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Basic fibroblast growth factor (bFGF) in rodent testis

Presence of bFGF mRNA and of a 30 kDa bFGF protein in pachytene spermatocytes

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We have previously described a 30 kDa basic fibroblast growth factor (bFGF)-like protein in rodent testicular homogenates and have shown that pachytene spermatocytes are the sites of predominant immunoreactivity for this bFGF-like protein (Mayerhofer, A., Russell, L.D., Grothe, C., Rudolf, M. and Gratzl, M. (1991) Endocrinology 129, 921–924). We have now addressed the question whether this 30 kDa bFGF-like protein is a large bFGF form and whether it is produced by pachytene spermatocytes. We detected bFGF mRNA in homogenates of isolated mouse spermatocytes (which consisted mainly of pachytene spermatocytes) using S1 nuclease protection assays. As shown by Western blot analyses, the bFGF mRNA in mouse spermatocytes is translated into bFGF of an approximate molecular weight of 30 kDa. Neither bFGF mRNA, nor bFGF itself, was observed in isolated mouse Leydig cells. These results indicate that the immunoreactive bFGF-like protein observed previously in germ cells of the murine testis is identical to bFGF. Thus, germ cells of the testis produce bFGF, which may exert regulatory function in the process of spermatogenesis.

Basic fibroblast growth factor; Testis; Mouse; mRNA; Germ cell

1. INTRODUCTION

The testis is a rich source of basic fibroblast growth factor (bFGF) [2,3]. However, until recently the localization of bFGF in the adult testis was unclear. We have previously shown that a bFGF-like protein of approximately 30 kDa exists in rodent testes [1]. Immunocytochemical procedures allowed us to localize this protein to germ cells in the rat and mouse testes. Thus, pachytene spermatocytes were the cells which revealed strongest immunoreactivity, but also some type A spermatogonia and spermatides during elongation were labeled [1]. Moreover, the bFGF-like immunoreactivity in the spermatocytes was restricted to certain developmental steps (stages), where it might exert specific functions. However, from this previous study it was not clear whether the bFGF-like protein in testicular germ cells is indeed bFGF or only an immunologically related factor. In addition, it was known whether germ cells are indeed the production site for bFGF. To answer these urgent questions, we have examined in the present study whether isolated spermatocytes contain both mRNA for bFGF and protein.

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2. MATERIALS AND METHODS

2.1. *Animals*

Adult male rats (Sprague–Dawley, Charles River, Sulzfeld, Germany) were killed under deep CO_2 anesthesia, testes were removed and rapidly frozen and stored frozen until isolation of RNA. Adult male mice (NMRI, Charles River, Extertal, Germany) were killed by cervical dislocation and spermatocytes and Leydig cells were isolated from mouse testes.

2.2. *Isolation of mouse spermatocytes*

Isolation procedure of spermatocytes from the testes of adult NMRI mice was based on the methods of Bellvé et al. [4], Wolgemuth et al. [5] and Djakiew and Dym [6]. In brief, testes of four mice were decapsulated and incubated in PBS containing 1 mg/ml collagenase (Worthington/Flow Laboratories, Bonn, Germany) and 2 μg DNAse I (Sigma, Munich, Germany) for 30 min at 34°C in a shaking water bath (120 cycles/min). Isolated tubules were washed three times with PBS and treated with 0.25% trypsin (Worthington/Flow Laboratories), 2 μg DNAse I, 0.07 mg dispase (Boehringer, Mannheim, Germany) in PBS for 15 min at 34°C. The resulting tubular fragments were further disrupted by repetitive pipetting (25 times) and centrifuged at 140 × *g* for 10 min. The cell pellet obtained was resuspended in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories) containing 0.5% bovine serum albumin (BSA, Sigma). After centrifugation at 50 × *g* for 10 min, the cell pellet was resuspended in 0.5% BSA/DMEM and passed through a nylon mesh (70 μm-openings).

The cell suspension (5×10^6 cells/ml) was loaded on top of a 2–4% BSA gradient (in PBS) in a separating funnel (1 l) at $1 \times g$ and was overlayed with 30 ml of 0.2% BSA/PBS. After 1.5 h, 50-ml fractions were collected from the bottom of the gradient and the purest fractions of spermatocytes were pooled (judged according to their morphological appearance; see Fig. 1). The cells were frozen (at −70°C) and stored until needed for Western blotting and/or RNA isolation for S1 nuclease assay.

2.3. *Isolation of mouse Leydig cells*

Mouse Leydig cells from an additional 6 adult mice were purified using discontinuous Percoll gradients as described previously by Sharpe and Fraser [7] with minor modifications. Testes were decapsulated and mechanically dispersed by repetitive pipetting in Dulbecco's modified Eagle medium (DMEM; Flow Laboratories), reducing the diameter of the pipette tip successively. Undispersed tissue and tubule fragments were allowed to settle and the supernatant (Leydig cells) was filtered through sterile gauze and then centrifuged at $120 \times g$ for 10 min. The pellet was resuspended in glucose-enriched PBS (6 g/l; G-PBS) and 10 ml of the cell suspension was loaded onto a discontinuous gradient of Percoll (Pharmacia, Freiburg, Germany) in G-PBS. The gradient consisted of 3 layers (each 10 ml) with specific weights of 1.08, 1.06 and 1.05 g/ml. After centrifugation at $1{,}100 \times g$ for 25 min, pure Leydig cells accumulated on top of the 1.08 g/ml layer and were aspirated; they were washed in G-PBS (with 0.03% BSA) and were resuspended in culture medium (DMEM with 10% heat-inactivated fetal calf serum, 2 mM glutamine and 20 μg gentamycin/ml) and were counted in a haemocytometer. Per animal the yields of Leydig cells were in the range of $5–8 \times 10^5$ cells and the purity ranged between 75–95% using 3β-hydroxysteroid dehydrogenase staining [7]. Freshly isolated cells were frozen until used for Western blotting/S1 nuclease assay.

2.4. *Western blot analyses*

Spermatocytes and Leydig cells were thawed and sonicated in 62.5 mM Tris–HCl buffer (pH 6.8) containing 10% saccharose, 5% 2-mercaptoethanol and 2% sodium dodecyl sulfate (SDS) and boiled for 5 min. Western blots were described previously in detail [1]. In brief, proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose and probed with a rabbit anti-bFGF antiserum (1:500) [8,9]. Immunoreaction was detected after incubation with a biotinylated goat anti-rabbit serum (Camon, Wiesbaden, Germany) using a commercial ABC kit (Vector, Burlington, CA, USA) and diaminobenzidine tetrahydrochloride (DAB, Aldrich, Milwaukee, WI) following the instructions of the manufacturer.

2.5. *Synthesis cRNA-probe, isolation of RNA and S1 nuclease protection assay*

The rat bFGF 820 bp cDNA encoding the entire rat bFGF was cloned with additional *Eco*RI linkers and has been previously characterized [10].

The 820 bp bFGF *Eco*RI fragment of this cDNA clone was subcloned in Bluescribe M13 (−) (Stratagene, La Jolla, CA, USA) and is referred to as clone bFGF (M13−). As a template for cRNA synthesis we used the cDNA clone bFGF (M13−). Synthesis of ^{32}P-labeled cRNA (spec. act. 3.96×10^7 cpm/μg) for S1 nuclease protection assay (S1-NPA) was carried out according to the T7 polymerase protocol of Promega Biotec (Madison, WI, USA) using 20 μCi of [α-^{32}P]UTP (spec. act. 15 TBq/mmol) and 2 μg of bFGF (M13−) linearized with *Rsa*I. The resulting cRNA probe contained 320 nucleotides of bFGF (225 bp translated and 96 nucleotides 3′ untranslated sequences) and 11 nucleotides of vector sequences (see Fig. 2A).

2.6. *S1 nuclease protection assay (S1-NPA) with a labeled cRNA-probe*

Total RNA was isolated by a modified guanidinium thiocyanate–CsCl method [11] from rodent testes and isolated pachytene spermatocytes (see Fig. 2B). A total of 20 μg of RNA (determined photometrically) and controls (consisting of H_2O or tRNA instead of total RNA) were hybridized with an excess of ^{32}P-labeled cRNA probe (5×10^4 cpm; spec. act. 3.96×10^7 cpm/μg) for 16 h at 58°C, as described previously [12]. The tRNA controls were used to verify the specificity of S1 nuclease hydrolysis. The RNA/cRNA hybridization was terminated by digestion with 680 U S1 nuclease (AGS, Heidelberg, Germany) for 2 h at 37°C. It should be mentioned that S1 nuclease, though more active in DNA hydrolysis, can also be used to hydrolyze single-stranded RNA templates. After phenol extraction and ethanol precipitation samples were separated electrophoretically on 0.3 mm-thick 5% polyacrylamide gels (8.3 M urea). Gels were dried and exposed to X-ray films at −70°C using intensifying screens.

3. RESULTS

Isolated mouse spermatocytes contained mRNA for bFGF, as did homogenates of whole testes of the rat

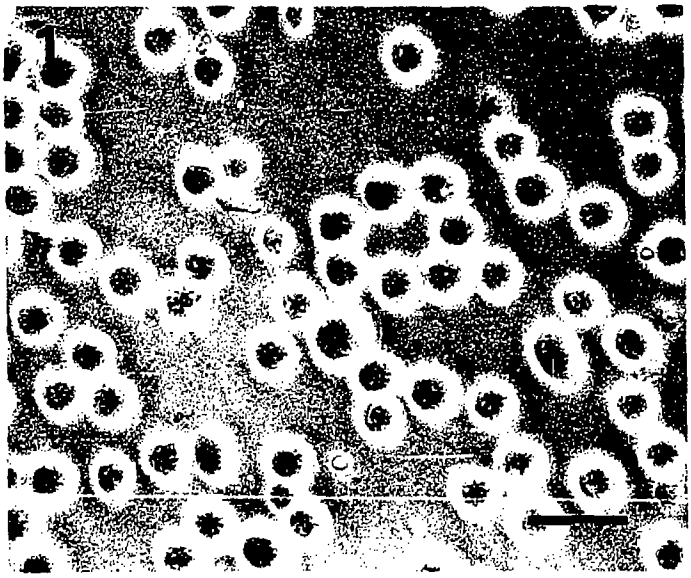

Fig. 1. Phase contrast micrograph of isolated mouse spermatocytes as used for the present study. Bar = 40 μm.

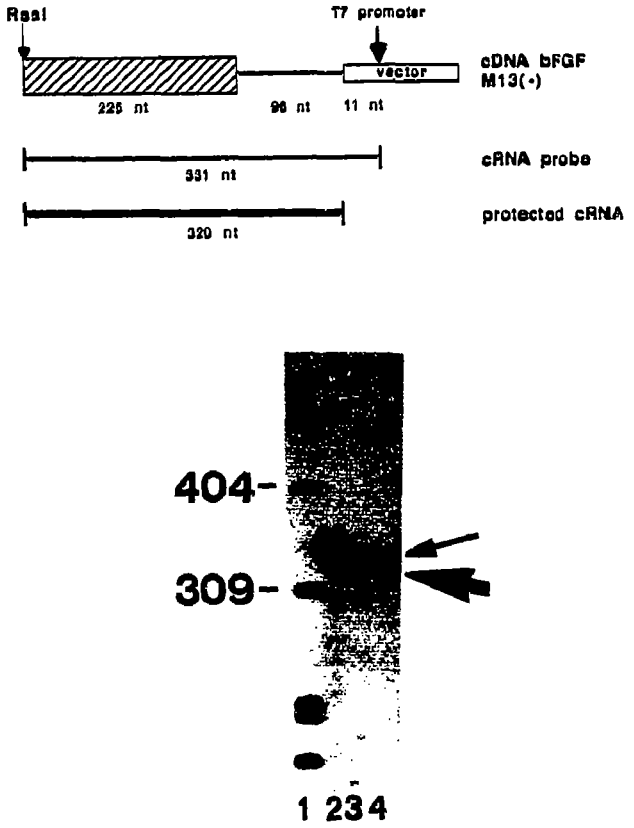

Fig. 2. (A) Scheme of single-stranded cRNA probe used for S1 nuclease protection assay. The cRNA probe (331 nucleotides) synthesized from cDNA bFGF M13(−) spans about one third of the whole rat bFGF translated and the total 3′ untranslated sequence. It originates at the 3′ end and extends towards its 5′ end up to the internal *Rsa*I restriction site. The 320 nucleotide RNA fragment protected from S1 nuclease hydrolysis is shown. The scaffolded box corresponds to the 3′ part of the bFGF coding region and the line to the 3′ untranslated sequence. The T7 promoter, the *Rsa*I restriction site and the vector sequences are indicated. (B) S1 nuclease protection analysis of extracted total RNA of rat testis and mouse spermatocytes bFGF mRNA is present in RNA isolated from rat testis and from mouse spermatocytes. Lane 3, pachytene mouse spermatocytes; lane 4, rat testis. As size marker, *Hpa*II-digested pBR322 was applied to lane 1, and the sizes are indicated on the left. The 331 nucleotide fragment in lane 2 represents undigested probe containing flanking vector sequences. The thin arrow depicts the undigested probe of 331 nucleotides, whereas the bold arrow corresponds to the protected cRNA fragment of 320 nucleotides.

(Fig. 2B). With S1 nuclease assays a protected band of 336 nucleotides was yielded, indicating the presence of bFGF mRNA co-linear with the probe in both testicular homogenates and isolated spermatocytes (Fig. 2B). No bFGF mRNA was found in isolated mouse Leydig cells and the tRNA control did not yield a band in the range of 336 nucleotides (not shown). The rat bFGF antisense RNA probe could be used to detect bFGF mRNA in the mouse spermatocytes, although there are some mismatches in the RNA/mRNA hybrids. The S1 nuclease does not hydrolyse within RNA/RNA hybrids unless there are more than three consecutive mismatches [13].

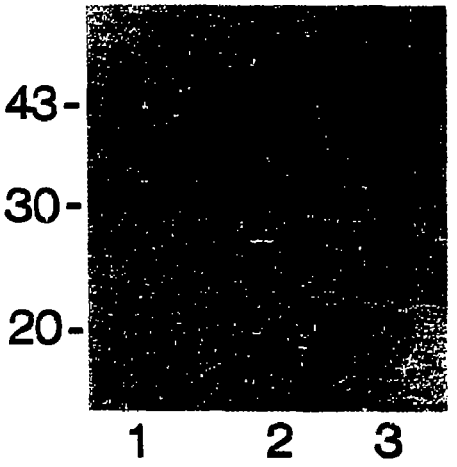

Fig. 3. Result of Western blot analysis. Immunoreactive bands of approximately 30 kDa were detected after probing blots of mouse testis homogenates (lane 1), isolated mouse spermatocytes (lane 2) with anti-bFGF (1:500), while no immunoreactive band was seen with isolated mouse Leydig cells (lane 3).

Western blotting revealed that in mouse spermatocytes mRNA is also translated into a 30 kDa form of bFGF, which is present in homogenates of whole mouse testes as well, but which cannot be detected in cultured mouse Leydig cells (Fig. 3).

4. DISCUSSION

The results of the present study extend our previous report on the localization of an immunoreactive 30 kDa bFGF-like protein in germ cells of rodent testes [1]. Here we now show that pachytene spermatocytes, which in both rat and mouse testes revealed strongest immunoreactivity for the bFGF-like protein, do indeed contain bFGF mRNA and, moreover, that bFGF mRNA is translated into a 30 kDa form of bFGF. These results, together with our previous detailed immunocytochemical study, indicate that pachytene spermatocytes are the main source of bFGF in the testis.

It is known that bFGF mRNA exhibits a strong sequence homology within several species. For example, the rat bFGF cDNA used for the present study has been successfully used in a taxonomically distant species, like *Xenopus*, and appears to be homologous to bFGF mRNA in *Xenopus* oocytes and the early *Xenopus* embryo [14,15]. This fact and the present results indicate that bFGF mRNAs in mouse and rat share a high sequence homology.

It is generally assumed that bFGF is a molecule of approximate molecular weight of 18 kDa (see [16]). However, at present several higher molecular weight

forms of bFGF (ranging from 20–25 kDa) [17–19] have been described and a large number of bFGF immunoreactive proteins are known (including 24-, 27-, 29-, 30- and 46-kDa forms [9,17,20,21]. Our results in the testis indicate that the 30 kDa pachytene spermatocyte-derived bFGF is another genuine high molecular weight form of bFGF. It is presently unclear whether this 30 kDa form itself has biological activity, or represents a precursor for the 18 kDa bFGF form. However, before this question can be addressed the target cells for bFGF in the testis must be identified. Because bFGF lacks a signal sequence, which is thought to mediate secretion [16], it is unclear how bFGF can leave the cell. Following secretion by spermatocytes it could act in a paracrine way on other cells within or maybe even outside the tubular compartment. Possible candidates for bFGF targets are Sertoli cells. In vitro, these cells reportedly respond to bFGF by expression of receptors for FSH or plasminogen activator [22] or by increases of c-*fos* mRNA [23]. However, it cannot be excluded that bFGF acts in an autocrine fashion on germ cells. Additional studies should address this question in view of the fact that germ cells are connected by intercellular bridges [24]. Thus, bFGF could also influence neighbouring germ cells without reaching the extracellular space.

The germinal epithelium in the adult testis is subjected to constant proliferation and differentiation. An array of locally produced growth factors (e.g. acidic fibroblast growth factor, nerve growth factor, seminiferous growth factor, insulin like-growth factor 1 and transforming growth factors alpha and beta) is assumed to be involved in the control of these processes (see [25–27]). Our data indicate that germ cell-derived bFGF is one of these factors, which in concerted action with others might participate in the regulation of spermatogenesis.

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