Development 120, 189-197 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994

A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the müllerian duct

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SUMMARY

The activin and TGF-β type II receptors are members of a separate subfamily of transmembrane receptors with intrinsic protein kinase activity, which also includes the recently cloned TGF-β type I receptor. We have isolated and characterized a cDNA clone (C14) encoding a new member of this subfamily. The domain structure of the C14-encoded protein corresponds with the structure of the other known transmembrane serine/threonine kinase receptors. It also contains the two inserts in the kinase domain that are characteristic for this subfamily. Using in situ hybridization, C14 mRNA was detected in the mesenchymal cells located adjacent to the müllerian ducts of males and females at day 15 (E15) of embryonic development. Marked C14 mRNA expression was also detected in the female gonads. In female E16 embryos, the C14 mRNA expression pattern remained similar to that in E15 embryos. However, in male E16 embryos C14 mRNA was detected in a circular area that includes the degenerating müllerian duct. The expression of C14 mRNA was also studied using RNase protection assays. At E15 and E16, C14 mRNA is expressed in the female as well as in the male urogenital ridge. However, at E19, a high C14 mRNA level in the female urogenital ridge contrasts with a lack of C14 mRNA in the male urogenital ridge. This correlates with the almost complete degeneration of the müllerian ducts in male embryos at E19. C14 mRNA expression was also detected in embryonic testes at E15, E16 and E19 using RNase protection assays, but at much lower levels than those found in the developing ovaries. In eleven other tissues no C14 mRNA was observed.

The results point to anti-müllerian hormone (AMH) being the most likely candidate ligand for C14. The embryonic C14 mRNA expression pattern in the urogenital ridge correlates with the expected site of AMH action, and C14 mRNA expression in the fetal ovary is in agreement with known effects of AMH on gonadal differentiation.

Postnatal C14 mRNA expression in rats was found to be confined mainly to the gonads. In the testis, C14 mRNA expression occurs in Sertoli cells. This testicular expression markedly increases during the first 3 weeks after birth, concurrent with the onset of spermatogenesis.

Key words: anti-müllerian hormone, activin, TGF-β, receptor, sex differentiation, testis, ovary

INTRODUCTION

The cloning of receptors for the peptide growth factors activin (Mathews and Vale, 1991; Attisano et al., 1992) and TGF-β (Lin et al., 1992, Ebner et al., 1993) has provided information about their overall structure and the molecular mechanism of signal transduction. The activin receptor types II and IIB and the TGF-β receptor types I and II, together with the *C. elegans* orphan receptor Daf-1 (Georgi et al., 1990) and several recently cloned rat orphan receptors (He et al., 1993), constitute a new subfamily of transmembrane protein kinase receptors. Based upon its primary structure, the kinase domain has a predicted specificity for serine and threonine residues. However, the mouse activin receptor type IIB has been shown to contain serine, threonine and tyrosine kinase activity (Nakamura et al., 1992).

The terms type II and type I refer to the nomenclature that is used for the different TGF-β receptors that have been described (Cheifetz et al., 1987). Crosslinking of radiolabelled TGF-β to cellular proteins allowed the identification of at least three different TGF-β binding proteins at the cell surface, named TGF-β receptor types I, II and III according to their relative molecular masses of 55, 80 and 280×10^3, respectively. The type III receptor is a betaglycan without a signalling motif (Wang et al., 1991), and is thought to play a role in the presentation and binding of TGF-β to the other receptor types

(López-Casillas et al., 1993). The TGF-β type I receptor cannot bind TGF-β in the absence of the type II receptor (Wrana et al., 1992, Ebner et al., 1993), and association with the type I receptor is essential for the TGF-β type II receptor to signal growth inhibition (Wrana et al., 1992). It is thought that this obligatory association between type I and type II receptors is a functional characteristic for all members of the serine/threonine kinase receptor family (Wrana et al., 1992). However, there are also indications that for some cellular responses to TGF-β the presence of only the type I or type II TGF-β receptor might be sufficient (Chen et al., 1993).

In this report, we describe the cloning and characterization of a rat cDNA, termed C14, which encodes a new member of the serine/threonine kinase receptor family. Based upon the cell-/tissue-specific C14 mRNA expression pattern, described herein, it will be discussed that the most likely candidate ligand of C14 is anti-müllerian hormone (AMH) (also called müllerian inhibiting substance; MIS).

AMH is a member of the activin and TGF-β family of peptide growth factors (Cate et al., 1986). These signalling molecules share numerous structural similarities. They are synthesized as large precursor molecules that form homo- or hetero-dimers. Each chain of the dimer is cleaved at approximately 110 amino acids from the C terminus, which generates the mature subunit and an N-terminal proregion. Sequence identity between members of this growth factor family is found mainly in the C-terminal mature subunits (25-80%; for review see: Massagué et al., 1990).

The known action of AMH in sex differentiation is very specific, when compared with the variety of functions that are performed by most other growth factors of the same family. For example, activin may regulate the secretion of follicle-stimulating hormone (FSH) by the pituitary gland (Vale et al., 1988), but also plays different roles in erythroid cell differentiation (Yu et al., 1987), neural cell survival (Schubert et al., 1990), and during embryogenesis (Thomsen et al., 1990; Hemmati-Brivanlou and Melton, 1992).

AMH is the earliest protein product known to be secreted by Sertoli cells in the fetal testis (Tran and Josso, 1982). Around day 15 of embryonic development in the male rat, AMH induces the regression of the müllerian ducts, which form the anlagen of the uterus, oviducts, and upper vagina. The Leydig cells in the developing testis produce testosterone, which stimulates the differentiation of the wolffian ducts into epididymides, vasa deferens and seminal vesicles. The window of sensitivity of the müllerian duct to AMH in the rat has been shown to be between E14.5-E15.5. After E16, exposure to AMH does not result in müllerian duct regression (Josso et al., 1977; Tsuji et al., 1992). In female embryos, the lack of androgens leads to wolffian duct regression, and the müllerian duct persists because the ovarian cells do not produce AMH. All these events form part of the earliest steps in sex differentiation following gonadal sex determination. In addition to its effect upon the müllerian duct, AMH may have a role to play in the development of ovaries and testes (Münsterberg and Lovell-Badge, 1991; Hirobe et al., 1992). The cellular mechanism by which AMH induces müllerian duct regression is poorly understood. Recent results show that AMH elicits its effect upon the müllerian duct epithelium most likely via the surrounding mesenchymal cells (Tsuji et al., 1992).

The expression of C14 mRNA during embryonic sex differentiation, described in the present report, is in accordance with the hypothesis that C14 encodes a type I/II receptor for AMH.

MATERIALS AND METHODS

Tissue and RNA preparations

Sertoli cells and peritubular myoid cells were isolated from 21-day-old rats and cultured as described previously (Themmen et al., 1991; Blok et al., 1992). Round spermatids were isolated from 35-day-old rats as described by Grootegoed et al. (1986). Total tissues, isolated cell fractions, or cultured cells were snap frozen in liquid nitrogen and stored at −70˚C until used for RNA isolation. Total RNA was isolated using the LiCl/urea method (Aufray and Rougeon, 1980).

Isolation of C14 from a rat Sertoli cell cDNA library

Sertoli cells were cultured for 6 hours without (−T) or with 0.6 μM testosterone (+T), in the presence of 50 μg/ml cycloheximide. These cells were used to prepare −T and +T cDNA libraries in Lambda ZAPII (Stratagene, Westburg, Leusden, The Netherlands), using standard molecular biology techniques (Sambrook et al., 1989). All radiolabelled agents were from Amersham (‘s Hertogenbosch, The Netherlands). A +T-enriched subtracted probe was prepared (Sive and St John, 1988), using in vitro synthesized cRNA from the −T cDNA library as driver RNA. RNA was synthesized using an in vitro RNA transcription kit (Stratagene) according to the instructions of the manufacturer. Clones from the +T cDNA library that showed a stronger hybridization signal with the subtracted probe than with a −T cDNA probe were isolated, and possible induction by androgens was tested on northern blots containing +T and −T total RNA. Northern blotting and random-primed labelling of the C14 cDNA with [^{32}P]dATP was performed according to Sambrook et al. (1989). C14 mRNA expression initially seemed to be androgen responsive, but this was not confirmed in later experiments (Fig. 1).

Sequencing

The complete 1.9 kb C14 cDNA clone was sequenced on both strands using the dideoxy chain termination method (Sanger et al., 1977). The sequence was analyzed using the sequence analysis program Microgenie (Beckmann, Mijdrecht, The Netherlands). Computer searches were carried out using the UWGCG program (Devereux, 1992).

RNase protection assay

A *Pst*I fragment containing bp 1243-1640 from C14 was subcloned in pBluescript KS (Stratagene) and used to generate [^{32}P]UTP-labelled anti-sense transcripts in vitro. The follicle-stimulating hormone receptor (FSHR) RNA probe was obtained using a 386 bp *Hin*dIII/*Pvu*II fragment from the rat cDNA (Sprengel et al., 1990). The activin receptor type II (ActRII) RNA probe was obtained using a 569 bp rat ActRII cDNA fragment (de Winter et al., 1992). The control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA probe was synthesized using a construct containing a 291 bp *Xba*I/*Sau*3AI fragment from rat GAPDH cDNA (Fort et al., 1985). Approximately 5×10^4 cts/minute of C14, FSHR, or ActRII probe, together with 5×10^4 cts/minute of GAPDH probe, was mixed with 5 or 10 μg of total RNA in a total volume of 30 μl hybridization mixture containing 40 mM Pipes pH 6.4, 1 mM EDTA, 0.4 M NaCl and 80% (v/v) formamide. The hybridizations were performed overnight at 55˚C. The RNase protection assay was performed as described by Sambrook et al. (1989).

In situ hybridization

The C14 subclone that was used for RNase protection assays was also used to prepare sense and anti-sense ^{35}S-UTP-labelled transcripts in vitro. The probes were dissolved in 5 μl 0.2 M DTT. Immediately prior to use, the probe solution was mixed with 5 μl of 30 μg/μl S-

ATP (Calbiochem, Omnilabo, Breda, The Netherlands), incubated at 100˚C for 1 minute and diluted in prewarmed (55˚C) hybridization mixture to a final concentration of approximately 2×10^5 cts /minute per µl. The hybridization mixture contained: 50% (v/v) deionised formamide, 0.3 M NaCl, 10 mM Tris pH 8, 1 mM EDTA, 1× Denhardt's solution, 1 mg/ml yeast tRNA, 50 mM DTT and 10% (w/v) polyethylene glycol 6000.

The lower body region of embryos at E15 and E16 was separated from the head region and fixed in fresh Bouin's fixative overnight at 4˚C. All in situ hybridization procedures (tissue embedding, slide preparations, pretreatments, hybridization and posthybridization incubations) were performed as described by Zeller and Rogers (1991). The tissue was embedded in paraffin, and 8 µM sections were mounted on slides which were coated with poly-L-lysine. Adjacent sections where mounted and stained with Mayer's haematoxylin and eosin for tissue and cell identification. The hybridizations were performed at 55˚C overnight in moist chambers. The slides were dipped in 1:1 (v/v) dilution of Kodak NTB-2 emulsion and dried at room temperature for 2 hours, followed by exposure at 4˚C for 2 weeks. After developing, the sections were counterstained with Nuclear fast red and mounted.

Genomic DNA isolation and PCR procedures

The head region of the embryos at E15 and E16 was snap frozen in liquid nitrogen and used for genomic DNA isolation according to Davis et al. (1986). This DNA was used in a DNA amplification reaction (Saiki et al., 1988) using primers for mouse Sbx and Sby (Mitchell et al., 1991, Kay et al., 1991) as described by Mitchell et al. (1991). The product obtained with the Sbx primers (approximately 250 bp) was used as a positive control to test the quality of the DNA, and the presence or absence of a product with the Sby primers (approximately 1000 bp) was used to determine the sex of the embryos (results not shown).

RESULTS

Selection of C14

C14 was isolated from a Sertoli cell cDNA library as described in Materials and methods. The 1.9 kb C14 cDNA probe hybridizes on northern blots to a major transcript of approximately 2.5 kb, which is expressed at high levels in cultured Sertoli cells (Fig. 1). However, there was no significant stimulation of the expression by testosterone. The tissue specificity of C14 mRNA expression was determined using an RNase protection assay. Fig. 2 shows that C14 mRNA expression in the adult rat is mainly restricted to the gonads. Based upon this marked tissue specificity of C14 mRNA expression, C14 was selected for further investigation.

C14 sequence analysis

The complete 1.9 kb C14 cDNA was sequenced. It contains a single open reading frame that encodes a 557 amino acid protein, starting from the first methionine codon at residue 60 (Fig. 3A). This ATG codon and its context form a potential functional initiation site (Kozak, 1987). Analysis of the protein sequence indicated the presence of a signal sequence with a predicted cleavage site before Gln^{16} (von Heijne, 1986). A hydrophobic region between amino acid residues 142-168 represents the single putative transmembrane domain (TMD). Thus, the mature protein consists of an extracellular domain of 126 amino acid residues, a TMD of 27 amino acid residues, and an intracellular domain of 389 amino acid residues. The extracellular domain is cysteine rich and contains 2 potential *N*-glycosylation sites.

EMBL and GenBank database searches revealed that the intracellular domain of C14 is most closely related to the intracellular domains of the mouse activin receptor types II (ActRII; Mathews and Vale, 1991) and IIB (ActRIIB; Attisano et al., 1992), the human TGF-β type II receptor (TGF-βRII; Lin et al., 1992), the mouse TGF-β type I receptor (TGF-βRI; Ebner et al., 1993), and the *C. elegans* orphan receptor Daf-1 (Georgi et al., 1990) (Fig. 3B). The ActRII, ActRIIB, TGF-βRII, TGF-βRI and Daf-1 together form a separate subfamily of receptors with intrinsic protein kinase activity (Mathews and Vale, 1991; Lin et al., 1992). Recently, several rat orphan receptors have been cloned, that are also members of this newly defined receptor family (He et al., 1993).

The C14 intracellular kinase domain contains the 12 subdomains, which are found in all protein kinases, in the proper order (Hanks et al., 1988), and also contains the two inserts located between subdomains VI-A and VI-B and between subdomains X and XI, that are characteristic for the subfamily (Mathews and Vale, 1991). The percentage identical amino acids of the kinase domain of C14 compared to the ActRII, TGF-βRII, TGF-βRI and Daf-1 kinase domains is 34%, 32%, 33% and 29%, respectively.

The sequences in the kinase subdomains VI-B and VIII indicate substrate specificity (Hanks et al., 1988). The relevant sequences in C14 within these subdomains are more in accordance with the serine/threonine kinase consensus sequence than with the tyrosine kinase consensus sequence, with the exception of serine residue 333. At this position in the kinase domain, a lysine residue is found in almost all known serine/threonine kinases including ActRII, ActRIIB, TGF-βRII, TGF-βRI and Daf-1.

The extracellular domains of C14, ActRII and TGF-βRII show a relatively low level of sequence identity (Fig. 3C). However, the position of most cysteine residues is conserved.

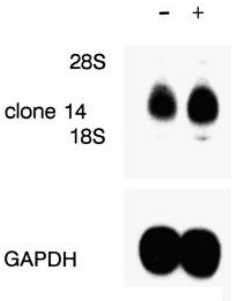

Fig. 1. C14 mRNA expression in cultured Sertoli cells. C14 cDNA probe was hybridized to a northern blot containing 20 µg total RNA isolated from Sertoli cells cultured in the absence (–) or presence (+) of testosterone (see also Materials and methods). Clone 14 indicates the 2.5 kb band of C14 mRNA, abundantly expressed under both culture conditions. The GAPDH hybridization indicates that equal amounts of mRNA were loaded in each lane. 18S and 28S indicate the locations of the respective rRNA bands.

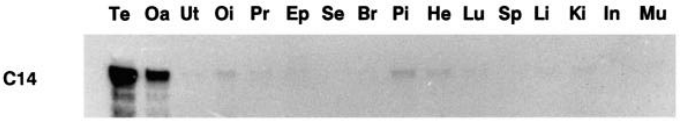

Fig. 2. Tissue specificity of C14 mRNA expression. RNase protection assays were used to study the expression of C14 mRNA in different tissues. From 4-week-old rats: Te, testis; Oa, ovary; Ut, uterus. From adult rats: Oi, oviduct; Pr, prostate; Ep, epididymis; Se, seminal vesicle; Br, brain; Pi, pituitary gland; He, heart; Lu, lung; Sp, spleen; Li, liver; Ki, kidney; In, intestine; Mu, muscle. 10 µg of total RNA was used for each lane. C14 indicates the position of the protected RNA fragment. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was present in all samples (not shown).

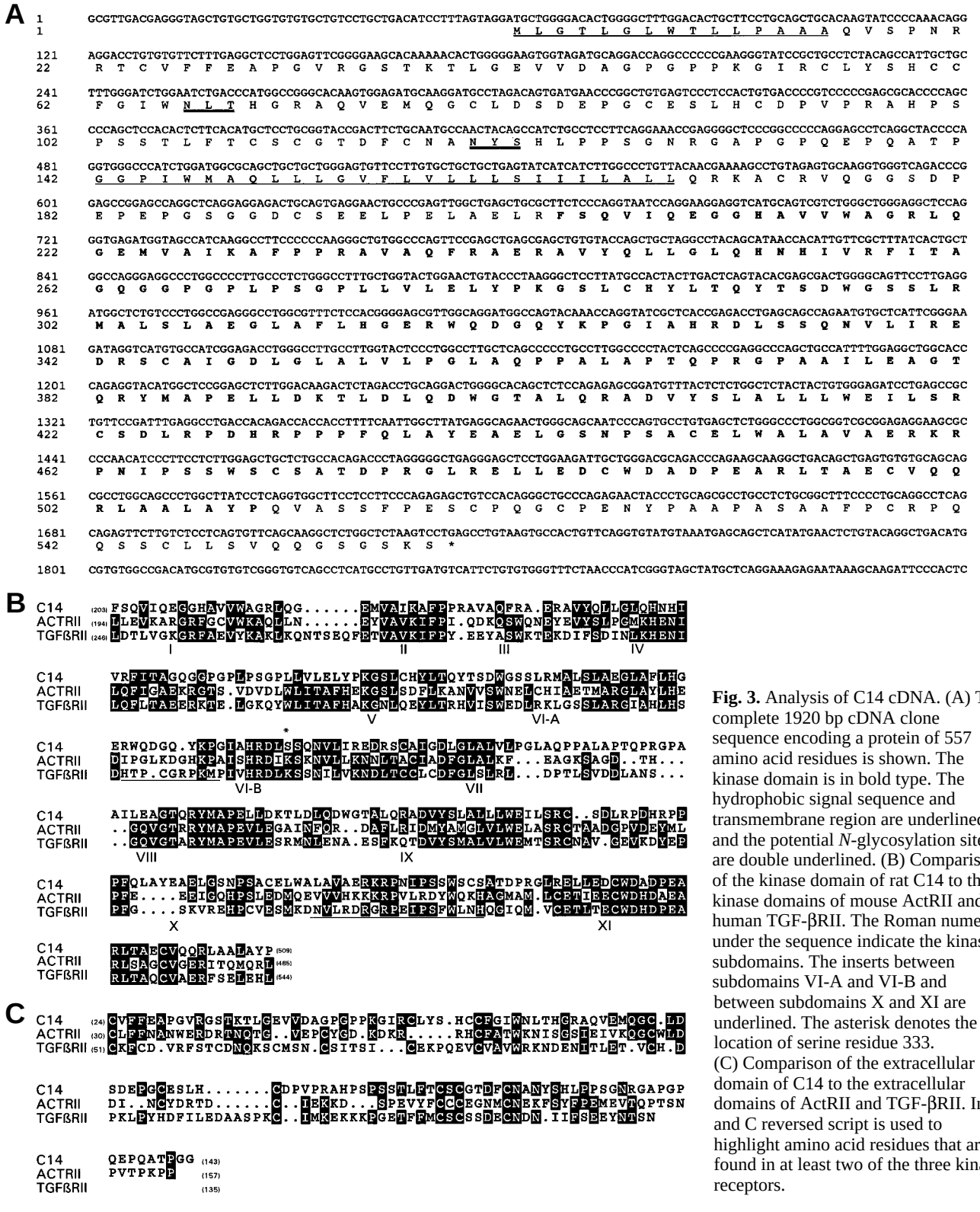

Fig. 3. Analysis of C14 cDNA. (A) The complete 1920 bp cDNA clone sequence encoding a protein of 557 amino acid residues is shown. The kinase domain is in bold type. The hydrophobic signal sequence and transmembrane region are underlined, and the potential *N*-glycosylation sites are double underlined. (B) Comparison of the kinase domain of rat C14 to the kinase domains of mouse ActRII and human TGF-βRII. The Roman numerals under the sequence indicate the kinase subdomains. The inserts between subdomains VI-A and VI-B and between subdomains X and XI are underlined. The asterisk denotes the location of serine residue 333. (C) Comparison of the extracellular domain of C14 to the extracellular domains of ActRII and TGF-βRII. In B and C reversed script is used to highlight amino acid residues that are found in at least two of the three kinase receptors.

C14 expression during embryonic sexual differentiation

The fact that C14 encodes a member of the activin and TGF-β type I/II receptor family, indicates that the C14 ligand will be a member of the activin and TGF-β-family of peptide growth factors. C14 is expressed mainly in the gonads in adult rats (present results), where anti-müllerian hormone (AMH) may perform specific functions (Hirobe et al., 1992; Münsterberg and Lovell-Badge, 1991). This pointed to AMH as an important candidate ligand for C14. To investigate this possi-

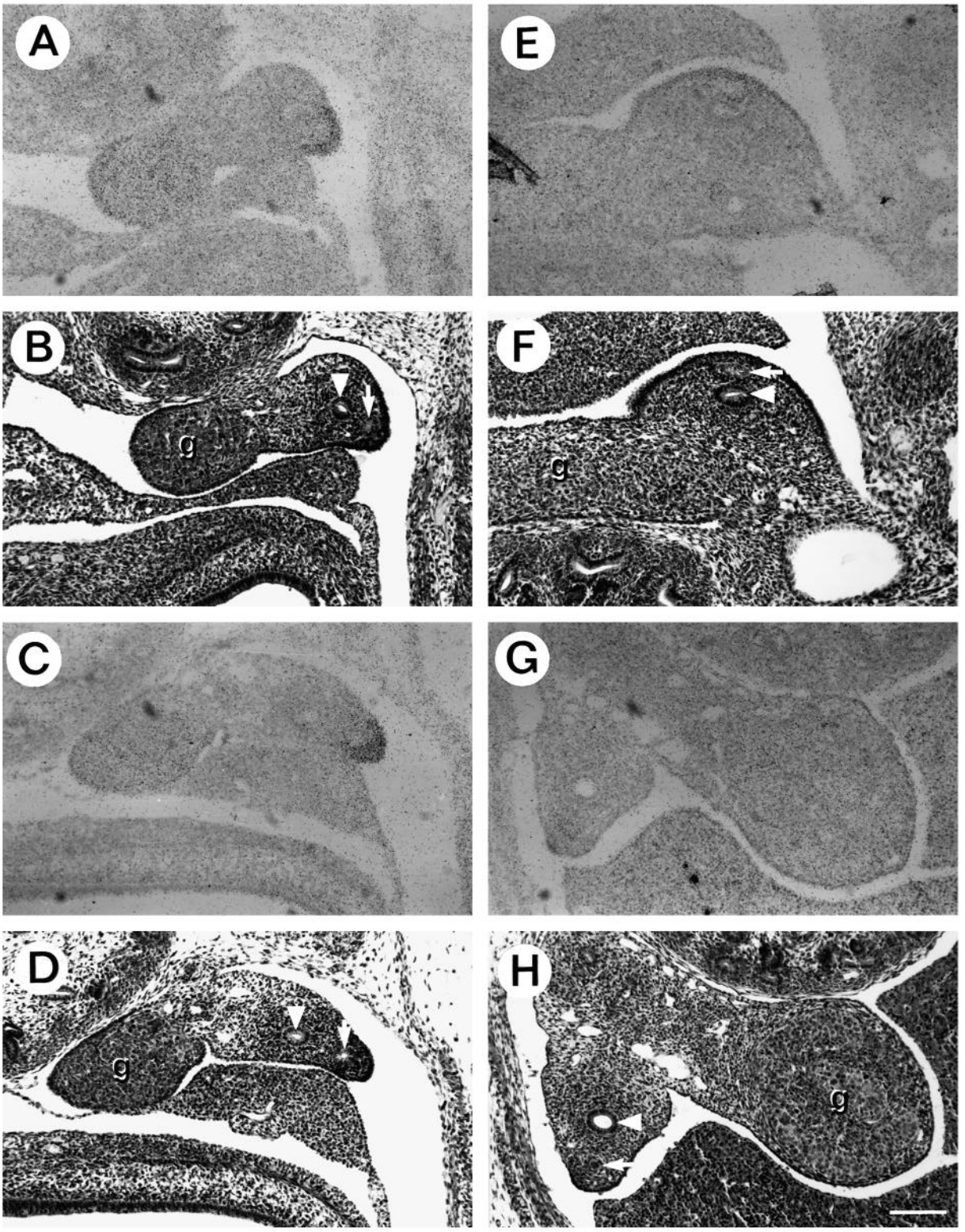

Fig. 4. Expression of C14 mRNA at E15 and E16. Bright-field photomicrographs from in situ hybridization of the antisense C14 RNA probe to paraffin sections (A,C,E,G) and from haematoxylin/eosin stained adjacent sections (B,D,F,H) of female embryos at E15 (A,B), E16 (C,D), and of male embryos at E15 (E,F) and E16 (G,H). Embryonal sex was determined as described in Materials and methods. g, gonad; arrowhead, wolffian duct; arrow, müllerian duct. Hybridization of the sense control C14 RNA probe did not result in signals above background (not shown).

bility, the expression pattern of C14 mRNA in the urogenital ridge of rat embryos during the induction of müllerian duct regression was studied.

Using in situ hybridization, marked C14 mRNA expression was detected in the female gonads, but not in the male gonads, of embryos at E15 and E16 (Fig. 4A-H). Furthermore, C14 mRNA is expressed in the female urogenital ridges at E15 and E16, in a sickle-shaped area that includes the mesenchymal cells between the müllerian duct and the coelomic epithelium (Fig. 4A-D). In the male urogenital ridges at E15, the expression of C14 mRNA is identical to the E15-E16 female pattern (Fig. 4E,F). However, this male expression pattern has clearly changed at E16, when C14 mRNA is present in a small circular area that includes the degenerating müllerian duct (Fig. 4G,H). No other sites of C14 mRNA expression above background were detected in the lower body halves of the embryos at E15 and E16.

RNase protection assays were used to study the expression of C14 mRNA in isolated urogenital ridges and gonads at E15, E16 and E19 (Fig. 5). C14 mRNA was detected at much higher levels in ovary than in testis at all embryonic stages tested. At E15, C14 mRNA expression in the urogenital ridge was detected at approximately equal levels in both male and female embryos. However, at E16, the C14 mRNA level in the male urogenital ridge is lower than in the female urogenital ridge. Finally, at E19, C14 mRNA was not detected in the male urogenital ridge, whereas the C14 mRNA level remains high in the female urogenital ridge. No C14 mRNA was detected in total RNA isolated from E19 intestine, skin, lung, liver, kidney, adrenal, stomach, heart, thymus, muscle, and brain (not shown).

Postnatal expression of C14 mRNA in testis and ovary

Different testicular cell types were isolated, to study the cellular location of C14 mRNA expression in the testis using RNase protection assay. It was observed that C14 mRNA is specifically expressed in Sertoli cells, at equal levels in cells isolated from 21-day-old or mature rats (Fig. 6A). No C14 mRNA was detected in round spermatids and peritubular myoid cells. ActRII mRNA, which is expressed at a high level in round spermatids (de Winter et al., 1992), was used to verify the integrity of the round spermatid mRNA. FSHR mRNA, which is expressed exclusively in Sertoli cells (Heckert and Griswold, 1991), was used to show the cellular colocalization of FSHR and C14 mRNAs and the absence of Sertoli cell contamination from the other isolated cell types.

C14 mRNA expression in total testis is very low at birth, and increases to a maximum at day 21 of postnatal development (Fig. 6B). Since we found similar levels of expression in Sertoli cells from immature and adult rats (Fig. 6A), the relative decrease in C14 mRNA expression in total testis between days 21 and 63 can be explained by the increasing population of C14-negative spermatids.

In the ovary of adult rats, marked expression of C14 mRNA was detected, using in situ hybridization, in the granulosa cell layers of small antral follicles (not shown).

DISCUSSION

Based upon the observation that in adult rats C14 mRNA expression is most abundant in the gonads, C14 was selected and characterized. C14 encodes a novel member of the activin and TGF-β type I/II serine/threonine kinase receptor family. Including C14, nine members of this gene family are presently known: Daf-1 encoding an orphan receptor (Georgi et al., 1990), TGF-βRII (Lin et al., 1992), TGF-βRI (Ebner et al., 1993), R2, R3 and R4, which are three orphan receptors cloned through PCR amplification of rat fetal urogenital ridge cDNA with a relatively high percentage of similarity to TGF-βRI in the kinase domain (He et al., 1993), and two genes encoding the different activin type II receptors, ActRII and ActRIIB (Mathews and Vale, 1991; Attisano et al., 1992). The sequence identity between ActRII and ActRIIB is 51% in the extracellular domain and 75% in the intracellular domain, and four different splice variants of ActRIIB have been described (Attisano et al., 1992).

There is complete structural homology between C14 and the other members of the serine/threonine kinase receptor family. The C14 kinase domain has the highest degree of amino acid identity with the kinase domain of ActRII (34%). However, the ActRII and TGF-βRII kinase domains are more closely related, showing 45% amino acid identity to one another. It has been shown by Nakamura et al. (1992) that ActRIIB is a dual specificity kinase, which can phosphorylate serine/threonine as well as tyrosine residues. It remains to be determined whether the change from the conserved lysine to a serine at position 333 in the C14 kinase domain has any significance with respect to substrate specificity or kinase activity.

Our hypothesis that AMH could be an important candidate ligand of C14 implies that C14 mRNA should be expressed in the urogenital ridge during the induction of müllerian duct degeneration by AMH, around day 15 of embryonic development in the rat (Jost, 1947; Tsuji et al., 1992). It was observed that C14 mRNA is expressed in an area that includes the mesenchymal cells between the müllerian duct and the coelomic epithelium at E15, in both male and female embryos. Recent results have shown that AMH can reduce DNA synthesis of cultured urogenital ridge mesenchymal cells but not of cultured müllerian duct epithelial cells (Tsuji et al., 1992). This suggests that AMH most likely elicits its effect upon the müllerian duct epithelium via the surrounding mesenchyme

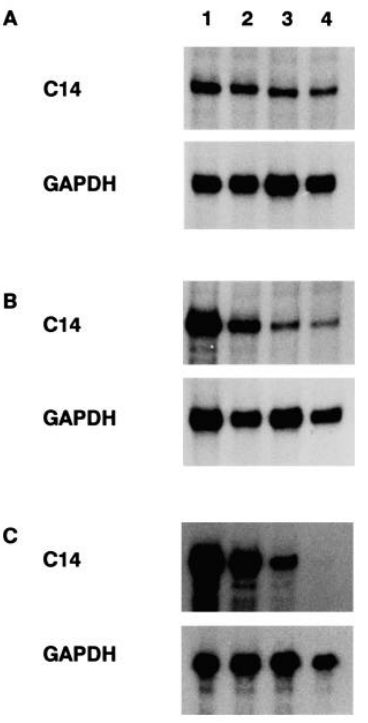

Fig. 5. Expression of C14 mRNA in different embryonal tissues at E15, E16 and E19. Embryos were collected at E15 (A); E16 (B) and E19 (C), and C14 mRNA expression was studied using RNase protection assays in: Lane 1, ovary; 2, female urogenital ridge; 3, testis; 4, male urogenital ridge. C14 and GAPDH indicate the positions of the respective protected fragments.

(Tsuji et al., 1992). Light and electron microscopic studies have demonstrated that the regression of the müllerian duct epithelium is closely associated with changes in the surrounding mesenchymal cells and changes in the epithelial-mesenchymal interface. One of the observed changes during müllerian duct regression is the formation of a dense circular whorl of mesenchymal cells around the epithelial duct (Trelstad et al., 1982). These observations on the cellular sites of AMH action and histological changes associated with müllerian duct regression, correlate with C14 mRNA expression.

The level of C14 mRNA in the urogenital ridges at E16 is relatively low in male compared to female embryos. At E19, C14 mRNA is expressed at a high level in the female urogenital ridge whereas at this stage it is no longer detected in the male urogenital ridge. This correlates with the gradual degeneration of the müllerian ducts in male embryos between E16 and E19 (Trelstad et al., 1982). Furthermore, in the female, the AMH receptor indeed persists after the critical period of induction of müllerian duct regression as can be concluded from the observation that AMH induces the formation of bulges in the cranial portion of the müllerian duct, in cultured urogenital ridges that were isolated from female embryos at E18.5 (Tsuji et al., 1992).

Using in situ hybridization at E15 and E16, marked C14 mRNA expression was detected in developing ovaries but not in testes. However, using RNase protection assays at E15, E16 and E19, C14 mRNA was detected at low levels in the testis (and at a high level in the ovary); the RNase protection assay appears to be more sensitive than in situ hybridization. In view of this C14 mRNA expression in fetal testis, AMH and its receptor might be involved in normal testis differentiation in the male. Overexpression of AMH in transgenic mice can induce formation of testis cord-like structures in fetal ovaries (Behringer et al., 1990). Furthermore, AMH exerts an inhibitory effect on aromatase activity in cultured fetal ovaries (Vigier et al., 1989). Aromatase catalyzes the conversion of testosterone to estradiol during female gonadal development, and inhibition of ovarian aromatase activity by AMH leads to production of testosterone rather than estradiol. These biological responses to AMH suggest that the AMH receptor indeed is present in fetal ovaries, where it is probably inactive due to the absence of AMH. Following day 3 of postnatal development, AMH is expressed in the ovary where it might play a role in oocyte maturation (Hirobe et al., 1992; Münsterberg and Lovell-Badge, 1991). In agreement with this, we observed postnatal ovarian expression of C14 mRNA in the granulosa cells of small antral follicles. Future studies will have to provide more information about the pattern and regulation of C14 mRNA expression in the ovary.

It is not known whether AMH plays a physiological role in the testis after birth. We have shown that C14 mRNA expression increases in Sertoli cells to a maximum during postnatal testis development, and remains high in the adult rat testis. With respect to testicular production of the ligand, it is known that testicular AMH mRNA expression decreases after birth, but persists at a low level throughout adulthood (Lee et al., 1992). Low levels of AMH may exert autocrine actions upon Sertoli cells in immature and mature testis. It is also possible that testicular AMH production varies at different stages of the spermatogenic cycle and hence involves local concentration gradients. Inherited defects in the biosynthesis of AMH do not seem to have a major effect on male fertility (Josso, 1992), but this does not exclude a quantitative defect in spermatogenesis. Moreover, other testicular factors (possibly members of the activin and TGF-β family of peptide growth factors) may partly compensate for a lack of AMH by binding to their specific receptors and activation of similar intracellular pathways.

The present results are in accordance with - but do not provide conclusive evidence for - the hypothesis that C14 encodes an AMH receptor. Evidence might be provided by showing AMH binding to cells that are transfected with a C14 expression vector. However, attempts that have been made in the past to show binding of AMH to its receptor on AMH-responsive cells have been hampered by the fact that radiolabelling of AMH seems to abolish its ability to bind to the receptor (Donahoe et al., 1977). Furthermore, conclusive results that identify a receptor ligand through a binding assay using a cell line that stably expresses the relevant receptor, might be difficult to obtain if this receptor behaves as a type I receptor; such a receptor would require cooperation with a specific type II receptor which may not be endogenously expressed in the cell line used. To resolve these problems, it will be necessary to develop a binding assay for AMH. Then the possible existence of different types of AMH receptors can be studied, and suitable model cell lines can be developed to test candidate receptors for ligand binding.

The persistent müllerian duct syndrome (PMDS) is a rare form of male pseudohermaphroditism that is characterized by

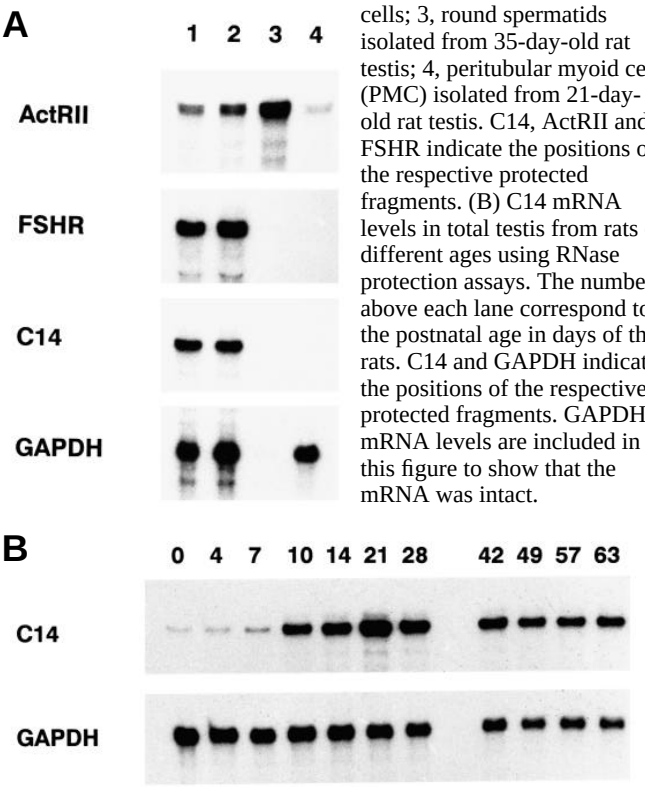

Fig. 6. C14 mRNA expression in testis. (A) The expression of C14, activin type II receptor (ActRII) and follicle-stimulating hormone receptor (FSHR) mRNAs in different testicular cell types, determined using RNase protection assays: Lane 1, 21-day-old rat Sertoli cells; 2, adult rat Sertoli cells; 3, round spermatids isolated from 35-day-old rat testis; 4, peritubular myoid cells (PMC) isolated from 21-day-old rat testis. C14, ActRII and FSHR indicate the positions of the respective protected fragments. (B) C14 mRNA levels in total testis from rats of different ages using RNase protection assays. The numbers above each lane correspond to the postnatal age in days of the rats. C14 and GAPDH indicate the positions of the respective protected fragments. GAPDH mRNA levels are included in this figure to show that the mRNA was intact.

the presence of uterus and fallopian tubes in otherwise normally virilized males (Josso et al., 1991). In some of these patients it has been shown that the phenotype is caused by a mutation in the AMH gene (Knebelmann et al., 1991), but other patients express a normal amount of bioactive testicular AMH (Guerrier et al., 1989). The existence of this type of PMDS indicates that mutation of the AMH receptor gene(s) can result in a complete loss of responsiveness to AMH. In the future, ultimate proof that C14 encodes an AMH receptor can be obtained through genetic analysis of PMDS, or when knock-out transgenic mice are generated that show the characteristics of PMDS.

This work was supported by the Dutch Science Foundation (NWO) through GB-MW (Medical Sciences). A. T. is a recipient of a fellowship of the Royal Netherlands Academy of Sciences (KNAW fellowship program). The authors wish to thank S. van Baal for the development of the DNA-RNA-protein analysis software.

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(Accepted 22 September 1993)