- He, W. W., Gustafson, M. L., Hirobe, S. and Donahoe, P. K. (1993). Developmental expression of four novel serine/threonine kinase receptors homologous to the activin/transforming growth factor- $\beta$  type II receptor family. *Dev. Dynamics* **196**, 133-142.
- Hemmati-Brivanlou, A. and Melton, D. A. (1992). A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature* **359**, 609-614.
- Hemmati-Brivanlou, A. and Melton, D. A. (1994). Inhibition of activin receptor signaling promotes neuralization in *Xenopus*. *Cell* **77**, 273-281.
- Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77**, 283-295.
- Hino, M., Tojo, A., Miyazono, K., Miura, Y., Chiba, S., Eto, Y., Shibai, H. and Takaku, F. (1989). Characterization of cellular receptors for erythroid differentiation factor on murine erythroleukemia cells. *J. Biol. Chem.* **264**, 10309-10314.
- Inouye, S., Guo, Y., De Paolo, L., Shimonaka, M., Ling, N. and Shimasaki, S. (1991). Recombinant expression of human follistatin with 315 and 288 amino acids: chemical and biological comparison with native porcine follistatin. *Endocrinology* **129**, 815-822.
- Kogawa, K., Nakamura, T., Sugino, T., Takio, K., Titani, K. and Sugino, H. (1991). Activin-binding protein is present in pituitary. *Endocrinology* **128**, 1434-1440.
- Kondo, S., Hashimoto, M., Etoh, Y., Murata, M., Shibai, H. and Muramatsu, M. (1989). Identification of the two types of specific receptor for activin/EDF expressed on Friend leukemia and embryonal carcinoma cells. *Biochem. Biophys. Res. Commun.* **161**, 1267-1272.
- Kondo, M., Tashiro, K., Fuji, G., Asano, M., Miyoshi, R., Yamada, R., Muramatsu, M. and Shiokawa, K. (1991). Activin receptor mRNA is expressed early in *Xenopus* embryogenesis and the level of the expression affects the body axis formation. *Biochem. Biophys. Res. Commun.* **181**, 684-690.
- Lawler, S., Candia, A. F., Ebner, R., Shum, L., Lopez, A. R., Moses, H. L., Wright, C. V. E. and Derynck, R. (1994). The murine type II TGF- $\beta$  receptor has a coincident embryonic expression and binding preference for TGF- $\beta$ 1. *Development* **120**, 165-175.
- Legerski, R., Zhou, X., Dresback, J., Eberspaecher, H., McKinney, S., Segarini, P. and De Crombrughe, B. (1992). Molecular cloning and characterization of a novel rat activin receptor. *Biochem. Biophys. Res. Commun.* **183**, 672-679.
- Ling, N., Ueno, N., Ying, S., Esch, F., Shimasaki, S., Hotta, M., Cuevas, P. and Guillemain, R. (1988). Inhibins and activins. *Vitamins and hormones* **44**, 1-46.
- Lu, R. Z., Matsuyama, S., Nishihara, M. and Takahashi, M. (1993). Developmental expression of activin/inhibin  $\beta$ A,  $\beta$ B and  $\alpha$  subunits and activin receptor IIB genes in preimplantation mouse embryos. *Biol. Reprod.* **49**, 1163-1169.
- Manova, K., Paynton, B. V. and Bachvarova, R. F. (1992). Expression of

- activins and TGF $\beta$ 1 and  $\beta$ 2 RNAs in early postimplantation mouse embryos and uterine decidua. *Mech. Dev.* **36**, 141-152.
- Mason, J., Berkemeier, L. M., Schmelzer, C. H. and Schwall, R. H. (1989). Activin B: Precursor sequences, genomic structure and in vitro activities. *Mol. Endocrinol.* **3**, 1352-1358.
- Mathews, L. S., Hairiri, A. and Vale, W. (1991). Characterization of activin A receptors by chemical cross-linking. *Proc. Endocrinol. Soc.* **73**, 1516.
- Mathews, L. S. and Vale, W. W. (1991). Expression cloning of an activin receptor, a predicted transmembrane serine kinase. *Cell* **65**, 973-982.
- Mathews, L. S., Vale, W. W. and Kintner, C. R. (1992). Cloning of a second type of activin receptor and functional characterization in *Xenopus* embryos. *Science* **255**, 1702-1705.
- Matzuk, M. M. and Bradley, A. (1992a). Cloning of the human activin receptor cDNA reveals high evolutionary conservation. *Biochem. Biophys. Acta* **1130**, 105-108.
- Matzuk, M. M. and Bradley, A. (1992b). Structure of the mouse activin receptor type II gene. *Biochem. Biophys. Res. Commun.* **185**, 404-413.
- Matzuk, M. M., Finegold, M. J., Su, J. G. J., Hsueh, A. J. W. and Bradley, A. (1992c).  $\alpha$ -Inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature* **360**, 313-319.
- Michel, U., Farnworth, P. and Findlay, J. K. (1993). Follistatins: more than follicle-stimulating hormone suppressing proteins. *Mol. Cell. Endocrinol.* **91**, 1-11.
- Millan, F. A., Denhez, F., Kondaiah, P. and Akhurst, R. J. (1991). Embryonic gene expression patterns of TGF $\beta$ 1,  $\beta$ 2 and  $\beta$ 3 suggest different developmental functions in vivo. *Development* **111**, 131-144.
- Mitrani, E. and Shimoni, Y. (1990). Induction by soluble factors of organized axial structures in chick epiblasts. *Science* **247**, 1092-1094.
- Mitrani, E., Ziv, T., Thomsen, G., Shimoni, Y., Melton, D. and Bril, A. (1990). Activin can induce the formation of axial structures and is expressed in the hypoblast of the chick. *Cell* **63**, 495-501.
- Moriya, N. and Asashima, M. (1992). Mesoderm and neural inductions on newt ectoderm by activin A. *Dev. Growth Differ.* **34**, 589-594.
- Mummery, C. L., Feijen, A., van der Saag, P. T., van den Brink, C. E. and de Laat, S. W. (1985). Clonal variants of differentiated P19 EC cells exhibit EGF receptor kinase activity. *Dev. Biol.* **109**, 402-410.
- Mummery, C. L., Feijen, A., van den Brink, C. E., Moolenaar, W. H. and de Laat, S. W. (1986). Establishment of a differentiated mesodermal line from P19 EC cells expressing functional PDGF and EGF receptors. *Exp. Cell Res.* **165**, 229-242.
- Mummery, C. L., Feijen, A., Freund, E. and Shen, S. (1990a). Characteristics of embryonic stem cell differentiation: A comparison with two embryonal carcinoma cell lines. *Cell Differ. Dev.* **30**, 195-206.
- Mummery, C. L., Slager, H., Kruijer, W., Feijen, A., Freund, E., Koornneef, I. and van den Eijnden-van Raaij, A. J. M. (1990b). Expression of transforming growth factor  $\beta$ 2 during the differentiation of murine embryonal carcinoma and embryonic stem cells. *Dev. Biol.* **137**, 161-170.
- Mummery, C. L. and van den Eijnden-van Raaij, A. J. M. (1993). Type  $\beta$  transforming growth factors and activins in differentiating embryonal carcinoma cells, embryonic stem cells and early embryonic development. *Int. J. Dev. Biol.* **37**, 169-182.
- Nakamura, T., Asashima, M., Eto, Y., Takio, K., Uchiyama, H., Moriya, N., Ariizumi, T., Yashiro, T., Sugino, K., Titani, K. and Sugino, H. (1992a). Isolation and characterization of native activin B. *J. Biol. Chem.* **267**, 16385-16389.
- Nakamura, T., Sugino, K., Kurosawa, N., Sawai, M., Takio, K., Eto, Y., Iwashita, S., Muramatsu, M., Titani, K. and Sugino, H. (1992b). Isolation and characterization of activin receptor from mouse embryonal carcinoma cells. *J. Biol. Chem.* **267**, 18924-18928.
- Nakamura, T., Sugino, K., Titani, K. and Sugino, H. (1991). Follistatin, an activin-binding protein associated with heparan sulfate chains of proteoglycans on follicular granulosa cells. *J. Biol. Chem.* **266**, 19432-19437.
- Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K. and Sugino, H. (1990). Activin-binding protein from rat ovary is follistatin. *Science* **247**, 836-838.
- Nishimatsu, S., Oda, S., Murakami, K. and Ueno, N. (1992). Multiple genes for *Xenopus* activin receptor expressed during early embryogenesis. *FEBS Lett.* **303**, 81-84.
- Paulusma, C. C., van der Kruijsen, C. M. M. and van den Eijnden-van Raaij, A. J. M. (1994). Localization of activin subunits in early murine development determined by subunit-specific antibodies. *J. Immunol. Meth.* **169**, 143-152.
- Pelton, R. W., Nomura, S., Moses, H. L. and Hogan, B. L. M. (1989). Expression of transforming growth factor  $\beta$ -2 RNA during murine embryogenesis. *Development* **106**, 759-767.
- Roberts, V. J., Sawchenko, P. E. and Vale, W. (1991). Expression of inhibin/activin subunit messenger ribonucleic acids during rat embryogenesis. *Endocrinology* **128**, 3122-3129.
- Roberts, V. J. and Barth, S. L. (1994). Expression of messenger ribonucleic acids encoding the inhibin/activin system during mid- and late-gestation rat embryogenesis. *Endocrinology* **134**, 914-923.
- Schmid, P., Cox, D., Bilbe, G., Maier, R. and McMaster, G. K. (1991). Differential expression of TGF $\beta$ 1,  $\beta$ 2 and  $\beta$ 3 genes during mouse embryogenesis. *Development* **111**, 117-130.
- Schubert, D. and Kimura, H. (1991). Substratum-growth factor collaborations are required for the mitogenic activities of activin and FGF on embryonal carcinoma cells. *J. Cell. Biol.* **144**, 841-846.
- Schubert, D., Kimura, H., Lacorbiere, M., Vaughan, J., Karr, D. and Fischer, W. H. (1990). Activin is a nerve cell survival molecule. *Nature* **344**, 868-870.
- Schulte-merker, S., Ho, R. K., Herrmann, B. G., and Nüsslein-Volhard, C. (1992). The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116**, 1021-1032.
- Shimasaki, S., Koga, M., Esch, F., Cooksey, K., Mercado, M., Koba, A., Ueno, N., Ying, S. Y., Ling, N. and Guillemin, R. (1988a). Primary structure of the human follistatin precursor and its genomic organization. *Proc. Natl. Acad. Sci. USA* **85**, 4218-4222.
- Shimasaki, S., Koga, M., Esch, F., Mercado, M., Cooksey, K., Koba, A. and Ling, N. (1988b). Porcine follistatin gene structure supports two forms of mature follistatin produced by alternative splicing. *Biochem. Biophys. Res. Commun.* **152**, 717-723.
- Shimonaka, M., Inouye, S., Shimasaki, S. and Ling, N. (1991). Follistatin binds to both activin and inhibin through the common beta-subunit. *Endocrinology* **128**, 3313-3315.
- Shinozaki, H., Ito, I., Hasegawa, Y., Nakamura, K., Igarashi, S., Nakamura, M., Miyamoto, K., Eto, Y., Ibuki, Y., and Minegishi, T. (1992). Cloning and sequencing of a rat type II activin receptor. *FEBS Lett.* **312**, 53-56.
- Shiozaki, M., Sakai, R., Tabuchi, M., Nakamura, T., Sugino, K., Sugino, H. and Eto, Y. (1992). Evidence for the participation of endogenous activin A/erythroid differentiation factor in the regulation of erythropoiesis. *Proc. Natl. Acad. Sci. USA* **89**, 1553-1556.
- Shukovski, L., Dyson, M. and Findlay, J. (1993). The effects of follistatin, activin and inhibin on steroidogenesis by bovine thecal cells. *Mol. Cell. Endocrinol.* **97**, 19-27.
- Smith, J. C., Price, B. M. J. van Nimmen, K. and Huylebroeck, D. (1990). Identification of a potent *Xenopus* mesoderm-inducing factor as a homologue of activin A. *Nature* **345**, 729-731.
- Sugino, K., Kurosawa, N., Nakamura, T., Takio, K., Shimasaki, S., Ling, N., Titani, K. and Sugino, H. (1993). Molecular heterogeneity of follistatin, an activin-binding protein. *J. Biol. Chem.* **268**, 15579-15587.
- ten Dijke, P., Ichijo, H., Franzen, P., Schulz, P., Saras, J., Toyoshima, H., Heldin, C. H. and Miyazono, K. (1993). Activin receptor-like kinases; a novel subclass of cell surface receptors with predicted serine/threonine kinase activity. *Oncogene* **8**, 2879-2887.
- ten Dijke, P., Yamashita, H., Ichijo, H., Franzen, P., Laiho, M., Miyazono, K. and Heldin, C. H. (1994). Characterization of type I receptors for transforming growth factor- $\beta$  and activin. *Science* **264**, 101-104.
- Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D. A. (1990). Activins are expressed early in *Xenopus* embryogenesis and can induce axial mesoderm and anterior structures. *Cell* **63**, 485-493.
- Tsuchida, K., Mathews, L. S. and Vale, W. W. (1993). Cloning and characterization of a transmembrane serine kinase that acts as an activin type I receptor. *Proc. Natl. Acad. Sci. USA* **90**, 11242-11246.
- Ueno, N., Ling, N., Ying, S. Y., Esch, F., Shimasaki, S. and Guillemin, R. (1987). Isolation and partial characterization of follistatin: a single chain Mr 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone. *Proc. Natl. Acad. Sci. USA* **84**, 8282-8286.
- Ueno, N., Nishimatsu, S. and Murakami, K. (1990). Activin as a cell differentiation factor. *Progr. Growth factor Res.* **2**, 113-124.
- Vale, W., Hsueh, A., Rivier, C. and Yu, J. (1990). The inhibin/activin family of hormones and growth factors. In *Peptide Growth Factors and Their Receptors II* (Eds. M. B. Sporn and A. B. Roberts), pp. 211-248. Berlin: Springer.

- van den Eijnden-van Raaij, A. J. M., van Achterberg, T. A. E., van der Kruijssen, C. M. M., Piersma, A. H., Huylebroeck, D., De Laat, S. W. and Mummery, C. L. (1991). Differentiation of aggregated murine P19 embryonal carcinoma cells is induced by a novel visceral endoderm-specific FGF-like factor and inhibited by activin A. *Mech. Dev.* **33**, 157-166.
- van den Eijnden-van Raaij, A. J. M., Feijen, A., Lawson, K. A. and Mummery, C. L. (1992). Differential expression of inhibin subunits and follistatin, but not of activin receptor type II, during early murine embryonic development. *Dev. Biol.* **154**, 356-365.
- van den Eijnden-van Raaij, A. J. M., van Zoelen, E. J. J., van Nimmen, K., Koster, C. H., Snoek, G. T., Durston, A. J. and Huylebroeck, D. (1990). Activin-like factor from a *Xenopus laevis* cell line responsible for mesoderm induction. *Nature* **345**, 732-734.
- van der Kruijssen, C. M. M., Feijen, A., Huylebroeck, D. and van den Eijnden-van Raaij, A. J. M. (1993). Modulation of activin expression by type  $\beta$  transforming growth factors. *Exp. Cell Res.* **207**, 407-412.
- Vassalli, A., Matzuk, M. M., Gardner, H. A. R., Lee, K. F. and Jaenisch, R. (1994). Activin/inhibin  $\beta$ B subunit gene disruption leads to defects in eyelid development and female reproduction. *Genes Dev.* **8**, 414-427.
- Wilkinson, D. G. and Green, J. (1990). In situ hybridization and the three-dimensional reconstruction of serial sections. In *Postimplantation Mammalian Embryos. A Practical Approach* (ed. A. J. Copp and D. L. Cockcroft) pp. 155-171. Oxford University Press.
- Wrana, J. L., Tran, H., Attisano, L., Arora, K., Childs, S. R., Massague, J. and O'Connor, M. B. (1994). Two distinct transmembrane serine/threonine kinases from *Drosophila melanogaster* form an activin receptor complex. *Mol. Cell. Biol.* **14**, 944-950.
- Xiao, S. and Findlay, J. K. (1991). Interactions between activin and follicle-stimulating hormone-suppressing protein and their mechanisms of action on cultured rat granulosa cells. *Mol. Cell. Endocrinol.* **79**, 99-107.
- Ying, S. (1988). Inhibins, activins and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocr. Rev.* **9**, 267-293.
- Ying, S. Y., Becker, A., Swanson, G., Tan, P., Ling, W., Esch, F., Ueno, N., Shimasaki, S. and Guillemin, R. (1987). Follistatin specifically inhibits pituitary follicle stimulating hormone release in vitro. *Biochem. Biophys. Res. Commun.* **149**, 133-139.

(Accepted 22 August 1994)