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Identification of a Novel Seven-Transmembrane Receptor with Homology to Glycoprotein Receptors and Its Expression in the Adult and Developing Mouse

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Using a PCR-based cloning strategy we have isolated a cDNA from mouse brain and named it fex, because it codes for a novel putative G protein-coupled receptor expressed in follicles. The deduced amino acid sequence shows a higher degree of homology to the family of glycoprotein receptors, namely those for FSH, LH, and TSH, than to other G protein-coupled receptors. With 18 leucine-rich repeats FEX exhibits features in its N-terminal portion characterizing it as unique within the glycoprotein receptor family. In the adult mouse fex expression was detected in the male and female gonads, the adrenal medulla, and the olfactory bulb of the brain. During embryonic development fex transcripts were detected transiently in various tissues, particularly in selected regions of the central nervous system, the developing face, the intervertebral discs anlagen, and the limb buds. Because fex was expressed during periods of active morphogenesis, it may be an important receptor for signals controlling growth and differentiation of specific embryonic tissues.

Key Words: **G protein-coupled glycoprotein receptor; expression pattern; mouse development; brain; gonads; mesenchyme.**

Vertebrate embryonic development involves a sequence of specific interactions between different tissues and cell types. A variety of different proteins is involved in these processes. Among those are members of the G protein-coupled receptor family which upon binding of a specific ligand transmit their signal by interaction with heterotrimeric G proteins (3). Some members of this family have been shown to be essential for proper embryonic development (7). All G protein-coupled receptors are characterized by seven transmembrane spanning regions preceded by an extracellular N-terminal portion of variable length. Depending on their ligands, extracellular domains, and specific sequence motifs they are grouped into different families. The receptors for the large glycoprotein hormones LH, TSH, and FSH show extended extracellular domains containing leucine-rich repeat regions, which are thought to be involved in ligand binding (8). We describe in this paper the isolation of a novel member of this receptor family which may be involved in signal transduction in the nervous system, in endocrine organs, and during embryonic development.

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MATERIALS AND METHODS

PCR amplification of the human fex fragment. In an attempt to identify fragments of genes coding for new G protein-coupled receptors expressed in brain, we performed PCR on a cDNA derived from uninduced NT2-cells (Stratagene) as template. NT2-cells are derived from a human teratocarcinoma and represent precursor cells committed to neuronal fates (2). Degenerate primers were designed from conserved regions of the extracellular loop 1 and the transmembrane domain VII of known heptameric receptors (12). The degenerate primers had the following sequence: sense, 5′-GGCC(G/T)TT(T/C) GG(G/C)(G/T)(A/C)I(G/C)(T/C)(C/G)(I/C)T(C/G)TG-3′ and antisense, 5′-(C/A)(C/G)GG AAG(C/G)CGTA(G/C)AG(G/C)A(T/G)IGG(G/A)TT-3′. The thermal cycling parameters were one minute of denaturation, followed by 35 cycles of 93°C (30 s), 50°C (30 s), and 72°C (30 s). Amplified DNA of the expected size of 600 bp was excised and cloned into the PCRII vector (Invitrogen). Several clones were sequenced and a data-bank search using the Blast algorithm (1) on the nonredundant NCBI data base was performed. One gene fragment, that revealed high homology to G protein-coupled receptors, was cloned in full length.

Cloning of the full-length fex cDNA. A newborn mouse brain cDNA library (Stratagene) was used as template. Gene-specific internal sense and antisense primers were designed from the cloned sequence, and anchored PCR was performed using either the T7 or the T3 primer. Products were cloned into the pGEM-T easy vector (Promega) and sequenced. The 3′-end of the cDNA was obtained in one step, the 5′-end required four sequential PCRs.

In situ hybridization. Embryos (E6.5–E18.5) from natural matings between inbred CD-1 mice (Charles River Laboratories, Wilmington, MA) were used. The midday of the occurrence of the vaginal plug was defined as E0.5. For *in situ* hybridizations on sections the animals were frozen on dry ice, and 10 μm sections were prepared on a cryostat (Leitz). Adult brains were sectioned at 15 μm. Antisense RNA probes, labeled with [α-^{35}S]UTP, were generated with Sp6 polymerase from the *Xho*I linearized 570-bp PCR clone correspond-

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   1 MDTSCVHMLL SLLALLQLVA AGSSPGPDAI PRGCPSHCHC ELDGRMLLRV
  51 DCSDLGLSEL PSNLSVFTSY LDLSMNNISQ LPASLLHRLC FLEELRLAGN
 101 ALTHIPKGAF TGLHSLKVLM LQNNQLRKVP EEALQNLRSL QSLRLDANHI
 151 SYVPPSCFSG LHSLRHLWLD DNALTDVPVQ AFRSLSALQA MTLALNKIHH
 201 IADYAFGNLS SLVVLHLHNN RIHSLGKKCF DGLHSLETLD LNYNNLDEFP
 251 TAIKTLSNLK ELGFHSNNIR SIPERAFVGN PSLITIHFYD NPIQFVGVSA
 301 FQHLPELRTL TLNGASHITE FPHLTGTATL ESLTLTGAKI SSLPQAVCDQ
 351 LPNLQVLDLS YNLLEDLPSL SGCQKLQKID LRHNEIYEIK GSTFQQLFNL
 401 RSLNLAWNKI AIIHPNAFST LPSLIKLDLS SNLLSSFPVT GLHGLTHLKL
 451 TGNRALQSLI PSANFPELKI IEMPSAYQCC AFGGCENVYK ISNQWNKDDG
 501 NSVDDLHKKD AGLFQVQDER DLEDFLLDFE EDLNALHSVQ CSPSPGPFKP
                    TM I
 551 CEHLFGSWLI RIGVWTTAVL TLSCNALVAL TVFRTPLYIS SIKLLIGVIA
      TM II                                  TM III
 601 VVDILMGVSS AVLAAVDAFT FGRFAQHGAW WEDGIGCQIV GFLSIFASES
                                          TM IV
 651 SIFLLTLAAL ERGFSVKCSS KFEVKAPLFS LRAIVLLCVL LALTIATIPL
                                 TM V
 701 LGGSKYNASP LCLPLPFGEP STTGYMVALV LLNSLCFLIM TIAYTKLYCS
                                TM VI
 751 LEKGELENLW DCSMVKHIAL LLFANCILYC PVAFLSFSSL LNLTFISPDV
                TM VII
 801 IKFILLVIVP LPSCLNPLLY IVFNPHFKED MGSLGKHTRF WMRSKHASLL
 851 SINSDDVEKR SCESTQALVS FTHASIAYDL PSTSGASPAY PMTESCHLSS
 901 VAFVPCL
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FIG. 1. Deduced amino acid sequence of the mouse orphan receptor FEX. The predicted signal peptide sequence is boxed, the seven transmembrane domains (TM) are overlined. Putative glycosylation sites are indicated by filled circles. Accession number.

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LRVDCSD-LG-LSELPSN--LS-VFT   48- 68
LDLSMNN-ISQLPASLLHR-LCFLEE   71- 94
LRLAGNA-LTHIPKGAFTG-LHSLKV   95-118
LMLQNNQ-LRKVPEEALQN-LRSLQS  119-142
LRLDANH-ISYVPPSCFSG-LHSLRH  143-166
LWLDDNA-LTDVPVQAFRS-LSALQA  167-190
MTLALNK-IHHIADYAFGN-LSSLVV  191-214
LHLHNNR-IHSLGKKCFDG-LHSLET  215-238
LDLNYNN-LDEFPTAIKT--LSNLKE  239-261
LGFHSNN-IRSIPERAF-VGNPSL--  262-283
ITIHFYDNPIQFVGVSAFQHLPELRT  284-309
LTLNGASHITEFPHLTGT--AT-LES  310-332
LTLTGAK-ISSLPQAVCDQ-LPNLQV  333-356
LDLSYN-LLEDLPSLSGCQK---LQK  357-328
IDLRHNE-IYEIKGSTFQQ-LFNLRS  329-402
LNLAWNK-IAIIHPNAFST-LPSLIK  403-326
LDLSSN-LLSSFPVTGLHG----LTH  327-448
LKLTGNRALQSLIPSANFPELKIIEM  449-473
LXLXXXXXLXXLXXXXXXXXLXXLXX  Consensus
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FIG. 2. Leu (Ile/Val)-rich repeats in the N-terminal region of FEX. Consecutive segments of the N-terminal region were aligned, gaps (dashed lines) where introduced to optimize the periodicity of the conserved leucine (isoleucine/valine) residues. These conserved residues are printed in bold. Amino acid positions are given at the right. Marked in black is the consensus sequence of the leucin-rich repetitive motif found in FEX.

ing to nucleotides 2140 to 2710 of the fex cDNA. A sense control was generated using T7 polymerase. *In situ* hybridization was performed as described (17).

For whole-mount *in situ* hybridizations (9) the 570-bp probe, described above, was labeled with digoxygenin-UTP (Boehringer-Mannheim). Adult tissues were cut into halves to facilitate the penetration of the probe and the antibodies. Specificity of the signals was verified by using sense probes.

RESULTS

Identification of fex. We constructed degenerate primers based on the conserved regions of the first extracellular loop and the seventh transmembrane domain of several G protein- coupled neuropeptide receptors. PCR was performed using an uninduced NT2-cell cDNA library as template. NT2 cells represent a committed neuronal precursor stage of differentiation and express neuroepithelial markers (10). We chose this cell line, because we wanted to identify receptors expressed in neuroepithelia. One of the subcloned PCR products coded for an amino-acid sequence with homology to the transmembrane domains of G protein-coupled glycoprotein receptors and was named fex, because of its follicular expression. A full-length murine cDNA sequence was obtained by PCR from a cDNA library derived from newborn mouse brain. The open

TABLE 1

Amino Acid Sequence Comparison of Murine FEZ with Its Closest Related Homologues Shown as Percentage Identity or Similarity (in Parentheses) of the Total Sequence and of the Transmembrane (TM) Domains

Receptor (organism)	HG38 (human)	FSH-R (rat)	LH-R (mouse)	TSH-R (mouse)	pGPCR (sea anemone)
Total sequence	86 (89)	27 (37)	27 (37)	30 (40)	28 (40)
TM domains	82 (87)	28 (40)	28 (39)	30 (41)	29 (41)

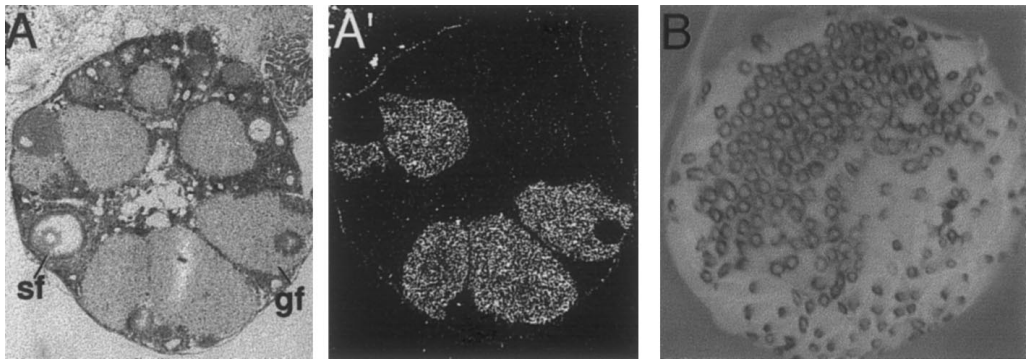

FIG. 3. Localization of the fex gene transcripts in the gonads of adult mice. A, A′ is a section hybridized with a ^{35}S-UTP labeled riboprobe, B is a whole-mount *in situ* hybridization using a digoxygenin-UTP labeled riboprobe. The darkfield photomicrograph of the same section is labeled with a prime (′). In a section of an ovary (A, A′) expression (white) is visible in the majority of the Graafian follicles (gf), but not in primary or secondary follicles (sf). (B) Testis were cut in halves to show hybridization (dark stain) in the seminiferous tubules.

reading frame of the isolated cDNA is 2721 bp long and codes for a protein of 907 amino acids. The deduced amino acid sequence (Fig. 1) and hydrophobicity analysis revealed seven putative transmembrane domains and a signal peptide at the amino terminus. The large amino-terminal extracellular domain contains 18 leucine-rich repeats (Fig. 2), the typical landmark for the binding domain of glycoprotein receptors (4). The FEX sequence revealed high homology to other vertebrate G protein-coupled glycoprotein receptors, such as the luteinizing hormone receptor (LH-R) (6), the thyroid stimulating hormone receptor (TSH-R) (16), and the follicle stimulating hormone receptor (FSH-R) (15) (Table 1). A homology in the same range was found to the invertebrate probable glycoprotein receptor (pGPCR) (13) from the cnidaria *Anthopleura elegantissima*. After this work was completed, a human receptor, termed HG38, was reported (11). Because of its 89% similarity

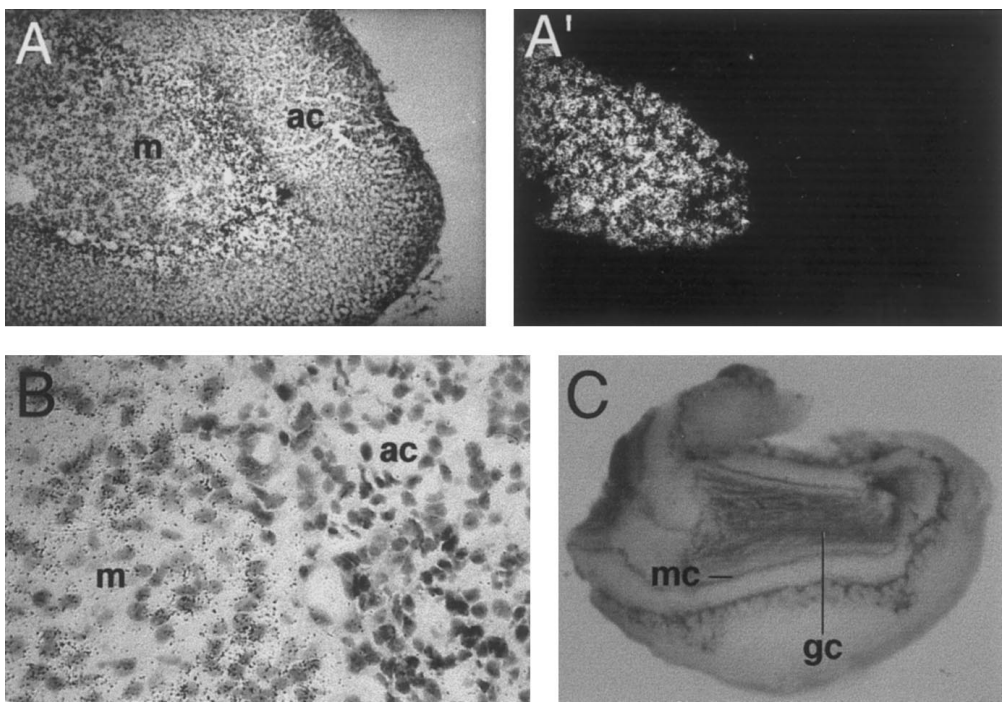

FIG. 4. Distribution of the fex mRNA in the adrenal gland (A, A′, B) and the olfactory bulb (C). A, A′, B are sections hybridized with a ^{35}S-UTP labeled riboprobe, C is a whole-mount *in situ* hybridization using a digoxygenin-UTP labeled riboprobe. In a section of an adrenal gland (A, A′) expression (white) is restricted to the medulla (m) while the adrenal cortex (ac) is not labeled. Phase contrast photomicrograph (B) shows a magnification of the border between the adrenal cortex (ac) and the medulla (m). Note the silver grains (black) associated with the chromaffin cells of the medulla. In the olfactory bulb (C) expression (dark stain) is found in all neuronal cell types and most prominently in the granule (gc) and mitral cell layer (mc).

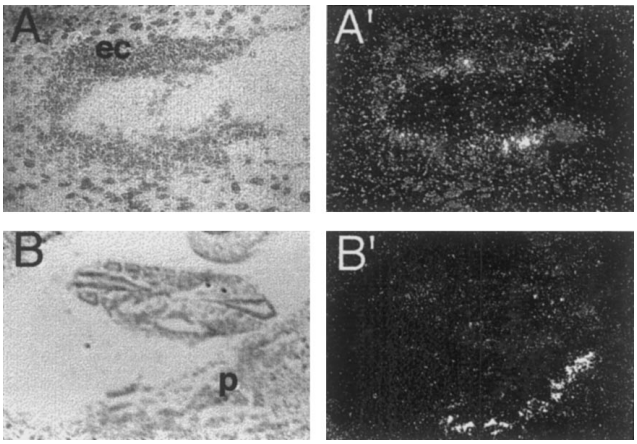

FIG. 5. Expression of fex during early mouse development. At E8.5 (A, A′) a few cells of the ectoplacental cone (ec) strongly express the Fez gene. At E9.5 (B, B′) signal intensity in the forming placenta (p) increased and the embryo proper exhibited only very weak hybridization signals.

to the murine FEX, HG38 may be the human orthologue of FEX.

Expression of fex in the adult mouse. The high homology of FEX to glycoprotein receptors suggested that it might have similar functions. Therefore we analyzed its expression in the gonads and other peripheral tissues of adult mice. Due to the fact that we had identified fex in a cDNA library derived from newborn mouse brain, we also analyzed its expression in adult brain. We found that in the ovary most of the Graafian follicles contained fex transcripts, while primary and secondary follicles were devoid of hybridization signals (Fig. 3A). In the testis hybridization signals were detected in a subpopulation of the seminiferous tubules (Fig. 3B). Because of the uneven distribution of transcripts in the seminiferous tubules, we assume that only specific stages of spermatocytes were labeled. Fex was expressed in the adrenal gland, where the chromaffin cells of the medulla were labeled (Figs. 4A and 4B). In the adult brain we detected transcripts exclusively in the olfactory bulb, not in other regions. All neuronal cell layers of the olfactory bulb exhibited hybridization signals, while fiber tracts did not express the fex gene (Fig. 4C).

Expression of fex during early embryogenesis. During early embryonic development fex was not expressed in the embryo proper up to embryonic day 9.5 (E9.5). A few cells of the ectoplacental cone exhibited hybridization signals at E8.5 (Fig. 5A) and at E9.5 a greater number of cells in the labyrinthine region of the forming placenta strongly expressed the fex gene (Fig. 5B). Very weak hybridization signals were seen over the whole embryo.

At E10.5 the distribution of transcripts in the embryo had become highly specific (Fig. 6A). Strong signals were detected in the facial area in the tissue overlaying the mandibular cleft and in the optic cup. In the central nervous system the neuroepithelium at the roof of the mesencephalon expressed fex (Figs. 6A and 6B), and the spinal cord was unevenly labeled. In the ventral and dorsal spinal cord a small stripe of cells extending laterally from the ventricular zone exhibited hybridization signals (Fig. 6A). At E11.5 in the central nervous system intense hybridization was detected over the neuroepithelium at the border between mes- and metencephalon (Fig. 7C). The neuroepithelium lining the fourth ventricle and the retina expressed fex (Figs. 7C and 8C). In the spinal cord the hybridization signals already observed at E10.5 (Fig. 6A) were still present. The ventral stripe was now broader than before and did not extend to the marginal zone (Fig. 8A). Outside the nervous system most prominent signals were seen over the mesenchyme over-

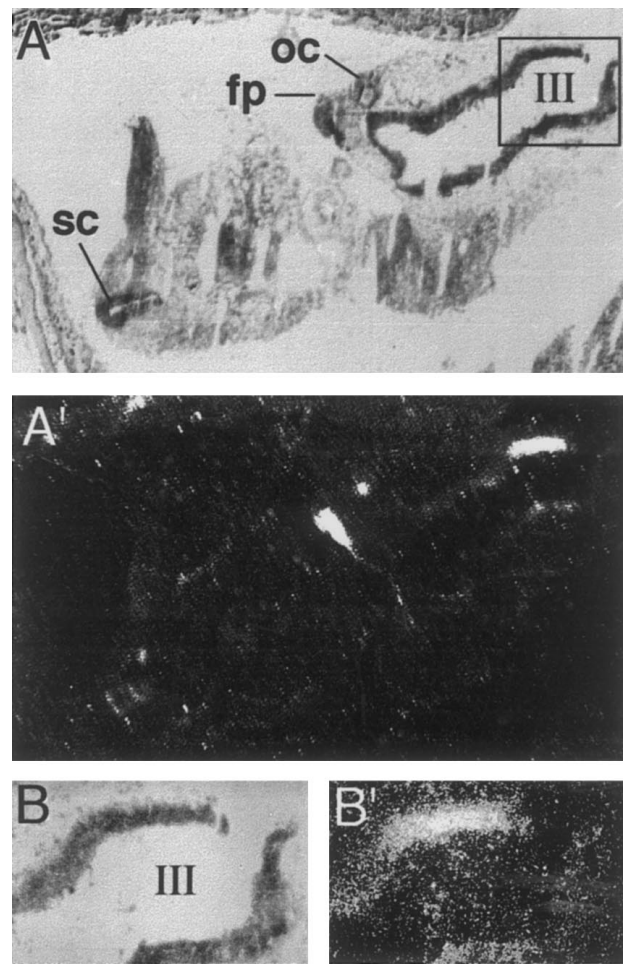

FIG. 6. Distribution of fex transcripts at E10.5. Brightfield (A, B) and their corresponding darkfield photomicrographs (A′, B′) are shown. A section through an E10.5 embryo *in utero* is seen in A, A′. Accumulation of transcripts was detected over the facial process (fp), the optic cup (oc), the neuroepithelium of the third ventricle (III), and cells in the ventral and dorsal spinal cord (sc). (B, B′) Magnification of the area framed in A, A′, showing strong hybridizing signals in the neuroepithelium of the third ventricle.

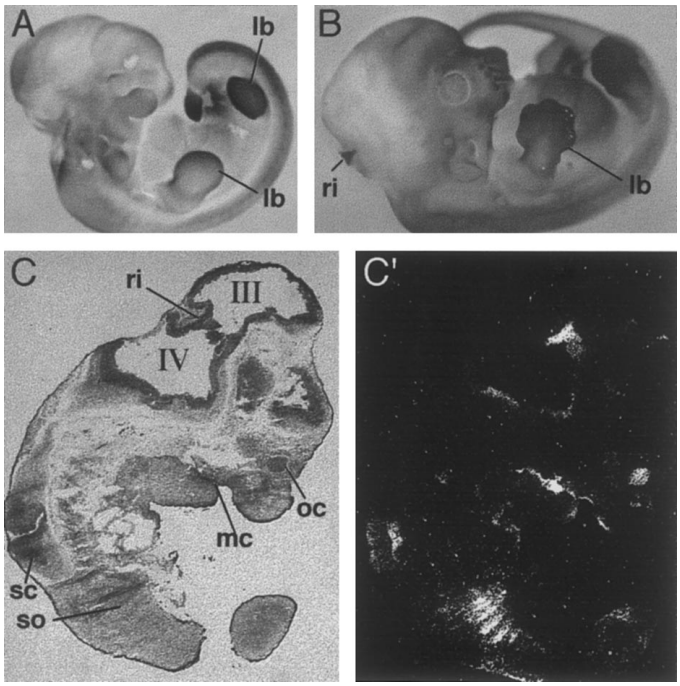

FIG. 7. Expression pattern of fex at E11.5 and E12.5. (A, B) Whole mount embryos at E11.5 (A) and E12.5 (B) were hybridized to a digoxygenin-UTP labeled riboprobe revealing transcripts in the condensing mesenchyme, the limb buds (lb), and the nervous system. At E12,5 the rhombic isthmus (ri) was highlighted by hybridization signals. (C, C′) A parasagittal section through an embryo at E11.5 shows an accumulation of transcripts over the rhombic isthmus (ri), the dorsal and ventral areas of the spinal cord (sc), the optic cup (oc), the neuroepithelium lining the IV. ventricle (IV), the mesenchyme surrounding the mandibular cleft (mc), and the condensing mesenchyme of the somites (so). The neuroepithelium lining the third ventricle (III) did not show significant hybridization signals.

laying the mandibular cleft (Fig. 7C). The distal limb buds, especially the hind limb buds were strongly labeled (Fig. 7A). In the rostral region of the embryo the perichordal mesenchyme, which will give rise to the intervertebrate discs, was marked by fex expression (Fig. 8B). Figure 7B shows that at E12.5 hybridization signals in the central nervous system were most prominent over the rhombencephalic isthmus. In the facial area the mesenchyme surrounding the olfactory epithelium and the forming vibrissae expressed fex. In the hind and front limb buds hybridization intensity had further increased and had spread to more proximal directions, but was restricted to the area were the digits develop (Fig. 7B).

Expression of fex during early organogenesis. At E13.5 the expression in the brain had decreased significantly (Fig. 9A). Only the border between mes- and diencephalon exhibited strong hybridization signals. The neuroepithelium of the met- and myelencephalon was weakly labeled (Fig. 9A), and in the pituitary transcripts were detected (Fig. 9C). The mesenchyme adjacent to the mandibular cleft was still strongly marked by hybridization, and the most lateral aspects of the tongue as well as the teeth anlagen were hybridizing (Figs. 9A–9C). The body wall and mesenchyme surrounding internal organs, e.g., the capsule of the liver, exhibited weak hybridization signals. A frontal section through the head region demonstrated that ectodermal as well as mesenchymal tissues expressed the fex gene (Fig. 9C). Figure 9D shows that this was also true for the trunk region. Epithelia and their adjacent mesenchyme were labeled by the fex probe. At

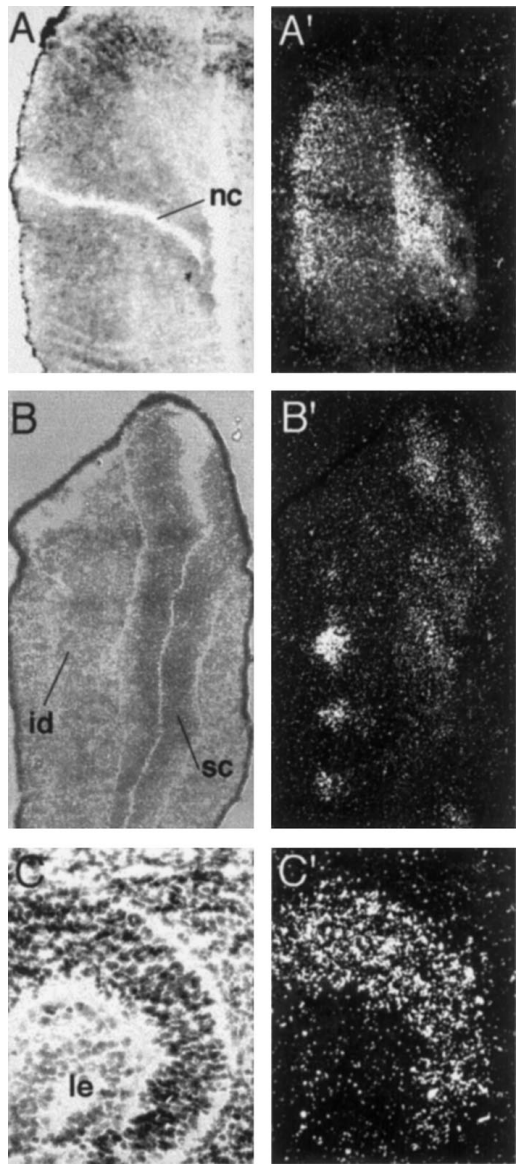

FIG. 8. Higher magnification of sections through spinal cord and eye at E11.5. (A, A′) In a sagittal section of the spinal cord transcripts were detected in the ventral and dorsal area extending laterally from the neuroepithelium lining the neural canal (nc). (B, B′) In a dorsal section through an embryo hybridization signals were found in the spinal cord and in the condensing mesenchyme of the intervertebrate disc anlagen (id). (C, C′) In a transverse section through an eye the neuronal retina is labeled, while no transcripts were detected in the lens (le).

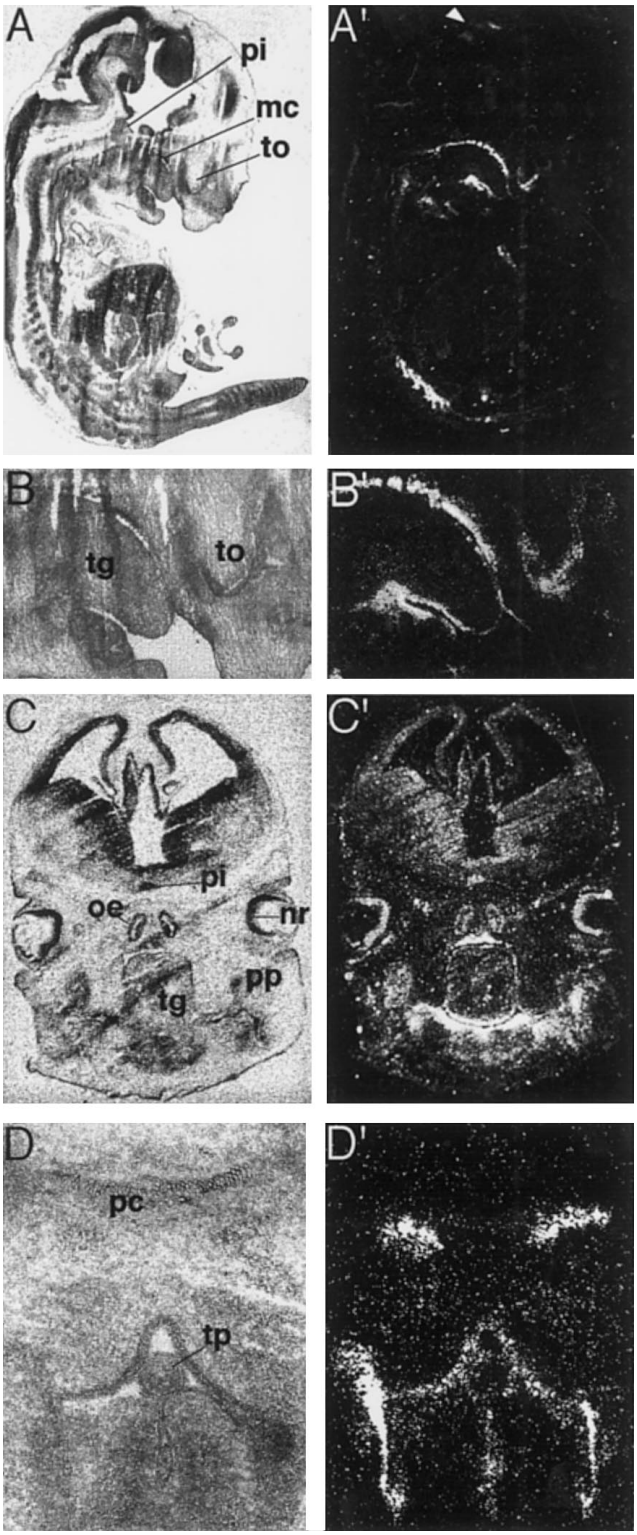

FIG. 9. Expression of fex in E13.5 mouse embryos. (A, A′) Sagittal section through an embryo shows hybridization signals at the border between di- and mesencephalon (arrow), the mesenchyme and epithelium surrounding the mandibular cleft (mc), the tooth anlagen (to) and the intervertebrate discs. (B, B′) A magnification of the facial region of the embryo shown in (A, A′) reveals prominent hybridization signals in the condensed mesenchyme and the ameloblast of the tooth anlage (to) and the mesenchyme of the mandible and the epithelium of the tongue (tg). (C, C′) Frontal section of the head shows additional expression of fex in the pituitary (pi), the neural retina (nr), the mesenchyme lining the olfactory epithelium (oe), and the mesenchyme of the palatine process (pp). (D, D′) In a transverse section through an embryo, transcripts were detected in epithelia and mesenchymal tissues under the parachordal plate (pc) and around the truncus pulmonaris (tp).

E14.5 signals were hardly detectable in the nervous system. In the body increasing signal intensity was detected in the perichondrium, but became rapidly weaker with ongoing age (data not shown). During all stages of embryonic development we could not detect hybridization signals over background in the gonads.

DISCUSSION

In the present study we describe the identification of a cDNA from mouse brain encoding a novel putative seven transmembrane receptor with an as yet unknown ligand. The gene is follicularly expressed, therefore we named it fex. Among the different types of G protein-coupled receptors fex shows the highest degree of homology to the family of glycoprotein receptors, namely 37–41% identity to the LH, FSH, and TSH receptors. Although, the identity is significantly lower than that between the already characterized glycoprotein receptors, FEX shows the typical features of a member of the glycoprotein receptor family. The seven transmembrane spanning region is preceded by a long extracellular domain containing 18 leucine-rich repetitive elements. For glycoprotein receptors with known ligands these repeats were shown to be involved in ligand binding (8). The number of repeats present is thought to reflect the size of the ligand. In the LH, TSH, and FSH receptor ten to eleven repeats are present in the extracellular domain. Because FEX contains more leucine-rich repetitive elements than any of the so far characterized receptors, it is tempting to speculate that its ligand has an unusually large size.

Expression of fex was studied in the adult mouse and during embryonic development. In the adult mouse prominent fex expression was found in the gonads, the adrenal gland, and the brain. In the central nervous system fex transcripts were restricted to the olfactory bulb, where only neuronal cells were labeled. Similarly, in the adrenal gland fex mRNA was detected in the neural-crest derived chromaffin cells of the medulla, but not in the cells of the adrenal cortex, hinting at a neurospecific expression. In the gonads fex transcripts were present at specific stages of sperm and oocyte development. Thus fex expression was high in Graafian follicles, but absent from primary and secondary follicles. This is in contrast to what was found for the LH and FSH receptors, which are expressed in all stages of follicular devel-

opment (14). The restricted expression of fex points at a function in oogenesis and spermatogenesis.

During very early murine embryonal development fex transcript could not be discovered in the embryo proper, but only in the placenta. Later, starting from E10.5 onward fex was expressed in the developing spinal cord and in the neuroepithelia of the myel-, met-, mes-, and diencephalon. Fex expression in the central nervous system was transient and the pattern changed rapidly, hinting at a role for FEX in inductive processes. The fact that fex was cloned from a NT2-cell cDNA library, and that NT2 cells represent neural precursor cells expressing neuroepithelial markers, is consistent with this notion.

With the onset of organogenesis fex was transiently expressed in a variety of regions of the embryo, predominantly in mesenchymal structures, including those surrounding the epithelia of internal organs, in a pattern resembling that of the activin/inhibin subunit βA (5). Members of the activin/inhibin family were originally isolated as gonadal proteins from follicular fluid regulating the release of FSH from the pituitary (18) and later shown to act as morphogens during embryonal development. This may suggest a similar role for FEX as morphogen receptor in early organ development.

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