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Expression and potential role of *Fsrg1*, a murine bromodomain-containing homologue of the *Drosophila* gene *female sterile homeotic*

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SUMMARY

We have isolated a cDNA which is a murine homologue of the *Drosophila* gene *female sterile homeotic* (*fsh*). This homologue, which we have designated *Fsrg1, contains two bromodomains and an ET motif characteristic of the Fsh sub-class of bromodomain-containing proteins. Northern blot hybridization analysis of adult tissues revealed that *Fsrg1* was expressed at low levels rather ubiquitously, but most abundantly in the testis and ovary. Polyclonal antibodies raised against an Fsrg1 fusion protein were used to characterize the *Fsrg1* gene product in tissues. Constructs were also generated in which the *Fsrg1* cDNA was tagged with epitopes for hemaglutinin and used in transfection experiments. Immunoblot analysis revealed that the Fsrg1 protein migrates with a relative molecular mass of ~110 kDa, although the cDNA sequence would predict a protein of ~88 kDa. The migration at ~110 kDa was observed for both in vivo protein and protein produced in cultured cells. The Fsrg1 protein was localized to the nucleus when expressed in cultured cells, consistent with the presence of a nuclear localization signal motif in the *Fsrg1* sequence. No kinase activity was detected for this nuclear protein as assessed in either autokinase or specific substrate assays. In situ hybridization analysis revealed strikingly high expression of *Fsrg1* in granulosa cells of growing follicles in the adult ovary and suggested its possible involvement in folliculogenesis. Additional clues to its potential function were provided by the demonstration of its high level of expression in epithelia of tissues which undergo hormonally-modulated remodeling.**

Key words: *Fsrg1*, *RING3*, *Drosophila fsh*, Bromodomain

INTRODUCTION

The *Drosophila* gene *female sterile homeotic* (*fsh*) was initially identified as a maternal effect gene (Gans et al., 1975) which also appears to be required later in development (Gans et al., 1980). Genes which share structural homology with *Drosophila fsh* have been identified in a variety of other species, including *Saccharomyces cerevisiae* (Chua and Roeder, 1995; Lygerou et al., 1994), *Caenorhabditis elegans* (Thorpe et al., 1996), *Xenopus* (Salter-Cid et al., 1996), chicken (Thorpe et al., 1996), and human (Beck et al., 1992; Nomura et al., 1994). In *Drosophila*, *fsh* has also been shown to interact synergistically with loci such as *Ubx* and *trx* in the production of homeotic transformations (Digan et al., 1986). The *Drosophila fsh* gene produces two transcripts of 5.9 and 7.6 kb, which potentially encode proteins of 110 kDa and 205 kDa, respectively (Haynes et al., 1989). The putative 205 kDa protein includes the full sequences of the 110 kDa protein along with an additional 95 kDa moiety at the C-terminal end.

A common feature of the polypeptide sequence of the *fsh*-related genes is the presence of two internal repeats, named bromodomains (Thorpe et al., 1996). The bromodomain, a motif initially identified in the *Drosophila* gene *brahma*, consists of an ~60 amino acid residue motif that has the potential to form two α-helices (Haynes et al., 1992). Although the function of the bromodomain remains to be elucidated, it has been suggested that it may mediate protein-protein interactions (Jeanmougin et al., 1997). This motif has been found in a number of proteins: 37 proteins in various species have been reported to date of which the *fsh*-related genes comprise a distinct subclass (Jeanmougin et al., 1997). An additional homology among the fsh sub-class has been observed at the extreme C-termini of the proteins and designated as the ET domain (extra terminal domain) (Lygerou et al., 1994). The functions are not yet known for either the bromodomain or the ET domain.

Although the *fsh*-related genes share structural homology, diverse roles for their protein products have been suggested. *Drosophila* fsh was initially believed to have a role in

*The designation of Frsg1 used in this manuscript has been suggested in consultation with the Mouse Gene Nomenclature Committee (Lois Maltais, The Jackson Laboratory, personal communication).

controlling homeobox gene expression, possibly as a membrane protein (Haynes et al., 1989). The yeast protein BDF1 was reported to be a transcription factor involved in the regulation of expression of the U2 snRNA gene (Lygerou et al., 1994). BDF1 has also been proposed as a component of chromatin, as some of the mutant phenotypes observed could be a consequence of alterations in chromatin structure (Chua and Roeder, 1995). A function as a nuclear kinase which mediates mitogenic stimulation has been postulated for the human *fsh*-related gene *RING3* (Denis and Green, 1996).

In the present study, we report the isolation of a murine homologue of *fsh* which we have named as *Fsrg1* (*female sterile homeotic-related gene-1*). *Fsrg1* contains two bromodomains and an ET domain at the C-terminal end. The mouse *Fsrg1* shares 95% identity with the human *RING3* at the protein level, suggesting that they may be functional homologues. We have studied the biological activity of Fsrg1, in particular, ascertaining whether Fsrg1 has a kinase activity as described for the RING3 protein (Denis and Green, 1996). Finally, the distribution of expression of *Fsrg1* suggests roles in certain aspects of the reproductive system, especially during development of ovarian follicles and in various epithelia which undergo remodeling in response to hormones.

MATERIALS AND METHODS

Sources of tissues, cell lines and DNA

Normal tissues were obtained from CD1 mice (Charles River, Wilmington, DE). Neonatal testes were obtained from mice at postnatal days 7 and 17 and adult tissues from mice at least 35 days old. The mouse mutant strains *atrichosis* (*at*; ATEB/Le *a*/*a d*/*d* + *at*/*eb* +) and *white spotting* (*W*/*W*^{v}; WBB6F$_1$/J-*W*/*W*^{v}) were obtained from The Jackson Laboratory (Bar Harbor, ME). Dissected tissue specimens were frozen in liquid nitrogen prior to RNA isolation. Dissected tissues for in situ hybridization and immunohistochemical analyses were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight at 4°C.

All radioactive nucleotides used in this study were obtained from New England Nuclear (Wilmington, DE). DNA probes for the cDNA library screening and the Southern blot hybridization analysis were labeled with [^{32}P]dCTP using the multiprime DNA labeling kit (Amersham, Arlington Heights, IL). Riboprobes were prepared using T7 or T3 RNA polymerases in the presence of [^{32}P]UTP for northern blot hybridization analysis and [^{35}S]UTP for in situ hybridization analysis. For the northern blot and in situ hybridization analyses of *Fsrg1* expression, the fragment from nucleotides 1698-2340, which contains *Fsrg1* specific sequence between the two bromodomains, was used to generate both sense and antisense riboprobes.

RatBla is a rat fibroblast cell line (generously provided by Dr J. Kitajewski, Columbia University; Taylor et al., 1995). HeLa is a human cervical carcinoma cell line. BOSC and BING cell lines (Pear et al., 1993) were obtained from Dr D. Baltimore, and were used for generation of high-titer, helper-free retroviruses by transient transfection.

A cDNA clone of human *RING3* (Beck et al., 1992) was obtained from Dr G. Denis. The retroviral expression vector pLNCX (Miller and Rosman, 1989) was used for transfection experiments. The haemagglutinin (HA) tag sequence was inserted into the C-terminal ends of mouse *Fsrg1* and human *RING3* proteins using the site-directed mutagenesis method. HA-tagged *RING3* (*RING3HA*) and *Fsrg1* (*Fsrg1HA*) were inserted into the *Not*I-*Stu*I site of pLNCX, so that the both genes were expressed under control of the CMV promoter.

Isolation and sequence analysis of murine Fsrg1

A testis cDNA library was screened as described previously (Rhee and Wolgemuth, 1997). Briefly, a mouse testis cDNA library was screened with reduced stringency following the protocols outlined by Sambrook et al. (1989), using a mixture of mouse *Cdc2*, *Cdk2*, *Cdk4*, *Cdk5*, *Pctaire-1*, and *Pctaire-3* cDNAs as probes. Filters were washed at a final stringency of 2×SSC, 0.1% SDS at room temperature for 2 hours. Positive clones were plaque-purified twice and inserts from the tertiary screen were in vivo-excised following the protocol of Stratagene (La Jolla, CA). Clones isolated from this screen were sequenced using an Applied Biosystems Model 373A DNA sequencer (Applied Biosystems, Foster City, CA).

The initial *Fsrg1* clone that we isolated was 1.8 kb in size. As survey northern blot analysis indicated that this clone recognized testicular mRNAs of ≥3.1 kb in size, we used this clone as a probe to re-screen the mouse testis cDNA library at high stringency to isolate additional cDNAs.

Southern blot hybridization analysis

Genomic DNA was purified from human, monkey, chicken, rat and mouse, following the protocols outlined in Sambrook et al. (1989). Ten μg of genomic DNA was digested with *Eco*RI, fractionated on a 1% agarose gel, transferred onto a nitrocellulose membrane and hybridized with *Fsrg1* probes (fragments encompassing nucleotides 1,698-2,340 or 2,633-3,236) in hybridization buffer (6×SSC, 5× Denhardt's solution, 0.5% SDS, and 10 μg/ml denatured salmon sperm DNA) at 65°C overnight. The membrane was washed at final stringency of 0.1×SSC/0.1% SDS at 68°C for 2 hours and exposed to X-ray film.

Northern blot hybridization analysis

RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) as described previously (Rhee and Wolgemuth, 1995). Total RNA was electrophoresed in a denaturing 0.85% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane, and hybridized with a *Fsrg1* riboprobe, according to our standard protocols (Chapman and Wolgemuth, 1993). Ethidium bromide staining of the 18S and 28S RNAs was used to indicate equal loading for each sample.

In situ hybridization analysis

Paraffin embedded tissues were cut into 5 μm sections and analyzed by in situ hybridization as described previously (Rhee and Wolgemuth, 1995). ^{35}S-labeled *Fsrg1* riboprobes were synthesized in the antisense orientation using T3 RNA polymerase or sense (control) orientation using T7 RNA polymerase. Slides were exposed to emulsion (Kodak type NTB-2) for 5 days, developed, stained with hematoxylin and eosin, mounted and viewed on a Leitz photomicroscope under bright-field and epiluminescence optics.

Antibodies

For generating an antibody specific to mouse Fsrg1, a fragment containing amino acids 51-651 of the *Fsrg1* open reading frame was cloned into the bacterial expression vector pET21 which contains a histidine tag (Novagen, Madison, WI). The fusion protein was purified by immobilized metal affinity chromatography (Novagen, Madison, WI), and injected into a rabbit (Pocono Farm & Laboratory, Canadensis, PA). The antisera were affinity-purified by incubation with a strip of nitrocellulose membrane blotted with the Fsrg1 fusion protein and eluted with 100 mM glycine, pH 3.0. Human RING3 antibody was obtained from Dr G. Denis (Denis and Green, 1996) and antibody 12CA5 was used for detection of the HA tag (Babco, Berkely, CA).

Immunoblot analysis

Samples from tissues and cell lysates were prepared and analyzed as

described previously (Rhee and Wolgemuth, 1997). Affinity purified anti-Fsrg1 antibody was diluted to 1:500, anti-RING3 antibody was diluted to 1:3,000, anti-HA tag was diluted to 1:50, and the secondary antibody was diluted to 1:5,000-25,000. The ECL western blotting detection reagents (Amersham, Arlington Heights, IL) were used according to the manufacturer's recommendations.

Introduction of DNA into cells

Expression constructs were introduced into cells using a retroviral infection method (Taylor et al., 1995). Initially, expression vectors were transfected into BOSC or BING cells by the calcium phosphate method (Pear et al., 1993). The transfection efficiency was usually 30~50%. Two days after transfection, the supernatant which contained packaged viral particles was collected and added to RatB1a or HeLa cells, respectively. G418 (400 μg effective mass/ml) was added into the culture medium 2 days after infection and cells were selected for 10 days. Cells resistant to G418 were analyzed for infected-gene expression.

Immunoprecipitation and autokinase assay

Both cytosolic and nuclear extracts were prepared using modifications of the procedures described by Dignam et al. (1983). In brief, cells were washed with PBS, suspended with buffer A (10 mM Tris-HCl, pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, 50 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mM DTT, 0.1% NP-40 and protease inhibitors), homogenized with a Dounce homogenizer, vortexed extensively, kept on ice for 1 minute to lyse membranes, and centrifuged at 5,500 rpm for 5 minutes. The supernatant was saved as a cytosolic extract. The pellet was suspended with buffer B (20 mM Tris-HCl pH 8.0, 420 mM NaCl, 1 mM EDTA, 50 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mM DTT, 25% glycerol, and protease inhibitors), vortexed extensively, kept on ice for 30 minutes with occasional vortexing, and centrifuged at 14,000 rpm for 10 minutes. The resulting supernatant was saved as a nuclear extract. All procedures were performed at 4°C.

For immunoprecipitation, aliquots of nuclear and cytosolic extracts were incubated with anti-Fsrg1, anti-RING3 or anti-HA antibodies in the immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 10% glycerol, 1% NP-40, 0.25% sodium deoxycholate, and protease inhibitors) for 1 hour at 4°C, followed by incubation with 3 μg of Protein A-Sepharose for 1 hour. Immune complexes were collected by centrifugation and washed four times with the same buffer and suspended with the SDS-PAGE sample buffer (Laemmli, 1970).

The autophosphorylation assay was carried out essentially as described by Denis and Green (1996), which was modified from Ferrell et al. (1989). In brief, the proteins were separated by SDS-PAGE, electroblotted onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was placed in the denaturing solution (7 M guanidine HCl, 50 mM Tris-HCl, 2 mM EDTA, 50 mM DTT, pH 8.3) with gentle rocking for 1 hour at room temperature, transferred into the renaturing solution (100 mM NaCl, 50 mM Tris-HCl, 2 mM EDTA, 2 mM DTT, 1% BSA, 0.1% NP-40, pH 7.5) overnight at 4°C, blocked in the blocking solution (5% BSA in 30 mM Tris-HCl, pH 7.5) for 1 hour at room temperature, and incubated with [γ-^{32}P]ATP (50 μCi/ml) in the autokinase buffer (30 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 2 mM $MnCl_2$) for 1 h. Subsequently, the membrane was washed with solution A (30 mM Tris-HCl, pH 7.5) for 10 minutes, solution B (30 mM Tris-HCl pH 7.5 and 0.05% NP-40) for 10 minutes and with solution A again for 10 minutes. Afterward, the membrane was treated with alkali by dipping into 1 N KOH solution for 10 minutes at room temperature, neutralized by dipping into 10% acetic acid, blotted excess solution with paper towel and exposed to an X-ray film for 1 to 4 days.

Serum stimulation of NIH3T3 cells

NIH3T3 cells were plated at 5×10^5 cells per 100 mm Petri dishes and were serum-starved for two days in DMEM containing 0.1% calf serum. The media was then changed to DMEM containing 20% calf serum. Serum-stimulation was stopped by washing cells with ice-cold PBS and processing them for cytoplasmic and nuclear fraction extraction as described above.

RESULTS

Isolation of the mouse *Fsrg1* cDNA

A clone of the mouse *Fsrg1* cDNA was isolated from a mouse testis library as part of a screen for new cyclin-dependent kinases (Rhee and Wolgemuth, 1997). The longest *Fsrg1* cDNA clone was 3.6 kb in size and contained an open reading frame of 798 amino acids. The GenBank access number of the sequence is AF045462. The mouse *Fsrg1* revealed a significant homology (32% and 43% identity at the DNA and protein levels, respectively) with *Drosophila fsh*, a gene which had been implicated in the establishment of segments during embryogenesis (Haynes et al., 1989). Like *Drosophila fsh*, the mouse *Fsrg1* gene encodes a protein with two bromodomains and an ET domain (Fig. 1). A search for sequences homologous to this clone in GenBank revealed that *Fsrg1* exhibited 75% identity at the DNA level to a human gene named *RING3*. At the protein level the similarity between the two proteins is 95% reaching 100% for the two bromodomains (Fig. 1). The human *RING3* gene had initially been identified during a chromosome walk in the region of the class II major histocompatibility complex (MHC) on chromosome 6 (Beck et al., 1992). Although the *RING3* gene is localized in the vicinity of the MHC genes, there has been no evidence reported suggesting a role in any immune function (Thorpe et al., 1996) and the tissue distribution of expression of *RING3* has not been reported.

We have named the mouse clone that we isolated as *female sterile homeotic-related gene* (*Fsrg1*), based on its structural similarity with *Drosophila fsh*. The same rationale has been used to designate another mouse bromodomain-containing gene as *Brg1* (mouse *brahma-related gene*) (Randazzo et al., 1994). The designation of *RING3*, corresponding to *really interesting new gene 3* (Beck et al., 1992), may actually be confusing since it may be inferred to reflect structural identity to other genes designated as containing RING motifs (Borden

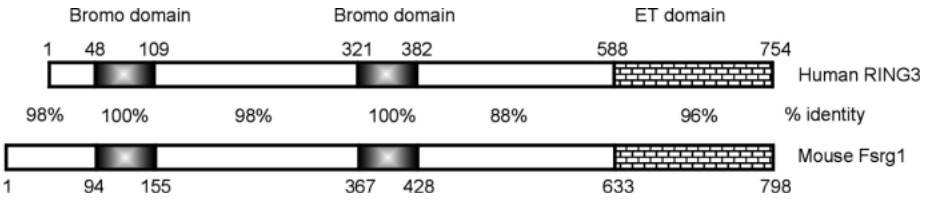

Fig. 1. Comparison of mouse Fsrg-1 protein and its putative human counterpart RING3. The two bromodomains and the ET domain are represented by filled boxes and the percents of identity between the two amino acid sequences are indicated for each region. The sequence analysis was performed with Megalign from DNAstar. The GenBank access number of the sequence is AF045462.

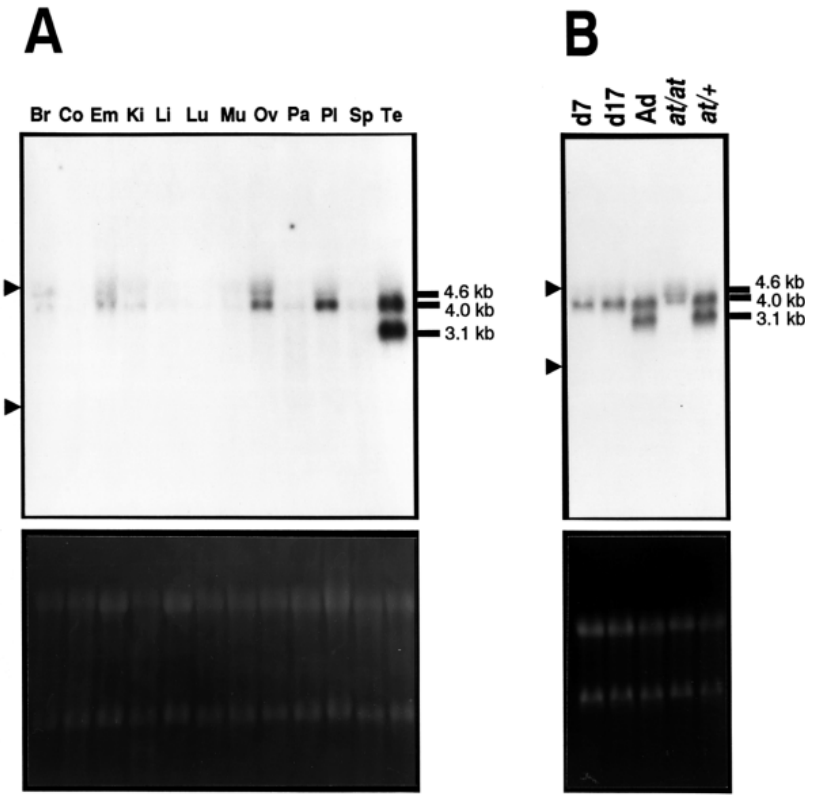

Fig. 2. Northern blot analyses of *Fsrg1* expression. Total RNA (10 μg) was isolated from various mouse tissues (A) or from testes (B) of immature (d7, d17), germ cell-deficient (*at*/*at*), or adult (Ad) mice. The sizes of the specific *Fsrg1* transcripts are indicated on the right side of the figures. Arrowheads mark positions of the 28S and 18S ribosomal RNA bands. The lower figures show the ethidium bromide-stained ribosomal RNA bands. Br, brain; Co, colon; Em, 12.5-day embryo; Ki, kidney; Li, liver; Lu, lung; Mu, muscle; Ov, ovary; Pa, pancreas; Pl, placenta; Sp, spleen; Te, testis. Exposure was for 12 hours.

and Freemont, 1996), which are quite distinct from the bromodomain genes of the *brahma* and *fsh* class.

To assess the level of conservation of *Fsrg1* among species, genomic Southern blot hybridization analysis was performed. Genomic DNA isolated from different species was hybridized with a mouse *Fsrg1* probe spanning nucleotides 1,698-2,340. A single band was detected in human, chicken and mouse, while double bands were detected in the monkey and rat DNAs (data not shown). The identical pattern of bands was detected when another *Fsrg1* probe, which spans nucleotides 2,633-3,236, was used (data not shown). This result suggested that *Fsrg1* is evolutionarily conserved among vertebrates (Thorpe et al., 1996). The single band in the human genome may correspond to the human *RING3*, which reveals identity at the nucleotide level with the mouse gene in the region we used as probe (Beck et al., 1992). Another human gene, *ORFX*, has been identified, which shares 41% and 60% identity with the mouse *Fsrg1* at the DNA and protein levels, respectively (Nomura et al., 1994). To the best of our knowledge, no studies beyond the observation that the *ORFX* gene is located on chromosome 9 have been reported (Thorpe et al., 1996). It is unlikely that the specific band in the human genomic DNA corresponds to *ORFX*, which is less homologous to mouse *Fsrg1* than human *RING3*.

Tissue distribution of *Fsrg1* expression

Northern blot hybridization analysis was carried out to determine the distribution of *Fsrg1* expression among several tissues of the mouse (Fig. 2A). *Fsrg1* was expressed in all tissues examined, but at varying levels. *Fsrg1* expression was readily detected in embryo, ovary, and placenta RNA and most abundantly in the testis. Three transcripts corresponding to *Fsrg1* were noted, of 4.6 kb, 4.0 kb and 3.1 kb in size. The 4.6 and 4.0 kb transcripts were detected in all tissues, while the 3.1 kb transcript was detected only in the testis.

As another murine bromodomain-containing gene, *Brg1*, has been shown to be expressed during development (Randazzo et al., 1994), the pattern of *Fsrg1* expression in embryos was examined. RNA was isolated from embryos of several gestation stages and examined by northern blot hybridization analysis. *Fsrg1* transcripts were more abundant in the mid-gestation rather than late embryos (Fig. 3), similar to the

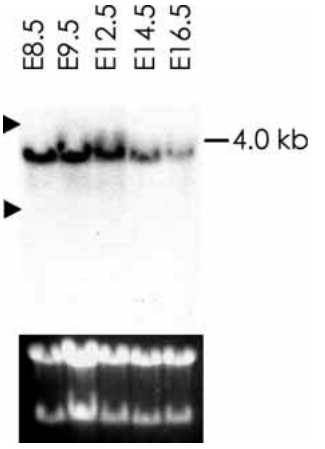

Fig. 3. Northern blot analyses of *Fsrg1* expression in embryos. Total RNA (10 μg) was isolated from total embryos of several gestation stages. The size of the specific *Fsrg1* transcript is indicated on the right side of the figure. Arrowheads mark positions of the 28S and 18S ribosomal RNA bands. The lower panel shows the ethidium bromide-stained ribosomal RNA bands. Exposure was for 3 days.

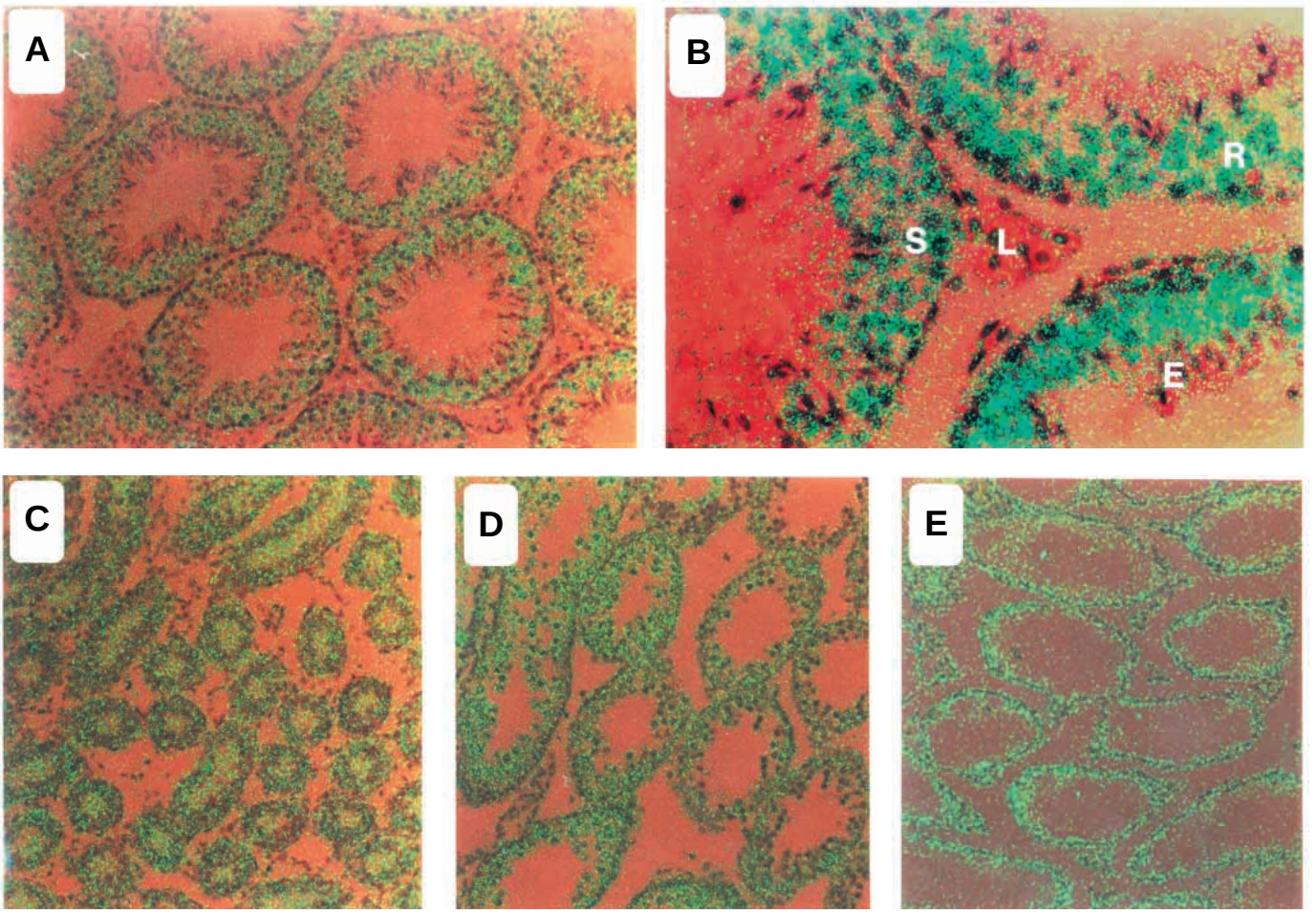

Fig. 4. In situ hybridization analysis of *Fsrg1* expression in testis. A ^{35}S-labeled antisense probe specific to *Fsrg1* was hybridized with sections of paraffin-embedded testis samples from adult (A,B), 7-day-old (C), 17-day-old (D), and germ cell-deficient (W/W^v) (E) mice. Exposure time was 5 days. The photomicrographs in this figure were taken using epiluminescence optics with a low bright-field background, so that signals were visualized with a bright green color. S, spermatocytes; R, round spermatids; E, elongating spermatids; L, Leydig cells.

pattern observed with *Brg1* (Randazzo et al., 1994). Preliminary in situ hybridization analysis has revealed that *Fsrg1* was expressed ubiquitously throughout the embryo; however, the signal was somewhat higher in the developing brain and in both cervical and dorsal root ganglia (data not shown).

Developmental and cellular distribution of *Fsrg1* expression in the testis and ovary

To determine the developmental and lineage specificity of *Fsrg1* expression in the testis, both northern blot (Fig. 2B) and in situ (Fig. 4) hybridization analyses with RNA and histological sections, respectively, from testes of immature and germ cell deficient mice were performed. The characteristic progression of spermatogenic differentiation in the postnatal mouse testis allows for the enrichment or elimination of particular germ cell types by utilizing testes of mice at specific days of development (Bellve et al., 1977). For example, testes from 7-day-old (d7) mice consist largely of Sertoli cells and spermatogonia. In the testes from 17-day-old (d17) mice, germ cells have entered meiotic prophase and have progressed to spermatocytes. Both d7 and dl7 testes lack postmeiotic spermatids (Bellve et al., 1977). Mice homozygous at the *atrichosis* (*at*) and *white-spotting* (*W*) loci are virtually devoid of germ cells, but have the normal complement of somatic cell types, including Leydig, Sertoli, and peritubular myoid cells (Hummel 1964; Mintz, 1957). The heterozygous littermates of both mutant strains have the normal somatic and germ cell complements and are fertile.

Northern blot hybridization analysis revealed *Fsrg1* expression in all the testis samples but in quite distinct patterns with regard to relative levels and subsets of transcript sizes (Fig. 2B). The 4.0 kb transcripts were detected in all testis samples while the 3.1 kb transcripts were detected only in normal adult testis samples, suggesting that the 3.1 kb transcripts are specific to postmeiotic germ cells. The 4.6 kb transcripts were detected in germ cell-deficient testis and at much reduced levels in the germ cell-containing testes, suggesting that these transcripts are predominantly expressed in the somatic compartment of the testis.

In situ hybridization was used to more precisely identify particular testicular cell types expressing *Fsrg1*. *Fsrg1* was expressed in spermatogonia, spermatocytes, and round spermatids of the adult testis (Fig. 4A,B). *Fsrg1* expression in

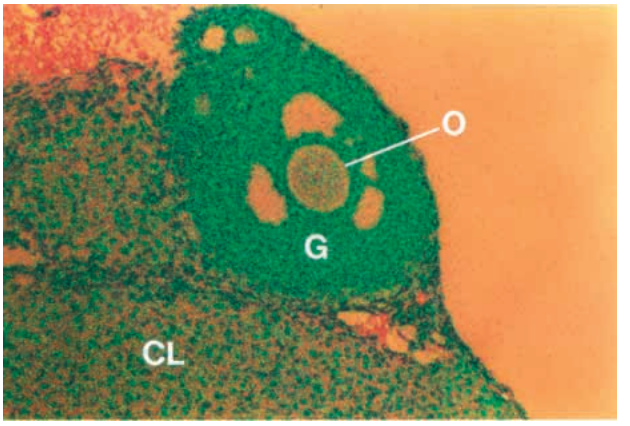

Fig. 5. In situ hybridization analysis of *Fsrg1* expression in ovary. A ^{35}S-labeled antisense probe specific to *Fsrg1* was hybridized with sections of paraffin-embedded ovary sample from an adult mouse. Exposure time was 5 days. O, oocytes; G, granulosa cells; CL, corpora lutea.

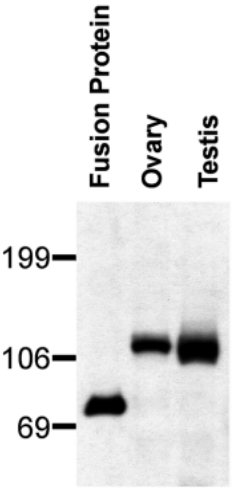

Fig. 6. Immunoblot analysis of Fsrg1. 30 μg of protein of total lysates from mouse ovary or testis were fractionated with 7.5% SDS-PAGE, along with 10 ng protein of bacterially expressed Fsrg1 fusion protein. The blot was incubated with an affinity-purified rabbit polyclonal antibody against the Fsrg1 fusion protein. The sizes of the protein markers are indicated on the left side of the figure.

spermatogonia and spermatocytes was apparent in the testes from d7 and d17 mice, respectively (Fig. 4C,D). Specific signals for *Fsrg1* were also detected in germ cell-deficient testis (Fig. 4E), indicating that *Fsrg1* was expressed in both germ cells and somatic cells of the testis.

The cellular distribution of *Fsrg1* was also examined by in situ hybridization in adult ovaries (Fig. 5). Although *Fsrg1* transcripts were detected in virtually all ovarian cell types, strikingly higher levels of *Fsrg1* were observed in the granulosa cells of growing follicles. Examination of *Fsrg1* levels in distinct corpora lutea revealed much lower levels and only relatively low amounts of *Fsrg1* transcripts were detected in oocytes. These observations suggest that *Fsrg1* may be involved in follicullar growth and differentiation.

Characterization of Fsrg1 protein

A rabbit polyclonal antibody was raised against a bacterially-expressed Fsrg1 fusion protein and affinity-purified. The specificity of the antibody was confirmed by immunoblot analysis against the Fsrg1 fusion protein, detecting a band of 75 kDa in size (Fig. 6, lane 1). The antibody was used for immunoblot analysis of lysates from the testis and ovary (Fig. 6, lanes 2,3) but was not suitable for immunohistochemical analysis (data not shown). A single, specific band of ~110 kDa in size was detected in the lysates from adult testis and ovary. The specific ~110 kDa band recognized by the Fsrg1 antibody migrated with a relative molecular mass that was larger than the calculated size of ~88 kDa based on the primary structure.

To ensure that this ~110 kDa band corresponded to bona fide Fsrg1 protein, transfection experiments with constructs in which the *Fsrg1* cDNA was tagged at the C-terminal end with the HA epitope were carried out. The transfected construct product was immunoprecipitated with anti-HA epitope monoclonal antibody 12CA5, and detected on immunoblot analysis with the anti-Fsrg1 antibody (Fig. 7A). A specific band of ~110 kDa in size was observed only in the nuclear fraction of the *Fsrg1HA*-

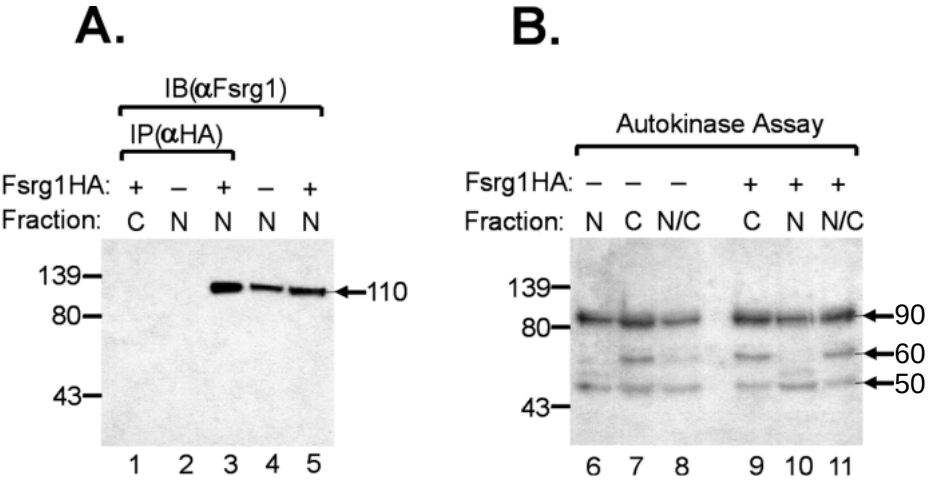

Fig. 7. Fsrg1 in RatB1a cells. (A) Detection of Fsrg1 protein. RatB1a cells were infected with *Fsrg1HA* (lanes 1,3,5) or with the vector DNA (lanes 2, 4). Cytosolic (C) or nuclear (N) fractions of infected RatB1a cells were immunoprecipitated with the HA antibody (lanes 1-3). The Fsrg1 proteins of resulting immunoprecipitates (lanes 1-3) and of total lysates (lanes 4,5) were detected using the affinity-purified Fsrg1 antibody. (B) Autokinase activity assay. Autokinase assay was carried out with samples from vector-infected (lanes 6-8) or *Fsrg1HA*-infected (lanes 9-11) RatB1a cells. The cytosolic (C), or nuclear (N) or combined (C/N) fractions of cell lysates were fractionated with SDS-PAGE and subjected to carrying out an autokinase assay as described in Materials and Methods. The sizes of protein markers and the sizes of specific bands are indicated on the left and right sides of the figures, respectively.

infected cells (lane 3), indicating that the Fsrg1/HA product also migrates as ~110 kDa protein upon SDS-PAGE analysis. The Fsrg1 antibody, but not the anti-HA antibody, also detected an ~110 kDa band in lysates of vector-alone infected cells (lane 4) and *Fsrg1HA*-infected (lane 5) RatB1a cells, suggesting that, like Fsrg1/HA protein, the endogenous rat Fsrg1 protein also migrates as an ~110 kDa band.

Potential biochemical activity of Fsrg1

It was recently reported that the human RING3 is a serine/threonine kinase which also exhibits an autokinase activity (Denis and Green, 1996). We therefore tested whether Fsrg1, which shares 95% identity with RING3 at the protein level, also has an autokinase activity (Fig. 7B). The same series of protein samples produced in the transfection experiments were subjected to an in-gel autokinase assay (Denis and Green, 1996). Autokinase activities were detected in both cytosolic and nuclear fractions of vector-transfected and *Fsrg1HA*-transfected RatBla cells, as three major bands of 90, 60 and 50 kDa in size (Fig. 7B). However, no autokinase activity was observed migrating at the size of the Fsrg1 protein, ~110 kDa in size (Fig. 7), even in overexposed autoradiograms (data not shown). We were also unable to detect any increase in autokinase activity in *Fsrg1HA*-transfected cells as compared to vector-transfected cells (Fig. 7). These results implied that, in this assay system, neither the endogenous rat nor the exogenous murine Fsrg1 has detectable autokinase activity.

Earlier experiments from Denis and Green (1996) suggested the existence of a RING3-activating kinase in HeLa nuclear extracts. To test the hypothesis that Fsrg1 autokinase activity might be induced in this cell line, HeLa cells were transfected with an HA-tagged human *RING3* (*RING3HA*) or *Fsrg1HA*, and used for immunoblot, immunoprecipitation and in-gel autokinase assay experiments (Fig. 8). A prominent band of ~110 kDa in size was detected with the Fsrg1 antibody in vector- and *RING3HA*-infected HeLa cells (Fig. 8A lanes 2,4,6), suggesting that, like Fsrg1, both exogenous and endogenous RING3 proteins migrate at ~110 kDa in size. When *RING3HA* and *Fsrg1HA* infected HeLa cell lysates were immunoprecipitated with the HA antibody, a specific band of ~110 kDa in size was detected with the Fsrg1 antibody (lanes 8,9).

Autokinase assays carried out with the samples described above detected a strong activity in a band migrating at ~90 kDa (Fig. 8B, lanes 10-14), but not at ~110 kDa, the size of bona fide Fsrg1. Concomitantly, we were unable to detect any kinase activity in immunoprecipitates of lysates from infected cells with the anti-HA antibody as well as the RING3 antibody (lanes 15-18). These results indicated that, under the conditions used in the present studies, the human RING3 migrates at ~110 kDa rather than at ~90 kDa (Denis and Green, 1996) and lacks autokinase activity. Although we did detect an ~90 kDa band with an in-gel autokinase activity, this activity could not be correlated with the size of the Fsrg1 protein detected either with HA or Fsrg1 antibodies. Finally, using NIH3T3 cells we examined the response to serum stimulation of the ~90 kDa proteins of unknown identity and the bona fide Fsrg1 protein migrating at ~110 kDa (Fig. 9). While the activity of the ~90 kDa band was strongly induced by serum stimulation, we did not detect any induction of activity at ~110 kDa. This suggested further that the Fsrg1 migrating at 110 kDa lacked detectable autokinase activity even in the presence of external stimulus.

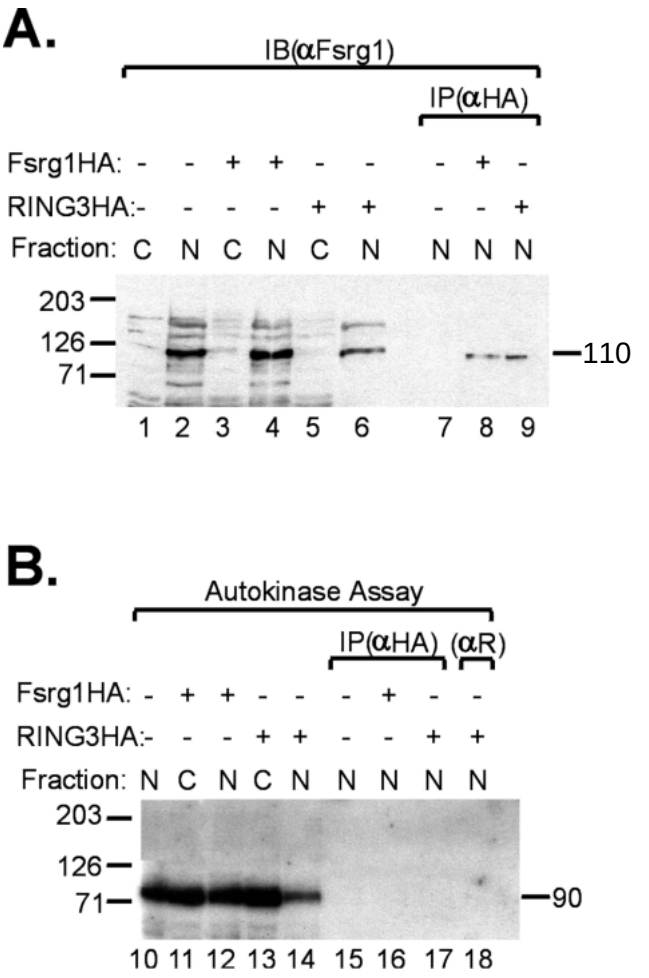

Fig. 8. Fsrg1 in HeLa cells. (A) Detection of Fsrg1 and RING3 proteins. HeLa cells were infected with *Fsrg1HA* (lanes 3,4,8), or with *RING3HA* (lanes 5,6,9), or with the vector DNA (lanes1,2,7). Cytosolic (C) or nuclear (N) fractions of infected HeLa cells were immunoprecipitated with the HA antibody (lanes 7-9). The Fsrg1 or RING3 proteins were detected with the affinity-purified Fsrg1 antibody. (B) Autokinase activity. Autokinase activity was carried out with samples from total lysates of vector-infected (lane 10), *Fsrg1HA*-infected (lanes 11,12), *RING3HA*-infected (lanes 13,14) HeLa cells. These infected cells were also immunoprecipitated with the HA antibody (αHA; lanes 15-17) or with the RING3 antibody (αR; lane 18) and used for the autokinase activity assay. The sizes of protein markers and the sizes of specific bands are indicated on the left and right sides of the figures, respectively.

Distribution of *Fsrg1* expression in hormonally modulated epithelia

Other bromodomain-containing proteins have been shown to associate with nuclear receptors of the steroid hormone superfamily, including the estrogen and retinoic acid receptors (Chiba et al., 1994) and the glucocorticoid receptor (Muchardt and Yaniv, 1993). To gain insight into whether Fsrg1 might have similar putative properties, we examined its expression in tissues from the male and female reproductive tract. In situ hybridization analysis revealed a remarkably specific expression of *Fsrg1* in these tissues, with highest levels being observed in the epithelium. Illustrated is the distribution of

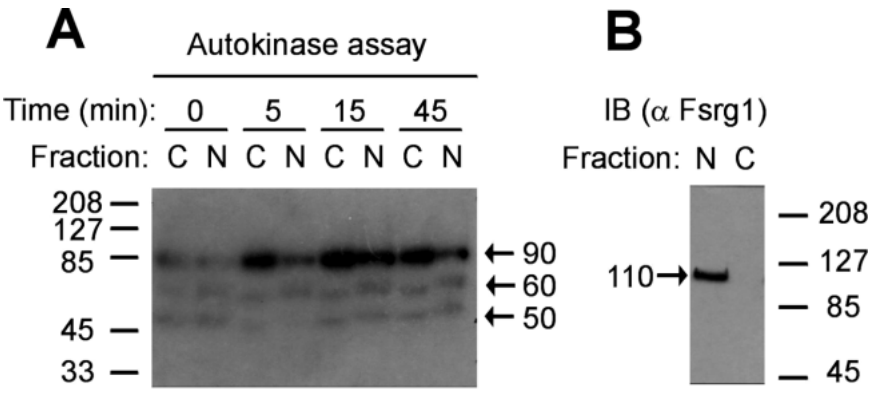

Fig. 9. Serum stimulation of NIH3T3 cells results in autokinase activity of ~95 kDa proteins but not Fsrg1. (A) Autokinase assay was carried out with samples from NIH3T3 cells either stimulated or not with 20% calf serum for various durations. The cytosolic (C) or nuclear (N) fractions of NIH3T3 cells were fractionated with SDS-PAGE and subjected to an autokinase assay as described in Materials and Methods. The sizes of protein markers and the sizes of specific bands are indicated on the left and right sides of the figures, respectively. (B) Detection of Fsrg1 protein. Cytosolic (C) or nuclear (N) fractions of NIH3T3 cells were separated on SDS-PAGE. The blot was incubated with affinity-purified rabbit polyclonal antibody against the Fsrg1 fusion protein. The sizes of the protein markers are indicated on the right side of the figure.

Fsrg1 in the epithelial lining of glandular ducts within the mammary gland (Fig. 10A), and in the epithelial lining of tubules within the epididymis (Fig. 10B). Strong signal was also seen in the epithelium of the vas deferens, the epithelial lining of fimbria of the oviducts, the endometrium (a specialized epithelium of the uterine horn), the cervical and vaginal epithelium in the female reproductive tract, and the epithelium of the prostate gland (data not shown).

DISCUSSION

In this paper, we report the identification and characterization of *Fsrg1*, a mouse gene homologous to the *Drosophila* gene *fsh*. This gene is believed to play an important function in development as mutations in Drosophila *fsh* result in sterility and homeotic transformations (Gans et al., 1975, 1980; Digan et al., 1986).

While there exists some genetic information regarding the function of *fsh* in *Drosophila*, very little is known about the function of its putative mammalian homologues. Recently, Denis and Green (1996) reported that an apparent human homologue of *fsh*, a gene designated *RING3* (Beck et al., 1992), encodes a serine/threonine kinase which can phosphorylate itself as well as other substrates such as Kemptide and myelin basic protein, in vitro. *RING3* was identified as the gene putatively encoding a ~90 kDa kinase which exhibited autophosphorylating activity in extracts from HeLa cells that had been transiently stimulated by certain mitogens. The ~90 kDa protein kinase was purified biochemically from the HeLa nuclear extracts and limited peptide sequencing revealed homology to RING3. This ~90 kDa nuclear kinase activity was also observed to be elevated in leukemic cells, suggesting that it is a component of a signal transduction pathway that becomes deregulated in certain tumor cells.

In the present study, however, the mouse homologue Fsrg1, which shares 95% identity at the protein level with RING3, was not found to contain any protein kinase activity. One critical difference between our results and those of Denis and Green (1996) may lie in the discrepancies in migration velocity of Fsrg1 and RING3 proteins in SDS-PAGE analysis. Our study showed that Fsrg1 and its human and rat homologues migrated as an ~110 kDa band as detected with the Fsrg1 antibody. When the products from *Fsrg1HA* and *RING3HA* constructs expressed in ratB1a cells were immunoprecipitated with an anti-HA antibody, they also migrated at ~110 kDa. In contrast, Denis and Green (1996) reported that the RING3 protein was detected as an ~90 kDa band. It is not clear why

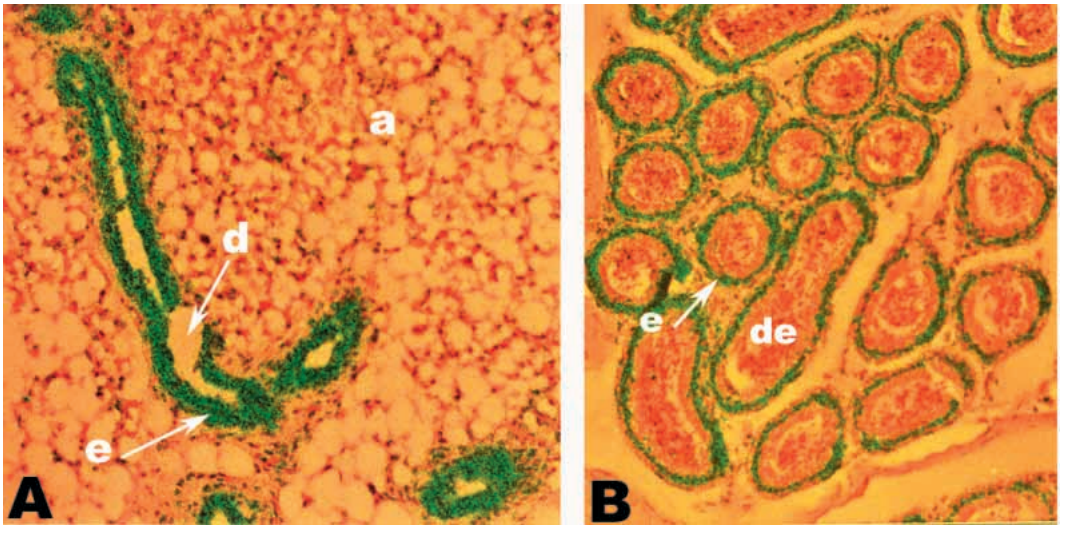

Fig. 10. In situ hybridization analysis of *Fsrg1* expression in mammary gland and epididymis. ^{35}S-labeled antisense probe specific to *Fsrg1* was hybridized with sections of paraffin-embedded tissues from mammary gland (A), and epididymis (B) of adult mice. Abbreviations: a, Adipose Tissue; e, Epithelia; d, Mammary Duct; de, Duct of Epididymis. Exposure 6 days.

Fsrg1 and RING3, whose predicted sizes are ~88 kDa, migrated as an ~110 kDa band upon SDS-PAGE. One possibility may be that Fsrg1 and RING3 are posttranslationally modified, such as by glycosylation (Jackson and Tjian, 1988). Removal of such a moiety while the nuclear samples were being prepared might yield migration at ~90 kDa, as reported in the study of Denis and Green (1996).

Two groups have reported the presence of an ~90 kDa protein which exhibited an autokinase activity upon induction by specific mitogens (Denis and Green, 1996; Rachie et al., 1993). Whether or not these reports dealt with the same kinase protein is not obvious. Rachie et al. (1993) reported the presence of the kinase activity in both nuclear and cytosolic fractions while Denis and Green (1996) observed that the ~90 kDa kinase activity was detected only in the nuclear fraction. In a recent article Ostrowski et al. (1998) reported the identification of a murine ~60 kDa mitogen-induced autokinase recognized by RING3 antibodies. These results suggest that the ~90 and ~60 kDa proteins might be members of a Fsrg1/RING3 family.

A kinase activity in the in-gel autokinase activity assay migrating at ~90 kDa and ~60 kDa was observed in our studies, using both the nuclear and cytosolic fractions, results similar to those observed by Rachie et al. (1993). However, our data indicated that these kinase activities were not derived from the bona fide Fsrg1. First, we detected a single Fsrg1 protein band as ~110 kDa rather than ~90 kDa in SDS-PAGE. Second, we could not detect an autophosphorylation activity in Fsrg1 immunoprecipitates. Third, we did not observe any increase in the kinase activity in cells which had been transfected with *Fsrg1HA* construct. Evidence supporting the role of RING3 as a serine/threonine kinase was suggested by the observation that the amino acid sequence of RING3 contains parts of conserved domains for protein kinases (Denis and Green, 1996). However, some of these conserved domains overlap within the bromodomain, a motif that has not been associated with any kinase activity to date. Further, amino acid residues critical for kinase activity, such as aspartate or arginine in the ATP binding site in subdomain VI (Hanks and Hunter, 1995), were not found in Fsrg1 (or RING3).

The question thus remains as to the potential biochemical function of Fsrg1. Several lines of evidence support the idea that *Drosophila fsh* and its vertebrate homologues may be involved in transcriptional regulation. First, *BDF1*, a yeast homologue of *fsh*, encodes a protein which has been shown to be a transcription factor (Lygerou et al., 1994). Second, the majority of proteins with bromodomains that have been characterized functionally are involved in some aspect of transcriptional control (Jeanmougin et al., 1997). For example, *Drosophila* brahma and its murine homologue Brg1 are components of transcription complexes of the so-called SWI/SNF class (Khavari et al., 1993; Tamkun, 1995; Wang et al., 1996). A postulated function of the bromodomain motif is for protein-protein interaction, including possible interactions with other bromodomain-containing proteins (Jeanmougin et al., 1997). The nuclear localization of the Fsrg1 protein demonstrated in our studies is consistent with a putative function in transcription complex formation. In this light, it is interesting to note that *Fsrg1* transcripts were not accumulated in elongating spermatids, cells in which the overall transcription activity is reduced significantly.

Bromodomain-containing proteins of the SWI/SNF class have further been shown to function as co-activators of nuclear hormone receptors. Co-activation has been shown for estrogen (Chiba et al., 1994), progesterone (Fryer and Archer, 1998), glucocorticoid (Muchardt and Yaniv, 1993), and retinoic acid (Chiba et al., 1994) receptors. Recent studies examining the role of chromatin configuration in transcriptional regulation have shown that chromatin remodelling by the glucocorticoid receptor requires the human bromodomain-containing protein BRG1 complex (Fryer and Archer, 1998). These receptors are known to be expressed in the epithelia of the reproductive tract tissues, in which we observed high levels of expression of *Fsrg1*. Glands from the mammary gland and prostate are also modulated through hormones, with estrogen stimulating growth of mammary glands and dihydrotestosterone with LH enhancing proliferation of prostate glands. The endometrium of the uterus undergoes cyclic regeneration and modulation under the influence of both estrogen and progesterone. The increased expression of *Fsrg1* in the epithelium of hormonally modulated tissues suggest a role for *Fsrg1* in either growth, differentiation and/or function of specialized epithelium.

The yeast homologue of *fsh*, *BDF1*, has been shown to be required for progression through the meiotic cell cycle (Chua and Roeder, 1995). Although nothing is known about the biochemical activity of the *Drosophila fsh*, genetic studies have shown its function in early development. Recently, it was reported that a rat homologue of *Fsh* was one of the genes whose expression was up-regulated in PC12 cells which were undergoing apoptosis (Wang et al., 1997). The exact role of *Fsrg1* in biological processes remains to be determined. The isolation of the mouse gene will enable functional analysis by gene mutation approaches, experiments which are currently underway in our laboratory.

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