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Activins are expressed in preimplantation mouse embryos and in ES and EC cells and are regulated on their differentiation

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SUMMARY

Members of the activin family have been suggested to act as mesoderm-inducing factors during early amphibian development. Little is known, however, about mesoderm formation in the mammalian embryo, and as one approach to investigating this we have studied activin expression during early mouse development.

Activins are homo- or heterodimers of the β_A or β_B subunits of inhibin, itself a heterodimer consisting of one of the β subunits together with an α subunit. Our results indicate that the oocyte contains mRNA encoding all three subunits, and antibody staining demonstrates the presence of both α and β protein chains. From the fertilized egg stage onwards, α subunit protein cannot be detected, so the presence of β subunits reflects the presence of activin rather than inhibin.

Maternal levels of activin protein decline during early cleavage stages but increase, presumably due to zygotic transcription (see below), in the compacted morula. By 3.5 days, only the inner cell mass (ICM) cells of the blastocyst express activin, but at 4.5 days the situation is reversed; activin expression is confined to the trophectoderm. Using reverse transcription-PCR, neither β_A nor β_B mRNA was detectable at the two-cell stage but transcripts encoding both subunits were detectable at the morula stage, with β_B mRNA persisting into the blastocyst.

We have also analyzed activin and inhibin expression in ES and EC cells. Consistent with the observation that activins are expressed in the ICM of 3.5-day blastocysts, we find high levels of β_A and β_B mRNA in all eight ES cell lines tested. F9 EC cells express only activin β_B, together with low levels of the inhibin α chain. When ES and EC cells are induced to differentiate, levels of activin fall dramatically. These results are consistent with a role for activins in mesoderm formation and other steps of early mouse development.

Key words: mouse, preimplantation embryos, ES cells, activin, inhibin.

INTRODUCTION

This paper considers the expression patterns of the activins in early mouse development. The activins are homo- or heterodimers of the β_A or β_B subunits of inhibin, itself a heterodimer consisting of one of the β subunits together with an α subunit (Ling et al., 1988). The inhibins were first isolated from mammalian follicular fluid through their ability to inhibit the release of follicle-stimulating hormone (FSH) from rat anterior pituitary cells; the activins, isolated from the same source, have the opposite effect and thus stimulate FSH release (Ling et al., 1986; Vale et al., 1986). The inhibin subunits are members of the TGF β family (Mason et al., 1985; Mayo et al., 1986; Esch et al., 1987) and are expressed in the gonads (Ling et al., 1988), in various adult tissues (Meunier et al., 1988) and during rat organogenesis (Roberts et al., 1991). Among the known functions of activin are the ability to induce the differentiation of erythroleukemia cells (Murata et al., 1988), to stimulate meiotic maturation of rat oocytes in vitro (Itoh et al., 1990) and to promote the survival of P19 cells induced to differentiate by retinoic acid (Schubert et al., 1990).

Recently, the activins have attracted particular attention through their ability 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). Mesoderm induction in amphibia occurs during blastula stages when signals from the vegetal hemisphere of the embryo cause overlying equatorial cells to form mesodermal rather than ectodermal cell types (Nieuwkoop, 1969; reviewed by Smith, 1989). As well as the activins, members of the fibroblast growth factor (FGF) family (Kimelman and Kirschner, 1987; Slack et al., 1987) have been implicated in mesoderm induction, although the actions of the two families differ. Activins tend to induce dorsal cell types from responding animal pole

tissue, while FGF induces predominantly posterior and ventral cell types (Green et al., 1990). In support of this role for FGF, if the action of the *Xenopus* FGF receptor is abolished, by over-expression of RNA encoding a mutated form, the embryos that develop lack tail structures (Amaya et al., 1991).

It is not known which members of the activin and FGF families are endogenous inducing factors. Jones and Woodland (1987) have shown that mesoderm induction begins around the 64-cell stage, well before the onset of zygotic transcription (Newport and Kirschner, 1982) and of gastrulation, and this indicates that mesoderm-inducing factors are maternal in origin. Maternal RNA and protein for basic FGF have been shown to be present (Kimelman et al., 1988; Slack and Isaacs, 1989; Shiurba et al., 1991), but activin RNA cannot be detected until the late blastula stage (Thomsen et al., 1990). Asashima et al. (1991) have, however, shown that activin-like proteins are present in the *Xenopus* egg and early embryo, and one possibility is that these are derived from the follicle cells during oogenesis (G.-D. Guex and J. C. Smith, unpublished data).

Little is known about activin expression or mesoderm induction in higher vertebrates. In the chick embryo, mRNAs coding both for FGF and for inhibin β subunits have been detected (Mitrani et al., 1990a,b), and both FGF and activin elicit morphological responses from cells of the epiblast (Cooke and Wong, 1991), but embryological manipulations have not demonstrated an interaction analogous to mesoderm induction in *Xenopus*. The mouse embryo is poorly suited to experimental manipulation, but one major advance in the understanding of mesoderm formation in mammals was the recent cloning of *Brachyury* (Herrmann et al., 1990). *Brachyury* is expressed by presumptive mesodermal cells as they pass through the primitive streak (Wilkinson et al., 1990) and, in the absence of the gene product, mesoderm formation is disrupted. The *Xenopus* homologue of *Brachyury* is expressed in an analogous fashion and its expression is induced in presumptive ectoderm by FGF and activin (Smith et al., 1991). This raises the possibility that these factors activate the gene in the mouse embryo as well. Consistent with this suggestion, ectopic expression of the mouse homologue of *goosecoid*, a gene expressed in the dorsal blastopore lip of *Xenopus*, can be induced by treatment of early mouse embryos with activin (Blum et al., 1992).

If mesoderm formation in the mouse resembles that in *Xenopus*, mesoderm induction would also occur well before gastrulation, and the timing of primitive streak formation and of mesoderm-specific gene expression would depend on an endogenous 'clock' rather than on the time of receipt of the inducing signal (Gurdon et al., 1985; Symes and Smith, 1987; Cooke and Smith, 1990). In this paper, therefore, we study inhibin subunit expression during early preimplantation mouse development. We first describe the cloning of the mouse α, β_A and β_B subunits of inhibin; as described above, activins and inhibins have opposite activities in some assays, so it is necessary to analyze the expression of all three subunits to know which of the dimers might be present. We go on to describe the expression patterns of these subunits during mouse development. mRNAs encoding the α, β_A and β_B chains are expressed in the oocyte, and immunocytochemical analysis shows that α and β protein subunits are also present. Zygotic transcription of β, but not α, subunit mRNA is detectable at morula and blastocyst stages, and the use of specific antibodies shows that β, but not α, subunit protein is present in all cells of pre-implantation embryos from fertilized eggs to compacted morulae. In early blastocysts this activin protein begins to disappear from trophectoderm cells, becoming restricted to the inner cell mass (ICM) by the expanded blastocyst stage. At 4.5 days, however, expression disappears from the ICM and reappears in the trophectoderm. In accord with these observations, activin expression in ES and EC cell lines is also regulated on their differentiation. These results are consistent with a role for activin in mesoderm formation and perhaps in other steps of early mouse development.

MATERIALS AND METHODS

Cells

All cells were grown in a humidified 37˚C incubator in an atmosphere of 5% CO_2. F9 stem cells were cultured with H-21/Pyr media (NIMR Biological Services) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Differentiation into parietal and visceral endoderm was according to Hogan et al. (1986). Differentiation times were 5 days for parietal endoderm and 11 days for visceral endoderm. When desired, cells were harvested and the RNA was extracted with guanidinium isothiocyanate (Chomczynski and Sacchi, 1987).

Embryonic stem (ES) cells were grown on mitomycin-treated STO fibroblasts on gelatin-coated plates as described by Robertson (1987). The medium used was Dulbecco's minimal essential medium (DMEM) supplemented with 20% FCS, antibiotics and 2-mercaptoethanol. The medium was changed daily and cells were passaged every three days. Before RNA extraction the cells were passaged once without STO cells to minimize contamination by feeder layer cells. One of the cell lines (TG2A) was grown without feeders in buffalo rat liver cell conditioned medium as a source of leukaemia inhibitory factor (LIF). RNA samples from the various ES cell lines were a gift from Dr Robin Lovell-Badge (NIMR, London). Embryoid bodies were made according to the method of Robertson (1987). After 2 and 5 days the cells were harvested by centrifugation and RNA was extracted as above. STO cells were grown and mitomycin-treated as described by Robertson (1987).

Mouse embryos

Mouse embryos were obtained from matings of the outbred strain MF1 or from crosses between F1 CBA × C57/Bl10 females and MF1 or F1 CBA × C57/Bl10 studs. Noon on the day of appearance of the vaginal plug is taken as 0.5 days of development. Superovulation was induced by intraperitoneal injection of 5 IU of FSH followed by injection of 5 IU of hCG 44 to 48 hours later. Embryos were recovered by flushing oviducts or uteri according to the stage required. For some experiments embryos were recovered and cultured in drops of M16 medium (Hogan et al., 1986) under paraffin oil in a CO_2 incubator.

Immunofluorescence of cells and embryos

The anti-β subunit antibody used in this study is a mouse monoclonal antibody prepared against amino acids 82-114 of the carboxy terminus of the human mature β_A subunit. All amino acids in this region are identical to the mouse sequence of the β_A sub-

unit shown in Fig. 2. This reacts with both inhibin and activin through binding to the β subunit (Groome and Lawrence, 1991). Recent results have shown that a β_B synthetic peptide representing the region homologous to the 82-114 peptide of the β_A subunit is approximately 20% as effective at inhibiting the binding of the anti-β antibody in ELISA procedures (N. Groome, unpublished observations). This β_B subunit peptide is also identical in amino acid sequence to the corresponding mouse sequence shown in Fig. 2. It is thus likely that this antibody reacts with both β_A and β_B subunits, so in this paper we refer to expression of activins rather than activin A, B or AB. The anti-α antibody is a sheep antiserum raised against amino acids 1-29 of the amino terminus of the bovine mature α subunit. This region is also identical in amino acid sequence to the corresponding region of the mouse α subunit shown in Fig. 2. This reacts with all molecular forms of inhibin (Groome et al., 1990). The anti-β antibody was used at 10 μg/ml and the anti-α antiserum was used at a 1/1000 dilution. Controls included use of mouse IgG in place of first antibody for the anti-β monoclonal and normal sheep serum in place of the anti-α polyclonal. In one experiment, the anti-β antibody was preincubated with the immunizing peptide and no specific staining was observed in the embryos. The secondary antibodies used were fluorescein-conjugated goat anti-mouse IgG (Sigma Immunochemicals) used at a 1/100 dilution and fluorescein-conjugated rabbit anti-sheep IgG (DAKO) used at a 1/50 dilution.

The whole-mount immunostaining procedure is based on that of Slager et al. (1991). Briefly, preimplantation embryos were flushed with Hepes (25 mM)-buffered DMEM containing 0.5% BSA (DMEM/BSA) and fixed for 30 minutes with 2% paraformaldehyde/0.1% glutaraldehyde in PBS. The embryos were then washed and quenched for 5 minutes with 0.05% sodium borohydride in PBS. They were washed again and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. After washing they were blocked with 2% BSA/3% normal serum in DMEM/BSA. After 1 hour they were transferred to the first antibody solution diluted in DMEM/BSA and left for 1 hour. They were then washed for 45 minutes and transferred to the second antibody solution diluted in DMEM/BSA containing 3% normal serum and left for 1 hour. Finally, the embryos were washed as described above and mounted on cavity slides in Cityfluor. The specimens were viewed under epifluorescence on a Zeiss Axiophot microscope and subsequently on a BioRad 600 laser confocal microscope. The whole mounts were repeated at least three times with more than 20 embryos per stage each time. The results obtained in Fig. 6 were obtained by staining fertilized eggs, 2-cell stage, morulae and 3.5-day blastocysts on the same day. The settings in the microscope were left constant to allow accurate comparison of fluorescence intensities. Staining of the 4.5-day blastocysts was done on a different day but with the same microscope settings. The patterns observed for these embryos were the same in at least three separate experiments. For quantitative comparisons, fertilized eggs, 2-, 4-, 8-cell and compacted morula embryos were recovered at approximately 12, 24, 31, 38 and 46 hours post-fertilization, respectively. The embryos were then submitted to the whole-mount protocol as they were recovered. For quantitation, ten equally-spaced optical sections were sampled from control and experimental embryos. The resulting Z-series was projected using the summation method of the associated CoMOS software, which averages the pixel values for all images in the file. The total pixel intensities for each image were then calculated, and multiplied by the area occupied by each embryo, thus giving an estimate of the fluorescence intensity of the whole embryo. Backgrounds obtained for each developmental stage were subtracted, and the results are presented as means ± s.e.m. At least ten embryos were sampled at each stage, and the experiment was performed twice with similar results.

ES cells (CCE) were grown on STO feeder layers on gelatin-coated glass coverslips as described above before being processed for immunofluorescence. Before staining, the coverslips were washed twice for 30 seconds with PBS and fixed for 20 minutes with 3% paraformaldehyde in PBS. The coverslips were washed twice for 4 minutes each with PBS and permeabilized with 0.2% Triton X-100 in PBS for 4 minutes. The coverslips were washed again in four changes of PBS for 4 minutes each time and blocked for 30 minutes with 2% BSA/3% normal serum in PBS. The first antibody was then applied to the coverslips and incubated for 45 minutes, washed with three changes of PBS for 5 minutes each and incubated with the second antibody. After 45 minutes the coverslips were washed as above and mounted with Cityfluor on glass slides.

Immunohistochemistry

Mouse ovaries were fixed in 4% paraformaldehyde in PBS overnight, embedded in wax and sectioned at 5 μm for immunohistochemistry. The sections were deparaffinized with xylene and rehydrated. Endogenous peroxidase activity was eliminated by incubation with 0.1% hydrogen peroxide in PBS. After 30 minutes the sections were washed twice with PBS for 5 minutes and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. The sections were then washed twice for 5 minutes with PBS and blocked with 10% normal serum for 30 minutes. The first antibody was applied diluted in 1% normal serum in PBS overnight at 4˚C. The sections were then washed three times for 10 minutes with PBS. Detection was with a Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions.

cDNA cloning of mouse α, β_A and β_B subunits

A commercial WEHI cDNA library in λZAP (Stratagene) was screened using a rat β_A cDNA (rat cDNAs for this subunit and for the β_B and α subunits were gifts of Dr S. Shimasaki, Salk Institute, La Jolla, USA). Initially about 300,000 plaques were screened. Filters were hybridized to the rat β_A cDNA, which had been labelled by random priming (Feinberg and Vogelstein, 1984), in 50% formamide, 5× SSC (20× SSC is 3 M NaCl/0.3 M sodium citrate, pH 7.0), 5× Denhardt's (100× Denhardt's solution is 2% (w/v) BSA, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrollidone), 0.2% SDS, and 150 μg/ml of sheared salmon sperm DNA at 42˚C overnight. Washes were at 50˚C with 0.2× SSC, 0.1% SDS twice for 10 minutes each. Hybridizing plaques were purified and the cDNAs were recovered by in vivo excision of the Bluescript plasmid.

In order to obtain cDNA clones for the α and β_B subunits, an ovary cDNA library was constructed. Total RNA from mouse ovaries was obtained by the method of Chomczynski and Sacchi (1987) and poly(A)+ RNA was prepared according to Sambrook et al. (1989). 4 μg of poly(A)+ RNA was used to make cDNA using a commercial kit (Stratagene). The cDNA was ligated to a Lambda UniZap vector and packaged according to the manufacturer's directions. This produced a primary library containing 5.2×10^6 independent recombinants. Approximately 1.4×10^6 plaques of the primary library were plated and duplicate lifts were done. The filters were first hybridized to random-primed rat β_B cDNA and washed as described above. Positive plaques obtained in the first round were purified and the cDNAs were recovered as Bluescript plasmids by in vivo excision as described above. The filters were then stripped of the β_B probe and hybridized to a random primed rat α subunit cDNA in the same conditions as described above. Positive plaques were purified and the cDNAs were recovered by in vivo excision as described above.

Sequencing

cDNAs corresponding to the α, β_A and β_B subunits were sequenced as double-stranded plasmids using a commercial kit

(Pharmacia) and T7 DNA polymerase (Pharmacia) using ^{35}S-dATP as label. Unidirectional nested deletions for both strands were generated with exonuclease III (Henikoff, 1984) and gaps were filled by use of specific oligonucleotide primers. Both strands of each cDNA were sequenced and at least one strand in each was sequenced with 7-deaza dGTP or 7-deaza dITP as substitutes for dGTP to minimize the occurrence of band compressions due to secondary structure. The data were analyzed with the GCG package (Devereux et al., 1984).

RNAase protection assays

These were performed according to the method of Krieg and Melton (1987). RNA was obtained by the method of Chomczynski and Sacchi (1987) or by extraction with LiCl/urea (Auffrey and Rougeon, 1979). The probes used were a 204 bases-protected fragment mouse β_A cDNA probe, a 245 bases-protected fragment mouse β_B cDNA probe, a 211 bases-protected fragment mouse α cDNA probe and a 67 bases-protected fragment human γ actin probe (Gunning et al., 1983). This latter probe was used as an internal loading control for the densitometric estimation of relative amounts of protected fragments when quantitation was needed. The regions in the cDNAs from where these fragments were derived are illustrated in Fig. 2.

Reverse transcription-PCR

Batches of 100-200 one-cell, two-cell, morula and blastocyst stages were collected by flushing oviducts or uteri with M2 medium. Embryos were extensively washed and RNA was extracted as described above. 10 μg of glycogen was added to each batch as carrier and the RNA was ethanol precipitated. The resulting RNA pellet was dissolved in DEPC-treated water and 5× reverse transcription buffer and nucleotides were added. The RNA was then treated with 1 unit of RNAase-free DNAase (Promega) for 30 minutes at 37°C prior to reverse transcription to eliminate any possible genomic DNA contamination. The samples were then heated to 95°C for 5 minutes to inactivate the DNAase. For reverse transcription, random hexamers were added to the samples which were then heated to 65°C for 5 minutes and cooled on ice for 3 minutes. After addition of 200 units of MoMuLv reverse transcriptase (Promega), the samples were incubated for 1 hour at 42°C. At the end of reverse transcription the samples were heated to 95°C for 5 minutes and cooled on ice. To check that the reverse transcription worked, one tenth (3 μl) of the reverse transcription reaction was submitted to PCR for 35 cycles using HPRT-specific primers. To detect inhibin and activin subunits, PCR was performed for 35 cycles on the equivalent of 98 oocytes, 77 two-cell embryos, 101 morulae and 50 blastocysts for each subunit. As a positive control reverse transcribed ovary RNA was used. Negative controls included primers in the absence of template and RNA samples in which reverse transcription was omitted. After PCR the samples were extracted with phenol/chloroform, 10 μg of glycogen was added and the samples were precipitated overnight at −70°C after addition of 5 μl of 3 M sodium acetate and 2.5 volumes of ethanol. The precipitates were recovered by centrifugation for 30 minutes and the resulting pellets were washed with 70% ethanol, dissolved in 10 μl water and loaded on a 1.8 % agarose gel. The gel was then blotted onto a nylon membrane and hybridized to specific cDNA probes for the α, β_A and β_B subunits. The primers used were:

HPRT: 5′-primer: 5′-CCTGCTGGATTACATTAAAGCACTG -3′
3′-primer: 5′-GTCAAGGGCATATCCAACAACAAAC -3′
(350 bp band on PCR)

α subunit: 5′-primer: 5′-GTCTGCCTCGAAGACATGCC -3′
3′-primer: 5′-CTGTACCAAGGACACAGGCA -3′
(341 bp band on PCR)

β_A subunit: 5′-primer: 5′-AAGAGACCCGATGTCACCCA -3′
3′-primer: 5′-CTCTCCCCCTTTAAGCCCAT -3′
(395 bp band on PCR)

β_B subunit: 5′-primer: 5′-TGGGCAGAGTGAGGCTGTTC -3′
3′-primer: 5′-TTTCGCCTGTGTGGGTCAAC -3′
(325 bp band on PCR).

PCR cycles were: 94°C, 30 seconds; 62°C, 30 seconds; 72°C, 30 seconds.

In situ hybridization

In situ hybridizations were carried out on 5 μm sections of ovary, and of morula and blastocyst embryos, as described by Wilkinson and Green (1990). For sectioning, morulae and blastocysts were flushed as described above and placed inside oviducts to facilitate wax embedding. ^{35}S-labelled probes in both the sense and the antisense orientations were derived from the regions of the α, β_A and β_B cDNAs indicated in Fig. 2. Exposure times for the ovary sections were 7 or 14 days while the morulae and blastocysts were exposed for 10, 14 or 21 days.

RESULTS

cDNA cloning of the mouse α, β_A and β_B inhibin subunits

A WEHI-3B cDNA library was screened in an attempt to find a cDNA for the β_A subunit. Approximately 300,000 plaques were screened and 16 hybridizing plaques were obtained. After plaque purification, cDNA rescue and sequencing, one 1.3 kb cDNA proved to encode most of the protein-coding region of the β_A subunit. Comparison with the rat sequence suggested that it lacked only the first 22 amino acids. To obtain a full length cDNA, a 300 bp fragment for the most 5′ region of this clone was used to screen 1.2×10^6 plaques from the same library. Twenty one hybridizing plaques were obtained and after three rounds of plaque purification the cDNAs were rescued and analyzed by restriction enzyme mapping. One 1.5 kb cDNA was selected to be sequenced, and it proved to contain the whole protein coding region of the activin β_A subunit. The initiation of the protein coding region at the ATG at base pair 178 was assigned by direct comparison with the published rat sequence and is in a favourable context for translation initiation (Kozak, 1987; not shown). The amino acid translation of this cDNA is depicted in Fig. 1. One potential N-glycosylation site is present at position 165 and the beginning of the amino terminus of the putative 14×10^3 M_r chain is indicated by an arrow.

WEHI cells do not express the α and β_B subunits (Albano et al., 1990; see also Fig. 3) so in an attempt to clone these subunits a mouse ovary cDNA library was constructed. Approximately 1.4×10^6 plaques of the primary library were screened using the rat cDNAs, and 11 hybridizing plaques for the β_B and 90 for the α subunits were obtained. After plaque purification of the strongest positives the cDNAs were rescued and analyzed by restriction enzyme mapping and dideoxy sequencing. A 2.4 kb cDNA for the β_B (β_B1) and a 1.3 kb cDNA for the α (α2) subunits were obtained.

The β_B1 cDNA does not encode the entire protein and attempts to find a clone that extended 5′ to this clone by conventional library screening were not successful. The

α chain

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1               10                  20                  30                  40
M V S Q R S L L L L L L T L R D V D S C Q G P E L V R E L V L A K V K A L F L D

                50                  60                  70                  80
A L G P P A M D G E G G D P G I R R L P R R H A V G G F M H R T S E P E E E D V S

            90                  100                 110                 120
Q A I L F P A T G A T C E D Q P A A R G L A Q E A E E G L F T Y V F R P S Q H I R

        130                 140             *   150                 160
S H Q V T S A Q L W F H T G L G R K S T A A A N S S A P L L D L L V L S S G G P M

        170                 180                 190                 200
A V P V S L V Q G P P R W A V L H L A A S A F P L L T H P I L V L L L R C P L C S

        210                 220                 230                 240
C S G R P E T T P F L V A H T R A R A P S A G E R A R R S T P S V P W P W S P A A

    250                 260             * 270                 280
L R L L Q R P P E E P A A H A F C H R A A L N I S F Q E L G W D R W I V H P P S F

    290                 300                 310                 320
I F H Y C H G S C G M P T S D L P L P V P G V P P T P V Q P L F L V P G A K P C C

  330                 340                 350                 360
A A L P G S M T S L R V R T T S D G G Y S F K Y E M V P N L I T Q H C A C I *
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β_A chain

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1               10                  20                  30                  40
M P L L W L R G F L L A S C W I I V R S S P T P G S E G H G S A P D C P S C A L A T

            50                  60                  70                  80
L P K D G P N S Q P E M V E A V K K H I L N M L H L K K R P D V T Q P V P K A A L L

        90                  100                 110                 120
N A I R K L H V G K V G E N G Y V E I E D D I G R R A E M N E L M E Q T S E I I T F

    130                 140                 150                 160     *
A E S G T A R K T L H F E I S K E G S D L S V V E R A E V W L F L K V P K A N R T R

  170                 180                 190                 200             210
T K V T I R L F Q Q Q K H P Q G S L D T G D E A E E M G L K G E R S E L L L S E K V

            220                 230                 240                 250
V D A R K S T W H I F P V S S S I Q R L L D Q G K S S L D V R I A C E Q C Q E S G A

        260                 270                 280                 290
S L V L L G K K K K K E V D G D G K K K D G S D G G L E E E K E Q S H R P F L M L Q

        300                 310                 320                 330
A R Q S E D H P H R R R R R G L E C D G K V N I C C K K Q F V F K D I G W N D W I I

  340                 350                 360                 370             380
A P S G Y H A N Y C E G E C P S H I A G T S G S S L S F H S T V I N H Y R M R G H S

            390                 400                 410                 420
P F A N L K S C C V P T K L R P M S M L Y Y D D G Q N I I K K D I Q N M I V E E C G

C S *
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β_B chain

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1               10                  20                  30                  40
R D R I R K R G L E C D G R T S L C C R Q Q F F I D F R L I G W N D W I I A P T G Y

            50                  60                  70                  80
Y G N Y C E G S C P A Y L A G V P G S A S S F H T A V V N Q Y R M R G L N P G P V N

        90                  100                 110                 120
S C C I P T K L S S M S M L Y F D D E Y N I V K R D V P N M I V E E C G C A *
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Fig. 1. Amino acid translation of the α, β_A and β_B cDNAs. Full-length precursor sequences of the α and β_A and a partial sequence of the β_B chains are depicted in the one letter code. Potential sites of Asn-linked glycosylation are indicated by (*). The amino termini of the 44×10^3 M_r and 18×10^3 M_r α subunits and of the 14×10^3 M_r β_A and β_B subunits are indicated by arrows.

amino acid sequence was assigned by comparison with the rat sequence and it also corresponds to the longest open reading frame in the nucleotide sequence (not shown). The amino acid sequence corresponds to 7 amino acids of the precursor and all of the 14×10^3 M_r subunit, the beginning of which is indicated by an arrow in Fig. 1. There is 100% amino acid identity between the β_B 14×10^3 M_r subunit obtained by us and the rat sequence described by Esch et al. (1987), indicating that we indeed cloned the mouse homologue of this subunit.

The first ATG of the α subunit was assigned in terms of

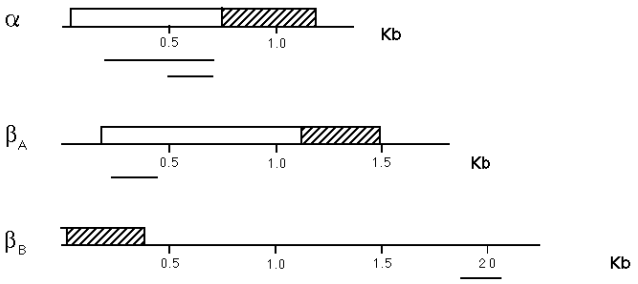

Fig. 2. Structures of the α, β_A and β_B cDNAs. Coding sequences are boxed and untranslated sequences are represented by lines. The scale is given in nucleotides from the 5′ ends of each cDNA. The open boxes represent the larger precursor sequences and the hatched boxes represent the putative mature subunits. The 510 and 211 base pair regions underlined in the α chain cDNA are used as in situ hybridization and RNAase protection probes, respectively. The 204 base pair region underlined in the β_A cDNA and the 245 base pair region underlined in the β_B cDNA are used both as in situ hybridization and RNAase protection probes.

comparison with the rat sequence and is in a favourable context for translation initiation (Kozak, 1987; not shown). The amino acid sequence for the mouse α subunit is shown in Fig. 1. There are two potential N-glycosylation sites at positions 147 and 269 of the α chain and the amino termini of the putative 44×10^3 M_r and 18×10^3 M_r α subunits are indicated by arrows. The amino acid sequence of the α subunit described in this paper from position 49 onwards is identical to that of the partial clone obtained by Tone et al. (1990).

The structure of the three cDNAs is shown in Fig. 2; the EMBL database accession numbers are: α subunit: X69618; β_A subunit: X69619; β_B subunit X69620. The precursor sequences are indicated as open boxes and the dimer forming processed subunits are hatched. The regions that were chosen to be used as probes for molecular studies are indicated. There is no significant nucleotide homology in these regions between these clones or with other members of the TGF β family.

Activins are expressed in ES and EC cells and regulated on their differentiation

Our first experiments investigated inhibin subunit expression in embryonic stem (ES) cells. ES cells provide an accessible in vitro model for the early stages of mouse development. They resemble most closely the inner cell mass (ICM) cells of 3.5-day blastocysts (Beddington and Robertson, 1989) and will differentiate into a variety of cell types, including those derived from mesoderm, when allowed to form embryoid bodies (Evans and Kaufman, 1981; Martin, 1981; Doetschman et al., 1985).

RNA from 8 different ES cell lines was analyzed by RNAase protection for expression of the α, β_A and β_B subunits of inhibin (Fig. 3A). All lines expressed high levels of mRNA for the β subunits but little α subunit RNA was detected. Most lines expressed higher levels of the β_A mRNA than β_B, but in two (CP1 and Sxr2), β_B transcripts were more abundant. The mitomycin-treated STO fibroblasts that were used as a feeder layer for the ES cells also express β_A RNA, but it is unlikely that these cells influ-

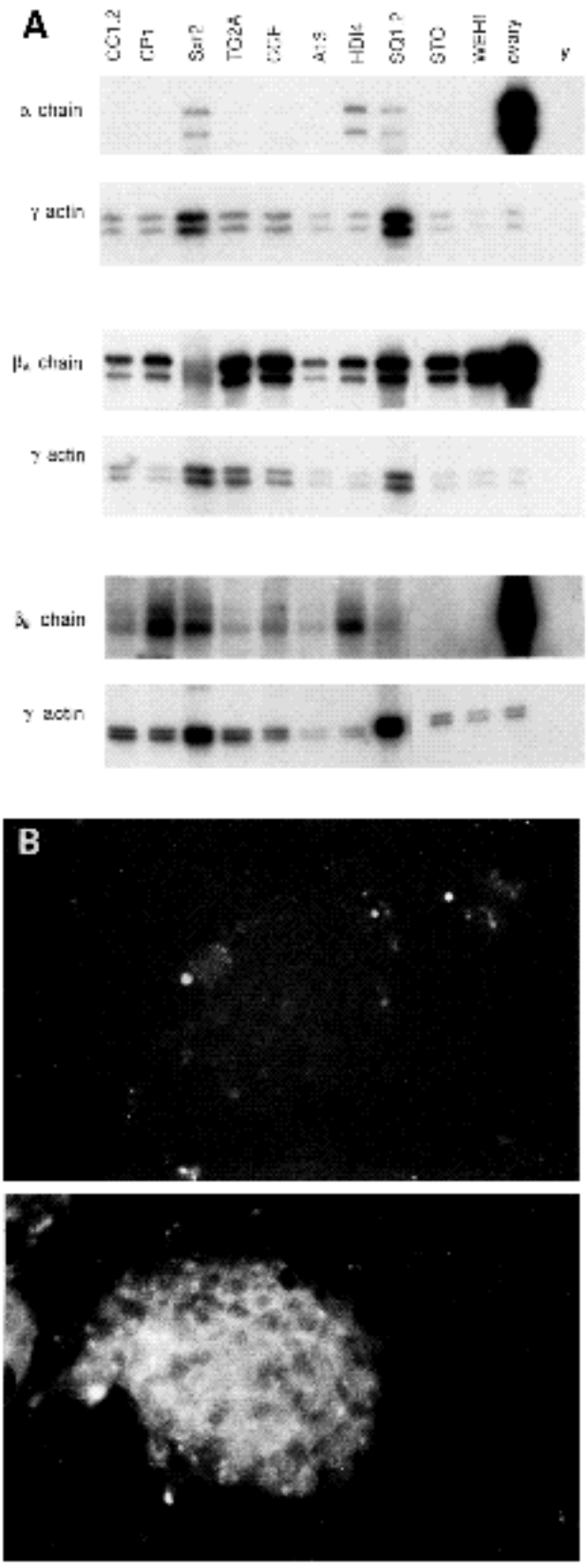

Fig. 3. Activins are expressed in ES cells. (A) Total RNA (15 μg) from 8 different ES cell lines, from mitomycin-treated STO fibroblasts (STO, 8 μg), from WEHI-3B cells (WEHI, 15 μg) and poly(A)+ RNA from mouse ovaries (ovary, 2 μg), were hybridized to in vitro transcribed RNA probes specific to human γ actin and to either the α, β_A or β_B cDNAs. RNAase protection analysis was carried out as described in Materials and methods. 20 μg of yeast t-RNA was used as a negative control. Exposures were 5 hours for γ actin and 3 days for α, β_A and β_B. (B) The CCE ES cell line was grown on gelatin-coated dishes on STO fibroblasts as described in Materials and methods and submitted to immunofluorescence using mouse IgG as primary antibody, top panel, or using the anti-β monoclonal antibody, bottom panel, as described in Materials and methods.

ence the observed levels of β_A RNA because the ES cells were passaged once in the absence of feeders before extraction of RNA. Furthermore, one of the lines (TG2A) was derived in DIA/LIF in the absence of feeder cells, and this too shows strong expression of β_A RNA. As positive controls, we analyzed RNA from WEHI-3B cells and ovary. As previously shown by northern blot analysis, WEHI cells contain only β_A transcripts (Albano et al. 1990), while ovary expresses all three subunits.

To investigate activin and inhibin protein synthesis in ES cells, we used a polyclonal antibody specific for the α subunit and a monoclonal antibody specific for the β subunits (see Materials and methods). No specific fluorescence was observed in the CCE ES cell line with the anti-α subunit polyclonal antibody, but a strong signal was obtained with the monoclonal anti-β subunit antibody (Fig. 3B). Weak fluorescence was also observed in the STO fibroblasts (not shown). Together with the results from RNAase protection, these observations indicate that CCE ES cells express activin but not inhibin.

We went on to analyze expression of the inhibin subunits in embryoid bodies derived from CCE ES cells and these results were analyzed by densitometry after normalizing each level to that of the γ-actin internal control for quantitation (Fig. 4). At both 2 and 5 days after embryoid body formation, α subunit RNA levels remained low. Levels of the β subunits are, however, strongly regulated: levels of both β_A and β_B RNA decrease 4- to 7-fold on differentiation.

Retinoic acid treatment of F9 teratocarcinoma cells provides a useful model for the differentiation of two extraembryonic cell lineages in the early postimplantation mouse embryo. When such cells are grown as a monolayer they form parietal endoderm (Strickland and Mahdavi, 1978; Strickland et al., 1980), but when grown in suspension as aggregates they form visceral endoderm (Hogan et al., 1981). We decided to compare inhibin subunit expression in these cells with that in differentiating ES cells. Total RNA from F9 stem cells, from cells differentiating as visceral endoderm at 5, 8 and 11 days after suspension, and from cells induced to become parietal endoderm, was analyzed by RNAase protection and the results were quantitated as described above (Fig. 5). The stem cells express only low levels of α subunit RNA but high levels of β_B RNA. No expression of β_A subunit RNA was detectable in stem, parietal or visceral endoderm-differentiated F9 cells. Differentiation of F9 cells to parietal endoderm causes down-regulation of both α and β_B RNA. Differentiation into visceral endoderm causes a 3-fold increase in α sub-

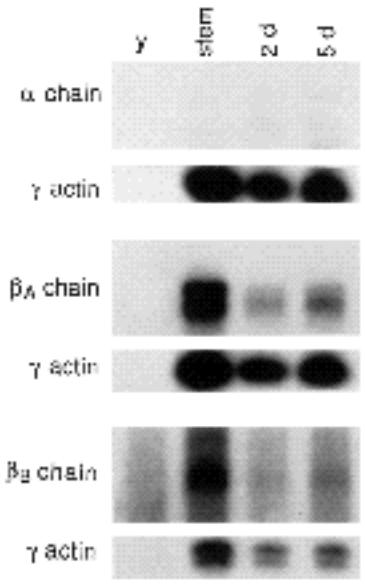

Fig. 4. Activins are regulated on differentiation of ES cells. CCE ES cells were differentiated as embryoid bodies as described in Materials and methods. 20 μg of total RNA from stem, 2-day differentiated, or 5-day differentiated cells was analyzed by RNAase protection as described in Materials and methods. Probes specific for human γ actin and for the α, β_A or β_B cDNAs were used. 20 μg of yeast t-RNA was used as a negative control. Exposure times were 5 hours for γ actin and 4 days for α, β_A and β_B.

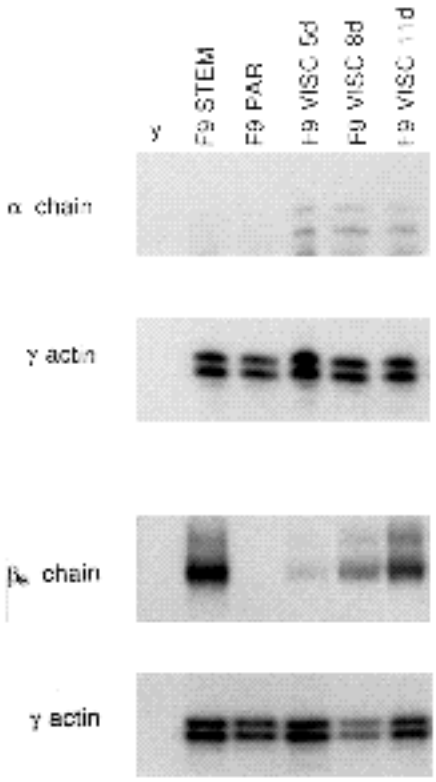

Fig. 5. Activins are regulated on differentiation of F9 EC cells. F9 EC cells were differentiated into parietal-like or visceral-like endoderm as described in Materials and methods. 20 μg of total RNA from stem cells (F9 stem), parietal endoderm differentiated cells (F9 par), and 5, 8 and 11 days of visceral endoderm differentiated cells, was analyzed by RNAase protection as described in the legend to Fig. 4. 20 μg of yeast t-RNA (y) was used as a negative control. Exposure times were 5 hours for γ actin, 3 days for α and overnight for β_B. No protected bands were observed for β_A mRNA.

unit RNA, which remained constant from 5 to 11 days of differentiation, and a down-regulation of β_B transcripts. The levels of β_B subunit RNA initially fell about 35-fold after 5 days of differentiation, but this increased after 8 and 11 days to 90% of the level expressed by stem cells. Embryoid bodies consist of an outer layer of visceral endoderm-like cells surrounding a population consisting largely of stem cells. At present it is not clear which cells are responsible for the progressive increase in β_B subunit RNA levels.

Activins are expressed throughout preimplantation development

Expression of activins by ES cells might reflect the behaviour of ICM cells of 3.5-day blastocysts, or it might be an artefactual response to in vitro culture. To address this question, we stained a developmental series of preimplantation embryos with the anti-α and anti-β subunit antibodies. The results were studied by whole-mount immunofluorescence allied to confocal laser microscopy, which allows both analysis of optical sections and quantitation.

The anti-β subunit antibody detected the presence of β chain in fertilized eggs and in all cells of the 2-cell embryo, morula and compacted morula (Fig. 6A-E). Blastocyst formation occurs when the outer cells of the morula begin to differentiate as trophectoderm. As this occurs, the trophectoderm cells cease to express β subunit protein while the ICM cells remain positive (Fig. 6F-H). In one experiment embryos cultured from the 2-cell stage to the morula stage were analyzed by immunofluorescence. The same results were observed, indicating that embryos are not taking up β subunit from a foreign source. No specific fluorescence was observed with the anti-α subunit antibody at these embryonic stages (Fig. 6I, J and K), so the presence of β subunits reflects the presence of activin rather than inhibin.

At 4.5 days of development, the blastocysts hatch from the zona pellucida and begin to implant. Some ICM cells begin to differentiate as endoderm and the remainder become specified as embryonic tissues (Pedersen, 1986). At this stage activins are no longer detected in the ICM but are now localized to the trophectoderm (Fig. 7C,D).

Comparison of the intensities of fluorescence during these stages indicates that levels of activin protein are highest in the fertilized egg and decrease at the 2- and 4-cell stages. By the early morula stage (8-cells), however, activin protein levels rise again, reaching a peak at the compacted morula stage as shown in Fig. 8. This suggests that maternal activin levels present in the fertilized egg decline and that expression is reactivated after the 4-cell stage.

Activin protein in the fertilized egg could derive directly from transcription of maternal mRNA or from transfer of protein to the oocyte from granulosa cells in the follicles. To investigate this, ovary sections were analyzed by

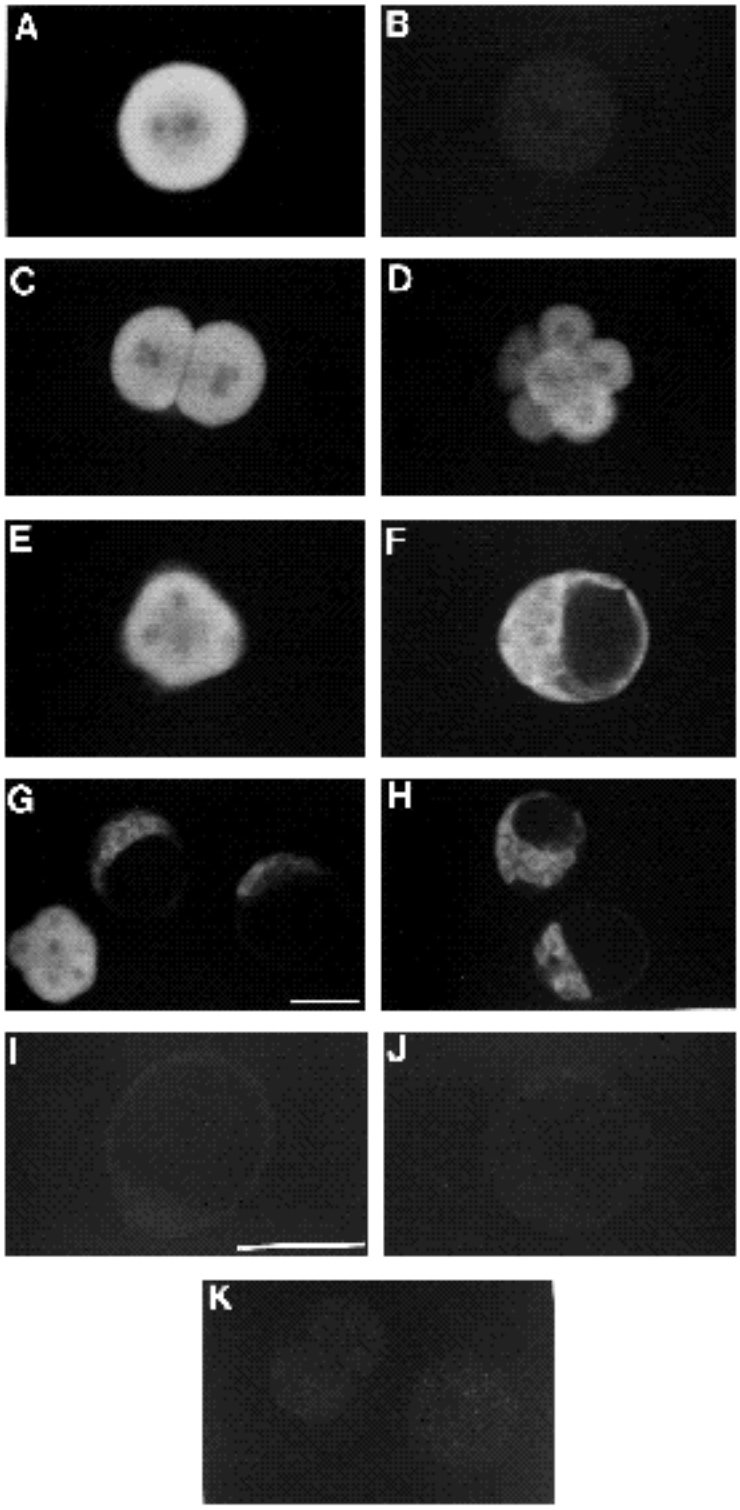

Fig. 6. Activins are expressed during preimplantation development. Preimplantation embryos were recovered from oviducts and uteri between 0.5 and 3.5 days of development and reacted with an anti-β subunit monoclonal antibody and an anti-α subunit polyclonal antibody as described in Materials and methods. (A,C-H) Experimental embryos reacted with anti-β_A antibody. (I-K) Experimental embryos treated with anti-α subunit antibody. (B) Control embryo reacted with mouse IgG in place of the monoclonal antibody. (A,B) Fertilized egg, (C) 2-cell stage, (D) morula, (E) compacted morula, (F) early blastocyst, (G) compacted morula and two late blastocysts, (H) early and late blastocysts, (I) late blastocyst, (J) late blastocyst, (K) fertilized egg and 2-cell stage embryo. Activins are present in all cells of preimplantation embryos until the early blastocyst stage. In later blastocysts they become restricted to ICM cells. No staining is observed with the anti-α subunit antibody. Bars, 50 μm.

immunohistochemistry using the anti-α and anti-β antibodies, and by in situ hybridization using probes specific for α, β_A and β_B subunits. Immunohistochemistry showed that oocytes contain high levels of the β subunit which showed a reticular pattern of staining when viewed at high power (Fig. 9B-D). Staining was also visible in the nuclei of oocytes (Fig. 9D). Surprisingly, staining of granulosa cells around the oocytes was weak. This contrasts with the results of Ogawa et al. (1991), who, using a polyclonal antibody directed against the N-terminal region of the β_A subunit, observed stronger staining in rat ovary granulosa cells. The difference might arise because the C-terminal epitope recognized by our monoclonal antibody is masked by the presence of follistatin, an activin- and inhibin-binding protein which interacts specifically with the β subunit (Shimonaka et al., 1991). Follistatin mRNA and protein are strongly

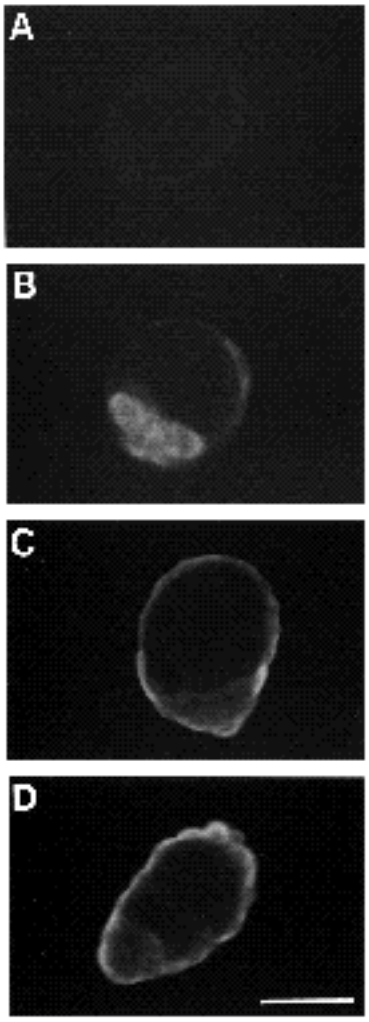

Fig. 7. Activins are expressed only in the trophectoderm of hatched blastocysts. Implanting blastocysts were recovered from uteri at 4.5 days of development and reacted with an anti-β_A subunit monoclonal antibody as described in Materials and methods. (A) Control embryo reacted with mouse IgG in place of monoclonal antibody. (B-D) Experimental embryos reacted with anti-β antibody. (A,C,D) 4.5-day blastocysts, (B) 3.5-day blastocyst. In expanded 3.5-day blastocysts, expression of activins is restricted to the ICM cells; by contrast, in hatched 4.5-day blastocysts the ICM cells become negative and staining is seen in trophectoderm cells. Bar, 50 μm.

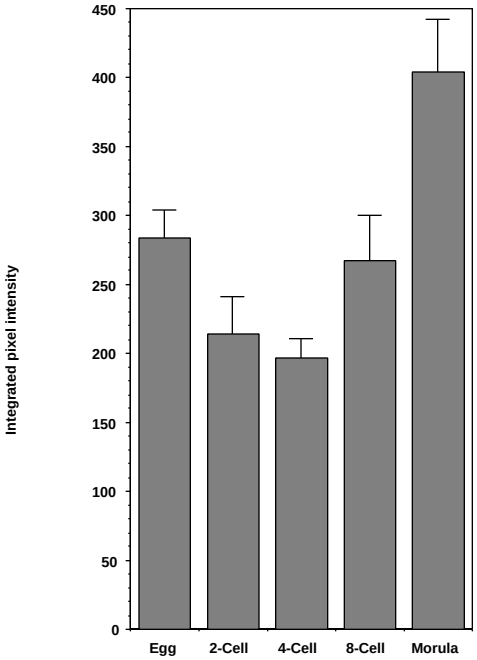

Fig. 8. Activin protein levels decline during early cleavage stages and increase in the compacted morula. Preimplantation embryos were recovered, submitted to whole-mount immunofluorescence, and fluorescence intensities were quantified on a confocal laser microscope as described in Material and methods. Bars represent s.e.m.

expressed in dominant, oocyte-containing follicles (Nakatani et al., 1991). Use of the anti-α subunit antibody showed high levels of expression in granulosa cells and weak staining in the oocyte (Fig. 9F-H). In this case, follistatin would not be expected to interfere with the binding of the anti-α subunit antibody.

In situ hybridization using probes specific for α, β_A and β_B subunits showed strong hybridization over the granulosa

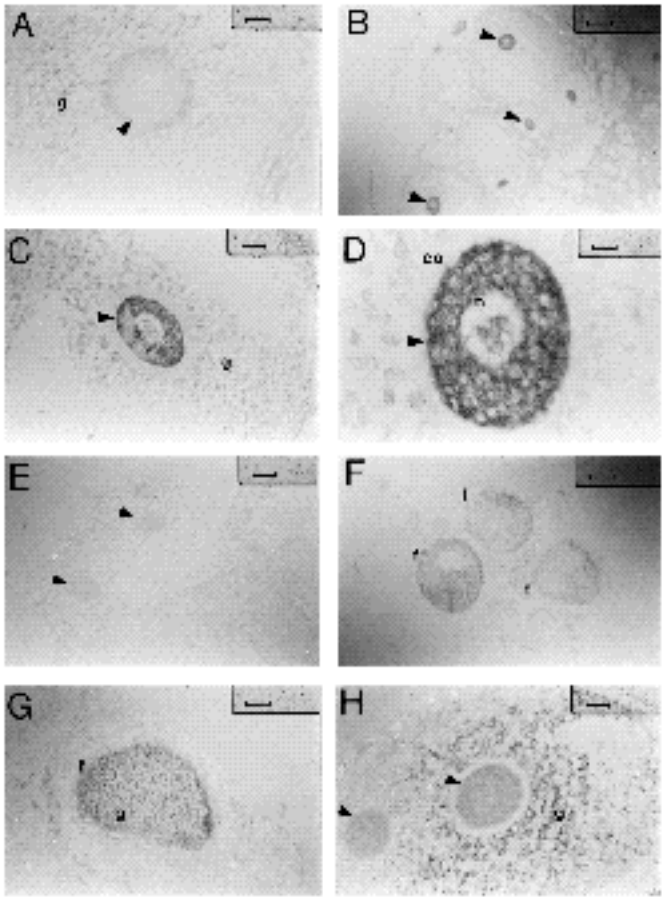

Fig. 9. Immunolocalization of α and β subunit proteins in mouse ovary follicles and oocytes. (A) Control histochemistry using mouse IgG in place of anti-β antibody. (B-D) Immunohistochemistry using anti-β antibody. (B) Low power view of stained oocytes. (C) An antral follicle containing an oocyte showing weak staining on the granulosa cells and strong staining on oocyte cytoplasm. (D) High power view of an oocyte showing weak staining of the cumulus oophorus, strong oocyte cytoplasmic staining and some intranuclear staining. (E) Control histochemistry using normal sheep serum in place of anti-α antibody. (F-H) Immunohistochemistry using anti-α antibody. (F) Low power view showing stained antral follicles. (G) A follicle showing staining on the granulosa cells. (H) An antral follicle showing staining on the granulosa cells and weak staining of the oocyte. Arrowheads, oocytes; f, follicles; g, granulosa cells; co, cumulus oophorus; n, nucleus. Bars in D, 10 μm; in A,C and H, 25 μm; in G and E, 50 μm; and in B and F, 100 μm.

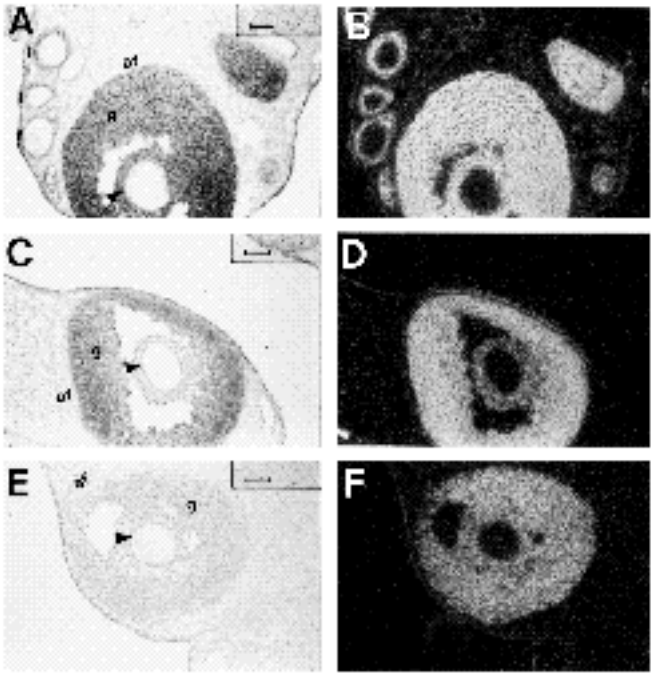

Fig. 10. mRNAs for the α, β_A and β_B subunits are present in mouse ovary follicles. In situ hybridization was performed on mouse ovary tissue sections using the α, β_A and β_B probes described in Fig. 2. Sense controls showed no hybridization (not shown). Hybridization with the α subunit probe is shown in bright field in (A) and in dark field in (B). Strong hybridization can be seen on the granulosa cells (g) of primary and secondary follicles (f) and of antral follicles (af) but not on oocytes (arrowheads). Hybridization with the β_A probe is shown in bright field in (C) and in dark field in (D). Silver grains can be seen on granulosa cells (g) of the antral follicle (af) but not on the oocyte (arrowhead). Hybridization with the β_B probe is shown in bright field in (E) and in dark field in (F). Silver grains can be seen on the granulosa cells (g) of the antral follicle (f) but the oocyte (arrowhead) shows no signal above background. Bars, 50 μm.

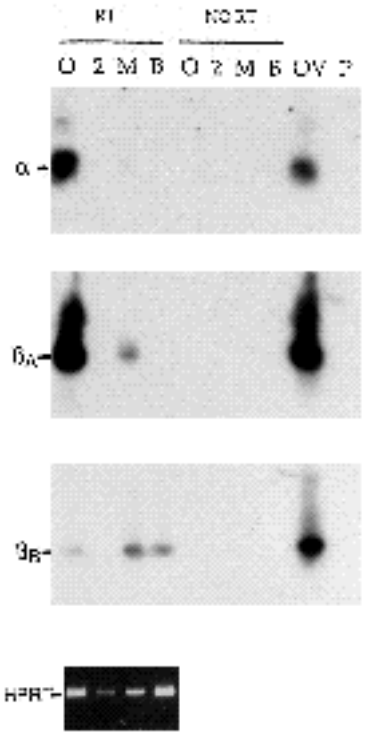

Fig. 11. Activin mRNA is expressed both maternally and zygotically in preimplantation embryos. Preimplantation embryos were recovered from oviducts and uteri and RNA was extracted, reverse transcribed and submitted to PCR using primers specific for α, β_A and β_B subunits. As a positive control, RNA from mouse ovaries was reverse transcribed and submitted to PCR with the same primers. HPRT-specific primers were used to assess the success of reverse transcription. RT, samples submitted to reverse transcription prior to PCR; NO RT, samples submitted to PCR without prior reverse transcription; O, oocytes/unfertilized eggs; 2, 2-cell embryos; M, morulae; B, blastocysts; OV, ovary; P, primers alone.

cells of follicles but no signal was detectable over oocytes (Fig. 10). No hybridization was observed when the same probes were used on sections of morulae and blastocysts (not shown). However, we were able to detect transcripts for the α, β_A and β_B subunits in eggs using the more sensitive technique of reverse transcription-PCR (Fig. 11). These transcripts were undetectable at the 2-cell stage but by the morula stage β_A and β_B mRNA were detectable. Only β_B transcripts were detected in blastocysts (Fig. 11).

Overall, these results indicate that mouse oocytes and eggs contain α and β subunit mRNA and protein, and that much of the β subunit protein is likely to be in the form of activin. Much of this material is degraded at the transition from maternal to zygotic control of development. The protein observed in later cleavage stages is probably due to translation of zygotic β subunit transcripts. The mRNA levels detected by RT-PCR are probably sufficient to account for the protein expression observed. Very low levels of IGF-II mRNA were observed in mouse preimplantation embryos by Rappolee et al. (1992) but it was shown that these transcripts are being translated and that they account for most of the IGF-II protein present; antisense oligonucleotides directed against these transcripts eliminated most of the immunoreactive IGF-II protein.

DISCUSSION

This paper describes the expression patterns of the α, β_A and β_B subunits of inhibin during early mammalian embryogenesis and in ES and EC cells. Oocytes and fertilized eggs contain high levels of β subunit protein with only low levels of α chain. This indicates that much of the β subunit protein is likely to be in the form of activin. Levels of activin then decrease during the first cleavage stages but increase at the 8-cell stage and peak at compaction, probably due to translation of zygotic mRNA. By 3.5 days, activin staining is visible in all cells of some early blastocysts, but in later blastocysts expression is not detectable in the trophectoderm, while it persists in the inner cell mass. At 4.5 days activin expression has decreased in the ICM but staining is now visible in the trophectoderm cells.

What might be the functions of activin during these stages of development? The high levels of protein in the oocyte and fertilized egg are consistent with the observation that activin A is a potent stimulator of oocyte maturation in vitro (Itoh et al., 1990). At morula and blastocyst stages activins might be acting, alone or with other members of the TGFβ family such as TGFβ1 (Rappolee et al., 1988) or TGFβ2 (Slager et al., 1991), to modulate growth or to inhibit differentiation. Consistent with the former sug-

gestion, activin stimulates DNA and protein synthesis in osteoblast cultures (Centrella et al., 1991) and modulates the growth of two gonadal cell lines which are also sensitive to TGFβ (Gonzales-Manchon and Vale, 1989). Activin also acts as a growth factor for P19 cells, an EC cell line which differentiates into neuronal or muscle cell types when treated with retinoic acid or DMSO, respectively (Rudnicki and McBurney, 1987), but in addition to this it inhibits their retinoic acid-induced differentiation (Hashimoto et al., 1990; Schubert et al., 1990; Schubert and Kimura, 1991; van den Eijnden-van Raaij et al., 1991). This role of activin as an inhibitor of differentiation is consistent with the spatial distribution of the molecule during early development and with data obtained using ES and EC cells. During development, the separation of blastomeres into two distinct lineages occurs at about 3.5 days of development with the differentiation of trophectoderm. These cells do not express activin but the ICM cells, which remain pluripotent, continue to do so until 4.5 days, when some give rise to primitive endoderm and the remainder become specified to form embryonic tissues (Gardner, 1989; Pedersen, 1986). Similarly, in both ES and EC cells, levels of activin mRNA fall dramatically on differentiation.

Recently, it was demonstrated that ectopic injection of activin B or *wnt-1* RNA could increase gap junctional permeability in early *Xenopus* embryos (Olson and Moon, 1992). In preimplantation mouse embryos, the onset of gap junctional communication is at the early compacted morula stage (late 8-cell embryo; Lo and Gilula, 1979), which also coincides with the second peak of expression of activin protein. Activins may be involved in inducing cell-cell communication in the early mouse embryo.

The expression of activins in the trophectoderm of 4.5-day embryos might suggest a role not in inhibition of differentiation but in implantation. Again, it is possible that activins interact with other growth factors, because TGFβ2 is also expressed in these cells and TGFβ1 mRNA and protein are expressed in luminal uterine epithelial cells during the pre- and periimplantation period (Tamada et al., 1990; Slager et al., 1991).

Finally, might activins be involved in mesoderm formation, as has been suggested for the amphibian embryo? By analogy with the amphibia, we should expect activins to be expressed some time before mesoderm formation, and this criterion is satisfied both in ES cells (see Fig. 4) and in developing embryos, where mesoderm formation starts at 6-6.5 days. To obtain further information about the role of activin in these early stages, it will first be necessary to study expression of the activin receptors (Mathews and Vale, 1991; Attisano et al., 1992). Another approach is to attempt to ablate inhibin subunit mRNA by use of antisense oligonucleotides (see Rappolee et al., 1992).

Results with ES cells (Fig. 4), and our preliminary observations on postimplantation embryos using RNAase protections and in situ hybridization (R.M. Albano and J.C. Smith, unpublished data), indicate that activins are not expressed by the embryo at significant levels during mesoderm formation itself. Even though activin mRNA was detected in the surrounding decidual cells of the 6.5-day embryo, nothing is known about the distribution of the protein (Manova et al., 1992; Albano and Smith, unpublished data). If, therefore, activins are involved in mesoderm induction they are likely to be triggering a patterning mechanism in the inner cell mass. Another member of a family of mesoderm-inducing growth factors, FGF-4, is also expressed in EC and ES cells and in the inner cell mass of blastocysts, and this could interact with activin to trigger patterning (Niswander and Martin, 1992). These two factors could, in turn, be inducing the expression of other molecules that are involved in fine-tuning the process. Evidence that such fine-tuning occurs comes from the observation that even as late as 6.7 days some individual cells can still give rise to derivatives in all germ layers (Lawson et al., 1991). Other candidates for factors involved in fine-tuning include other members of the FGF and TGFβ family. FGF-5 and two FGF receptors are expressed in the early egg cylinder (Haub and Goldfarb, 1991; Hébert et al., 1991; Orr-Urtreger et al., 1991), while bone morphogenetic protein 4 (BMP-4) is expressed in the posterior mesoderm of the mouse embryo (Jones et al., 1991). Interestingly, BMP-4 has recently been shown to 'ventralize' the response to activin in the *Xenopus* embryo (Dale et al., 1992; Jones et al., 1992).

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