Cloning and regulation of the rat activin β_E subunit

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ABSTRACT

Using a combination of polymerase chain reaction (PCR) procedures, we have cloned and sequenced the rat activin β_E subunit cDNA. The putative protein corresponding to the prepro-activin β_E subunit was predicted to comprise 350 amino acids which, when cleaved between amino acid residues 236 and 237, would yield a mature polypeptide of approximately M_r 12 500 with a predicted pI of 5.1. Two cDNA transcripts for activin β_E were identified; these differed by 738 bp in the 3'-untranslated region. Activin β_E mRNA transcripts were expressed only in rat liver and lung tissue as assessed by Northern blotting and PCR analysis. Relatively higher levels of both transcripts were found in the liver, whereas the lung contained lower levels that were detectable by PCR only. *In situ* hybridisation data showed that, within the liver,

activin β_E mRNA was localised to hepatocytes. *In vivo* treatment with lipopolysaccharide as a means of activating the immune system and the hepatic acute-phase response resulted in stimulated activin β_E mRNA levels, compared with untreated, control rats. This increased expression was accompanied by a preferential increase in the amount of the long activin β_E transcript over the shorter transcript. These findings suggested that the two activin β_E mRNA transcripts may be products of alternative splicing events or use alternative polyadenylation sites which are differentially regulated during inflammation. These data provide evidence of a role for activin β_E in liver function and inflammation in the rat.

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INTRODUCTION

Activins and inhibins are homo- and heterodimeric proteins with regulatory functions in many areas of biology, including spermatogenesis, ovarian function, erythropoiesis, tumorigenesis, bone growth, cardiac morphogenesis and immune function (Vale *et al.* 1994, de Paolo 1997, Yu & Dolter 1997). Activins are composed of two β subunits ($\beta\beta$), covalently linked by a single disulphide bond, whereas the inhibins are composed of the same β subunit disulphide bonded to an α subunit ($\alpha\beta$). To date, a single α subunit and two β subunit proteins (β_A and β_B) have been purified. However, within highly differentiated cells, the presence of three additional β subunits (β_C , β_D and β_E) have been postulated on the basis of mRNA data (Hotten *et al.*

1995, Oda *et al.* 1995, Fang *et al.* 1996). Biological functions have not been delineated for these three novel β subunits. Microinjection of synthetic activin β_D mRNA transcribed from cDNA into *Xenopus* embryo blastomeres, however, led to secondary body axis formation, whilst microinjection into pole caps led to mesoderm induction. This finding suggested that the activin β_D subunit might be involved in embryogenic development and more specifically in mesoderm induction (Oda *et al.* 1995). Analysis of the predicted amino acid sequence homologies between the known activin β subunits showed a 50% overall homology, and suggests that the β_C , β_D and β_E subunits form a separate subset within the activin β subunit 'family' of the transforming growth factor- β superfamily (Fang *et al.* 1996).

Amongst other functions, activin A ($\beta_A\beta_A$) appears to act as an autocrine regulator of hepatic parenchymal cell function. Activin A potently inhibits hepatocyte proliferation and acute-phase protein secretion and induces cell apoptosis (Schwall *et al.* 1993, Yasuda *et al.* 1993, Hully *et al.* 1994, Russell *et al.* 1999). These effects can be antagonised by co-administration of the activin-binding protein, follistatin (Schwall *et al.* 1993, Russell *et al.* 1999). Activin A also appears to be a modifier of the immune response, acting at the signal transduction level to antagonise many of the effects induced by interleukin-6 (IL-6) (Brosh *et al.* 1995, Yu *et al.* 1998, Russell *et al.* 1999). In addition, the β_B and β_C subunits of activin/inhibin have also been found in liver tissue, but their functions remain unknown (Hotten *et al.* 1995, de Bleser *et al.* 1997, Zhang *et al.* 1997a). The cDNA sequence of the activin β_E subunit has been reported for the mouse only, where its expression, as determined by Northern analysis, was restricted to the liver (Fang *et al.* 1997). The function and regulation of activin β_E within the liver, however, remains completely unknown. In this study, we describe the cloning strategy used for the determination of the cDNA sequence of the rat homologue of activin β_E and provide preliminary data on the role of activin β_E in hepatic function.

MATERIALS AND METHODS

Chemicals and enzymes

Unless stated otherwise, chemicals were obtained from Sigma Chemical Company (St Louis, MO, USA). Enzymes and buffers used for molecular biology were purchased from Promega Corporation (Madison, WI, USA). All primers were obtained from Geneworks (Adelaide, Australia) with the exception of those used for 3' rapid amplification of cDNA ends (RACE) experiments which were purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA).

Experimental animals and tissue processing

Outbred adult male Sprague–Dawley rats (80–100 days) were obtained from the Central Animal House, Monash University. Experimental procedures were approved by the Monash University Standing Committee on Ethics in Animal Experimentation and conformed to the NH&MRC/CSIRO/AAC Code of Practice for the Care and Use of Animals for Experimental Purposes. For *in situ* hybridisation studies, rats were anaesthetised using ether and then fixed by perfusion with Bouin's fluid

via the descending aorta (O'Bryan *et al.* 2000). Excised tissues were immersion-fixed in Bouin's fixative for 3 h prior to being processed into paraffin using standard embedding procedures.

For the determination of the normal tissue distribution of activin β_E mRNA, rats were anaesthetised with ether, tissues were removed and immediately snap frozen and stored at -70°C until required. In the experiment on the effect of acute inflammation on activin β_E mRNA levels, rats were treated and tissues collected as described previously (O'Bryan *et al.* 2000). Briefly, rats were given a single intraperitoneal injection of 0.1 mg/kg *E. coli* lipopolysaccharide (LPS, serotype 0127:B8) in saline, or endotoxin-free saline alone. LPS has been extensively used as an activator of the immune response (Cybulsky *et al.* 1988) and the dose was chosen as representative of a minor bacterial infection (O'Bryan *et al.* 2000). The body temperature and level of physical activity of the rats receiving this dose of LPS returned to control values within 12–18 h. Animals (three per group) were killed at 0, 3, 6, 12, 18, 24 and 72 h after the LPS injection and the livers and lungs were removed and stored at -70°C for subsequent RNA extraction. The activin β_E mRNA content was also assessed in the human prostate cell lines DU145 and LNCaP (AATCC, Rockville, MD, USA). Cells were grown as previously described (McPherson *et al.* 1997).

Cloning of rat activin β_E cDNA

The rat β_E cDNA was cloned using a combination of reverse transcriptase–polymerase chain reaction (RT-PCR) protocols. Primers used in these procedures are given in Table 1. Total RNA was purified using the method of Chomczynski & Sacchi (1987) from normal adult rat liver and reverse-transcribed to cDNA using avian myeloblastosis virus (AMV) RT as outlined by the manufacturer. The equivalent of 0.25 μg hepatic RNA was used for the PCR amplification of a 974 bp activin β_E cDNA product using the activin β_E specific primers, β_E1 and β_E2 , designed from the published mouse sequence (Fang *et al.* 1996) and a DNA polymerase with proof-reading capability (Stratagene, La Jolla, CA, USA) in a touch-down PCR protocol. Cycling conditions were 30 cycles of denaturation at 94°C for 30 s, followed by annealing at $60\text{--}45^\circ\text{C}$ (at 0.5°C increments) for 70 s, followed by extension at 72°C for 3 min (Katsahambas & Hearn 1997). Following the touch-down protocol, the signal was further amplified by ten rounds of cycling: denaturation at 94°C for 30 s, annealing at 45°C for 70 s and extension at 72°C for 3 min followed by a

TABLE 1. PCR primer sequences used in the cloning and analysis of the rat β_E activin cDNA

Primer	Length	Nucleotide position	Sequence
β_E 1	21	323–343	GGAGCTAGCCAAGCAGCAAAT
β_E 2	21	1276–1295	GGAACCCCCAACTTGAACCTT
β_E 3	21	669–689	GAGCACCAAAACCACTTCCTCT
β_E 4	21	918–938	CCTACAACATAAGGGGGTCTC
β_E 5	20	349–368	GCGATCTACTCTCAGTCTTC
β_E 6	21	349–368	GCTGGTTAGGTGCAGTCCCTC
β_E 11	20	1088–1107	TAGCCTCCTCAAAGCCAACA
β_E 12	20	1198–1217	AGACCGATGTGCCAGACTATG
β_E 17	20	2495–2514	CGTGGGCTGTTTCCAAACAA
RTP	51	3' RACE	CCTCTGAAGGTTCCAGAATCGATAGCTTAAGT ₍₁₈₎ VN
AP3	25	RACE anchor	CCTCTGAAGGTTCCAGAATCGATAG
GAP-F	21	GAPDH sequence	ATCACTGCCACCCAGAAGACT
GAP-B	21	GAPDH sequence	CATGCCAGTGAGCTTCCCGTT

final extension of 5 min at 72 °C. The 974 bp product was gel purified using the Wizard PCR Preps DNA Purification System and subcloned into pCR-Script Amp SK(+) as outlined by the manufacturer (Stratagene), and designated as the activin $\beta_{E1,2}$ clone.

The sequence of the 5'-region of the rat activin β_E mRNA/cDNA was obtained by RT-PCR using a forward primer taken directly from the mouse β_E sequence (Fang *et al.* 1996) and a reverse primer based on the sequence obtained from the activin $\beta_{E1,2}$ clone. A rat activin β_E fragment was obtained from hepatic cDNA using primers β_E 5 and β_E 6 in a touch-down PCR protocol. Cycling conditions were as outlined above with the exception that a 2-min extension time was employed. The resultant 369 bp product was gel purified using a Wizard column and subcloned into pCR-Script Amp SK(+). The cloned product was designated as the activin $\beta_{E5,6}$ clone.

The sequence of the 3'-region of rat activin β_E mRNA/cDNA was obtained using the RACE method essentially as described elsewhere (Troutt *et al.* 1992). Rat hepatic total RNA was reverse transcribed using the primer RTP and AMV reverse transcriptase (Table 1). The equivalent of 0.15 μ g reverse transcribed RNA was then amplified in a touch-down PCR protocol. An oligonucleotide designed to the 3'-region of known internal rat activin β_E sequence (β_E 11) was used as the forward primer. The reverse primer was the anchor primer 3 (AP3). The cycling conditions were as outlined above with the exception that a 6-min extension time was used. The resultant PCR products were purified using Wizard purification columns and subcloned into pCR-Script Amp SK(+).

The inserts of all plasmids were sequenced at the Monash University Microbiology Department

DNA Sequencing Facility (Clayton, Australia) from dye-terminator PCR reactions using an ABI Model 330 gene sequencer (Perkin Elmer, Warrington, Cheshire, UK). In order to ensure the veracity of sequences, both DNA strands and inserts from multiple colonies of each plasmid were sequenced. The Blast-N and Blast-P programs (Altschul *et al.* 1990) were used to compare the nucleotide and deduced amino acid sequences obtained for clones with the information stored in the genomic and protein sequence databases. Signal peptide prediction was carried out using the SignalP program (Nielsen *et al.* 1997) and the predicted pI and M_r were calculated using the Compute pI/MW program (Bjellqvist *et al.* 1993, 1994).

Rat activin β_E expression studies

RT-PCR analysis of the tissue distribution of activin β_E in the rat

The tissue expression pattern of β_E mRNA in the rat was determined using an RT-PCR approach. Tissue and total RNA were purified as outlined above from rat prostate, testis, liver, epididymis, kidney, uterus, small intestine, lung, ovary, brain, spleen and seminal vesicles and from the human prostate cell lines DU145 and LNCaP. The mRNA was reverse transcribed and the activin β_E cDNA was specifically amplified using the primers β_E 1 and β_E 2 as described above. Products were visualised on 1% agarose gels and bands of approximately the predicted size of 974 bp were subcloned into pCR-Script Amp SK(+) and sequenced. The presence of cDNA in each RT reaction was confirmed by amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using the primers GAP-F and GAP-B using the conditions that are outlined above. The

tissue distribution was confirmed in three separate experiments using RNA from two different preparations.

As described in the results, the sequencing of activin β_E rat clones indicated that two different length activin β_E transcripts were present, the longer of which completely contained the shorter transcript plus an additional 738 bp at the 3' end. The existence and identity of the longer transcript was further investigated in the rat liver and lung using an activin β_E long transcript specific PCR. Any long activin β_E transcript was specifically amplified from liver or lung with the PCR primers β_{E12} and β_{E17} using the touch-down protocol outlined above with a 4-min extension time. Specific amplification gave a product of 1320 bp that was subcloned into pCR-Script Amp SK(+) (clone $\beta_{E12,17}$) and the sequence was confirmed to be activin β_E by automated sequencing. The presence of the short form of activin β_E in the lung was determined using the 3' RACE method as described above. Results were obtained from three separate experiments.

Digoxigenin (DIG)-labelled riboprobe preparation and in situ hybridisation

The expression of activin β_E mRNA within the rat livers ($n=2$) was determined by *in situ* hybridisation with two different DIG-labelled antisense cRNA probes using a previously described method (O'Bryan *et al.* 1998). The plasmids used for the generation of riboprobes were $\beta_{E3,4}$ and $\beta_{E5,6}$. The PCR $\beta_{E3,4}$ product corresponded to a 270 bp fragment of the protein coding region, and the $\beta_{E5,6}$ product corresponded to the 5'-untranslated region of the β_E cDNA. Plasmid inserts were prepared using the primers listed in Table 1. Labelled sense and antisense cRNAs were synthesised by incubating either the Not1 (antisense) or HindIII (sense) linearised template (1 μ g) with DIG-labelled UTP (3.5 μ mol/l) in the presence of T3 or T7 RNA polymerase, appropriate for the respective restriction enzyme employed, for 2 h at 37 °C. Hybridisation was performed overnight at 50 °C. This step was followed by sequential 15-min washes in SSC solutions to a maximum stringency of $0.1 \times$ SSC at 50 °C.

Northern blotting

Approximately 20 μ g liver and lung RNA from saline-treated control and LPS-treated rats were fractionated by electrophoresis on a 1% agarose formaldehyde gel and transferred to Nytran+ membrane (Schleicher & Schuell, Keene, NH, USA) in $10 \times$ SSC. RNA was fixed onto membranes by baking at 80 °C for 2 h. α^{32} P-Labelled cDNA probes, corresponding to either the $\beta_{E1,2}$ or

$\beta_{E5,6}$ clone inserts, were prepared using the Strip-EZ DNA probe synthesis kit (Ambion Inc., Austin, TX, USA). Unincorporated nucleotides were removed using Probe Quant micro-columns (Pharmacia Biotech, Uppsala, Sweden). Membranes were prehybridised in Ultrahyb (Ambion Inc.) at 42 °C for 4 h prior to hybridisation overnight at 42 °C in fresh prehybridisation buffer containing 1×10^6 c.p.m./ml α^{32} P-labelled cDNA. Membranes were washed to a maximum stringency of $0.1 \times$ SSC, 0.1% SDS at 65 °C prior to autoradiography. The amount of RNA loaded in each lane was equalised by comparison with GAPDH expression. GAPDH mRNA expression was determined by Northern blotting as outlined above using a 650 bp cDNA fragment of the rat GAPDH sequence for each treatment group. Northern analysis for the activin β_E mRNA was repeated for three rats per time-point. Signals were detected using a phosphorimager screen and read in a Fuji BAS1000 MacBAS Bio-Imaging Analyzer (Berthold, Sydney, Australia). Phosphorimager results were generated using MacBAS version 2.4 software supplied by the manufacturer. After background subtraction, the luminescence intensity of each rat activin β_E mRNA band was corrected for sample loading differences against the GAPDH mRNA band for the corresponding sample. The corrected data were normalised against the samples collected from untreated rats, which were assigned an arbitrary value of 1.0. Values for each β_E mRNA transcript, as well as the ratio between the band intensity of long and short mRNA transcripts for each animal, were analysed by ANOVA, followed by Dunnett's test for comparison of multiple means using Sigmasat software version 1.0 (Jandel Corp., San Rafael, CA, USA).

RESULTS

Cloning of rat activin β_E cDNA and sequence analysis

Rat activin β_E cDNA was cloned using a combination of PCR techniques (Fig. 1). The cDNA sequence of the rat activin β_E encodes a putative prepro-protein of 350 amino acids which, when proteolytically cleaved between amino acids 236 and 237, would yield a mature polypeptide of approximately M_r 12 500 with a pI of ~ 5.1 . Comparison with the published mouse activin β_E sequence (Fang *et al.* 1997) showed 93% homology at the nucleotide level, 93% at the level of the predicted prepro-protein and 96% homology at the level of the predicted mature polypeptide. The rat β_E gene was found to encode for two cDNAs with lengths of

[illegible]

FIGURE 1. The cDNA sequence of the rat actinin β_E subunit. The numbers on the left-hand side indicate the relative position of nucleotides. The numbers on the right-hand side indicate the relative position of predicted amino acids. Two β_E transcripts were isolated which were found to vary in the 3'-untranslated region. The shorter transcript is an orthologue of that reported for the mouse and was completely contained within the longer transcript. The 3'-most nucleotide of the shorter transcript is indicated by $\perp$. The highlighted areas indicate potential poly-adenylation consensus sites. ** Indicates a predicted proteolytic cleavage location which would, on the basis of data from other actinin subunits, give rise to the mature actinin β_E subunit. The boxed amino acids indicate a potential N-linked glycosylation site.

1.87 and 2.61 kb respectively in both the liver and lung (Fig. 1). (These sequence data have been submitted to DDBJ/EMBL/GenBank databases

under accession number AF089825. Subsequent to submission of our rat β_E cDNA sequence to GenBank, an additional submission of the Wistar rat

FIGURE 2. An RT-PCR analysis of the activin β_E expression in adult rat tissues and in two human prostate cell lines. Specific β_E cDNA was amplified using the β_{E1} and β_{E2} primers and yielded products of 974 bp (upper panel). The identities of bands were confirmed by sequencing. The presence of cDNA in each RT mix was confirmed by specific amplification of GAPDH (lower panel). Lanes contain RT-PCR products from: (1) prostate, (2) testis, (3) liver, (4) epididymis, (5) DU145, (6) LNCaP, (7) kidney, (8) uterus, (9) small intestine, (10) lung, (11) ovary, (12) brain, (13) spleen and (14) seminal vesicle.

protein coding region was made by W Rossmanith, B Peter & R Schulle-Hermann (accession number AF40032). The identity between the two sequences was 99.6% at the nucleotide level and 99.4% at the protein level.) The longer transcript contained the smaller transcript plus an additional 738 bp at the 3'-end. Four putative poly-adenylation consensus sequences were detected in rat β_E sequence, two of which were in the appropriate position to generate the short and long transcript, i.e. approximately 10–30 nucleotide 5' to the start of the poly-A tail (Sachs & Wahle 1993) (Fig. 1).

Similar to other activin β subunits, the amino acid sequence of the putative rat activin β_E prepro-protein contained a predicted signal peptide of 24 amino acids, one potential N-linked glycosylation site, and 13 cysteine residues. Homology alignment of the cysteine residues of the C-terminal region of the rat activin β_E prepro-protein with the amino acid sequences of other activin β subunits is consistent with the presence of a canonical cystine knot for the mature rat activin β_E polypeptide and a single cysteine available for disulphide bond formation with other β subunits (McDonald & Hendrickson 1993). A proteolytic cleavage site was predicted between amino acids 236 and 237. By analogy with the known activin β_A and β_B subunits sequences, cleavage at this point would give rise to the mature rat activin β_E subunit (Fig. 1).

Rat activin β_E expression studies

The activin β_E transcript content was assessed by RT-PCR in 12 rat tissues and two human prostate

cell lines. The only tissues that were found to contain activin β_E mRNA using this protocol were rat liver and lung (Fig. 2). cDNA sequence data from 3' RACE indicated that two different activin β_E transcripts were present in rat liver and lung. This result was confirmed using the long transcript specific PCR primer and sequencing (PCR product $\beta_{E12,17}$) and Northern blot analysis (Figs 3 and 5). *In situ* hybridisation on liver sections from normal adult, male rats indicated that the activin β_E transcript was found within hepatocytes and tended to be more concentrated (as indicated by staining intensity) in hepatocytes that were in closer proximity to the central vein of the hepatic lobules (Fig. 4). Parenchymal cells further from the central vein contained less or no activin β_E mRNA. Under these *in situ* hybridisation conditions, Kupffer cells, hepatic stellate cells and endothelial cells did not contain detectable activin β_E mRNA. *In situ* hybridisation using a sense β_E cRNA did not result in any staining of rat liver sections, thus indicating the specificity of the antisense cRNA hybridisation. With rat lung, activin β_E mRNA was not detected by *in situ* hybridisation or Northern blot analysis, presumably due to low mRNA abundance.

Within the liver, the longer activin β_E mRNA transcript appeared to be present at slightly higher abundance than the shorter transcript (Fig. 5, time 0). The injection of 0.1 mg/kg LPS into adult male rats resulted in a significant increase in hepatic activin β_E mRNA expression (Fig. 5A) following normalisation of loading using GAPDH mRNA expression levels (Fig. 5B). Levels of the long

FIGURE 3. An RT-PCR analysis of rat liver (H) and lung (L) tissue for the rat activin β_E long transcripts run on an ethidium bromide-stained 1% agarose gel. Easily detectable levels of the long (β_E L) activin β_E transcripts were found within the liver (lane 3), whereas lung tissue contained lesser amounts of the longer β_E transcript (lane 2). Lane 1 contains DNA size markers with their respective sizes indicated.

transcript increased by more than twofold at 6 h post-injection (Fig. 5C). A similar profile was also observed for the β_E short transcript (Fig. 5D, time 6); however, there was a preferential stimulation of the short transcript, so that the short transcript was slightly more abundant than the longer transcript after LPS treatment (Fig. 5E). Although the levels of both transcripts appeared to return to normal thereafter, the increased ratio of long-to-short transcript persisted for at least a further 72 h. Similar Northern analysis results were obtained using the rat activin $\beta_{E1,2}$ and the $\beta_{E5,6}$ plasmid inserts as probes (data not shown).

Northern blotting of LPS-treated rats also revealed the size of the full-length β_E transcripts as approximately 3.1 kb and 2.25 kb. The difference of approximately 850 bp between the short and long transcripts is consistent, within the resolution of agarose gel electrophoresis, with that predicted from RT-PCR sequencing data. The possibility remains however, of further transcripts with alternatively processed 5'-untranslated regions.

DISCUSSION

The rat activin β_E transcript has been cloned, sequenced and found to be expressed only in liver and lung tissue. Consistent with the species relationships for activin β_A and β_B , rat activin β_E was found to be highly homologous to the mouse sequence at both the nucleotide and the predicted amino acid level. Both lung and liver possessed two transcripts, which differed by virtue of a 738 bp extension in the 3'-untranslated region of the longer transcript. These data are suggestive of either the involvement of alternative polyadenylation consensus sites or an alternative splicing event in response to different stimuli. Although the significance of this 3'-extension remains to be determined, it could, however, contribute to changes in mRNA stability, translational delay or modulation of the 20S ribosomal assembly. The observed tissue distribution of activin β_E in the rat is consistent with the results of Fang *et al.* (1996) who found activin β_E only in the liver of the mouse using the less sensitive method of Northern analysis. Injection of the inflammatory mediator LPS into rats resulted in a significant elevation in β_E transcript levels in the liver 6 h post-injection. These significant differences in relative abundances of the two different length transcripts following LPS injection suggest that a degree of differential regulation occurs in response to this inflammatory stimulus.

The increase in activin β_E mRNA levels at 6 h post-LPS injection correlates with a pattern of an acute-phase response in the liver (Moshage 1997). The stimulation may arise directly through the action of LPS on hepatocytes or via the mediation of proinflammatory cytokines, such as tumor necrosis factor- α , IL-1 β or IL-6.

As yet the function and biological actions of the activin β_E subunit remains unknown. Moreover, its potential to heterodimerise with other members of the activin family, to produce either heterodimeric activins or inhibin, or to interact with the binding proteins such as follistatin and α_2 -macroglobulin which are also expressed within the liver (Lorent *et al.* 1994, Zhang *et al.* 1997b) remains to be clarified. The activin β_A , β_B and β_C subunits and follistatin are known to be expressed in the liver of various species (Yasuda *et al.* 1993, Fang *et al.* 1996, de Bleser *et al.* 1997). β_D has not been found in mammals. If all forms of the activin β subunits known to be present in mammalian tissues were able to undergo homo- and heterodimerisation with each other, this process would potentially allow the formation of ten different members of the activin sub-family, some or all of which may have different biological actions. The restriction in expression of

FIGURE 4. The expression of the activin β_E mRNA in the adult rat liver. The activin β_E mRNA was detected by *in situ* hybridisation using two antisense cRNA probes generated from plasmids $\beta_{E3,4}$ (C and D) and $\beta_{E5,6}$ (E). The specificity of the *in situ* hybridisation technique was confirmed by hybridising sections with sense cRNA probes generated from the $\beta_{E3,4}$ plasmid (A) and the $\beta_{E5,6}$ plasmid (B). The activin β_E mRNA was found within hepatocytes in close proximity to the central veins (arrows). Those further away from the central vein contained less or no β_E transcript. Endothelial cell, Kupffer cells and hepatic stellate cells did not appear to contain β_E mRNA. Panel C, 40 $\times$ magnification. Panels A, B, D and E, 100 $\times$ magnification.

FIGURE 5. Representative Northern blot of activin β_E (A) and GAPDH (B) expression in rat liver samples following LPS injection. Arrows indicate the calculated size of detected activin β_E transcripts. Samples collected at each time-point after injection were analysed for the relative expression of the long (C) and short (D) normalised against GAPDH mRNA expression and the ratio of long-to-short activin β_E transcripts plotted (E). Values are means $\pm$ S.E.M. (n =three rats at each time-point). *Significantly different from untreated rats, i.e. 0 time-point.

activin β_E to the rat liver, with lower levels of expression in the lung, would suggest that activin β_E may be important in hepatic function. The present investigation indicates a role for activin β_E in inflammation or the acute-phase response. Further

clarification of this role will be of vital importance in order to unravel the function of activin β_E given the known expression of other activin subunits in the liver and the large number of processes in which activins have been implicated (Vale *et al.* 1994, de Paolo 1997).

Activins are known to have biological actions within the liver, for example, activin A ($\beta_A\beta_A$) is an autocrine regulator of parenchymal cell apoptosis (Yasuda *et al.* 1993). It can be proposed that activin A acts in concert with the mitogen hepatic growth factor (Zarnegar & Michalopoulos 1989) to maintain hepatic mass (Yasuda *et al.* 1993). This conclusion was supported by the relatively lower levels of activin A in areas of rapid cell division in livers from treated rats compared with control rats, following a reduction in hepatic mass due to carbon tetrachloride (CCl_4) exposure (de Bleser *et al.* 1997). In this previous study, the most rapidly dividing hepatocytes were those in contact with hepatic stellate cells which were producing hepatocyte growth factor. In terms of sequence homology, however, the activin β_E subunit shares the highest degree of similarity with the activin β_C subunit. Following partial hepatectomy, a different mRNA expression response occurs for the β_C subunit compared with that of activin β_A . This observation was suggestive of different biological roles for activin A and activin C in hepatic function (Zhang *et al.* 1997a). The results from the present study indicate that activin β_E may also have a different function from that of β_A in the liver.

In summary, we have cloned and sequenced the rat homologue/orthologue of the activin β_E subunit and found its expression to be largely restricted to the liver. Sequence data confirm that two transcripts exist for rat activin β_E , putatively encoding for the same protein, which differ by a 3' extension in the longer form.

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REFERENCES

- Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ 1990 Basic local alignment search tool. *Journal of Molecular Biology* **215** 403–410.

- Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F, Snachez JC, Frutiger S & Hochstrasser DF 1993 The focusing position of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* **14** 1023–1031.
- Bjellqvist B, Basse B, Olsen E & Celis JE 1994 Reference points for comparison of two-dimensional maps of proteins from different human cell types defined in a pH scale where isoelectric points correlate with polypeptide compositions. *Electrophoresis* **15** 529–539.
- de Bleser PJ, Niki T, Xu G, Rogiers V & Geerts A 1997 Localization and cellular sources of activins in normal and fibrotic rat liver. *Hepatology* **26** 905–912.
- Brosh N, Sternberg D, Hongigwachs-Sha'anani J, Lee C-C, Shav-Tal Y, Tzehoal E, Shulman LM, Toledo J, Hacham Y, Carmi P, Jiang W, Sasse J, Horn F, Burstein Y & Zipori D 1995 The plasmacytoma growth inhibitory restrictin-P is an antagonist of interleukin 6 and interleukin 11. *Journal of Biological Chemistry* **270** 29594–29600.
- Chomczynski P & Sacchi N 1987 Single step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159.
- Cybulsky MI, Chan MKW & Movat HZ 1988 Acute inflammation and microthrombosis induced by endotoxin, interleukin-1, and tumor necrosis factor and their implication in gram-negative infection. *Laboratory Investigation* **58** 365–378.
- Fang J, Yin W, Smiley E, Wang SQ & Bonadio J 1996 Molecular cloning of the mouse activin β_E subunit gene. *Biochemical and Biophysical Research Communications* **228** 669–674.
- Fang J, Wang SQ, Smiley E & Bonadio J 1997 Genes coding for mouse activin β_C and β_E are closely linked and exhibit a liver-specific expression pattern in adult tissues. *Biochemical and Biophysical Research Communications* **231** 655–661.
- Hotten G, Neidhardt H, Schneider C & Pohl J 1995 Cloning of a new member of the TGF β family; a putative new activin β_C chain. *Biochemical and Biophysical Research Communications* **206** 608–613.
- Hully JR, Chang L, Schwall RH, Widmer HR, Terrell TG & Gillet NA 1994 Induction of apoptosis in the murine liver with recombinant human activin A. *Hepatology* **20** 854–862.
- Katsahambas S & Hearn MTW 1997 Application of an optimised reverse transcriptase–polymerase chain reaction method for the determination of the cDNA nucleotide sequence of porcine fibroblast growth factor. *Journal of Biochemical and Biophysical Methods* **33** 231–243.
- Lorent K, Overbergh L, Delabie J, van Leuven F & van den Bergh H 1994 Distribution of mRNA coding for alpha-2-macroglobulin, the murinoglobulins, the alpha-2-macroglobulin receptor and the alpha-2-macroglobulin receptor associated protein during mouse embryogenesis and in adult tissues. *Differentiation* **55** 213–223.
- McDonald NQ & Hendrickson WA 1993 A structural superfamily of growth factors containing a cystine knot motif. *Cell* **73** 421–424.
- McPherson SJ, Thomas TZ, Wang H, Gurusinge CJ & Risbridger GP 1997 Growth inhibitory response to activin A and B by human prostate tumor cell lines, LNCaP and DU145. *Journal of Endocrinology* **154** 535–545.
- Moshage H 1997 Cytokines and the hepatic acute phase response. *Journal of Pathology* **181** 257–266.
- Nielsen H, Engelbrecht J, Brunak S & von Heijne G 1997 Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* **10** 1–6.
- O'Bryan MK, Loveland LL, Herszfeld D, McFarlane JR, Hearn MTW & de Kretser DM 1998 Identification of a rat testis-specific gene encoding a potential rat outer dense fibre protein. *Molecular Reproduction and Development* **50** 313–322.
- O'Bryan MK, Schlatt S, Phillips DJ, de Kretser DM & Hedger MP 2000 Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels *in vivo*. *Endocrinology* **141** 238–246.
- Oda S, Nishimatsu S & Ueno N 1995 Molecular cloning and functional analysis of a new activin beta subunit: a dorsal mesoderm-inducing activity in *Xenopus*. *Biochemical and Biophysical Research Communications* **210** 581–588.
- de Paolo LV 1997 Inhibins, activin, and follistatins: the saga continues. *Proceedings of the Society for Experimental Biology and Medicine* **214** 328–339.
- Russell CE, Hedger MP, Brauman JN, de Kretser DM & Phillips DJ 1999 Activin A regulated growth and acute phase proteins in the human liver cell line, HepG2. *Molecular and Cellular Endocrinology* **148** 129–136.
- Sachs A & Wahle E 1993 Poly(A) tail metabolism and function in eucaryotes. *Journal of Biological Chemistry* **268** 22955–22958.
- Schwall RH, Robbins K, Jardieu P, Chang L, Lai C & Terrell TG 1993 Activin induces cell death in hepatocytes *in vivo* and *in vitro*. *Hepatology* **18** 347–356.
- Trout AB, McHeyzer-Williams MG, Pulendran B & Nossal GJV 1992 Ligation-anchored PCR: a simple amplification technique with single-sided specificity. *Proceedings of the National Academy of Sciences of the USA* **89** 9823–9825.
- Vale W, Bilezikjian LM & Rivier C 1994 Reproductive and other roles of inhibins and activins. In *The Physiology of Reproduction*, edn 2, pp 1861–1878. Eds E Knobil & JD Neill. New York: Raven Press Ltd.
- Yasuda H, Mine T, Shibata H, Eto Y, Hasegawa Y, Takeuchi T, Asano S & Kojima I 1993 Activin A: an autocrine inhibitor of initiation of DNA synthesis in rat hepatocytes. *Journal of Clinical Investigation* **92** 1491–1496.
- Yu EW, Dolter KE, Shao L-E & Yu J 1998 Suppression of IL-6 biological activities by activin A and implications for inflammatory arthropathies. *Clinical and Experimental Immunology* **112** 126–132.
- Yu J & Dolter KE 1997 Production of activin A and its roles in inflammation and hematopoiesis cytokines. *Cellular and Molecular Therapy* **3** 169–177.
- Zarnegar R & Michalopoulos G 1989 Purification and biological characterization of human hepatopoietin A, a polypeptide growth factor for hepatocytes. *Cancer Research* **49** 3314–3320.
- Zhang YQ, Shibata H, Schrewe H & Kojima I 1997a Reciprocal expression of mRNA for inhibin β_C and β_A subunits in hepatocytes. *Endocrine Journal* **44** 759–764.
- Zhang YQ, Kanzaki M, Shibata H & Kojima I 1997b Regulation of the expression of follistatin in rat hepatocytes. *Biochimica et Biophysica Acta* **1354** 204–210.

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