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Spef1, a conserved novel testis protein found in mouse sperm flagella

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Abstract

We describe the cloning and characterisation of *Spef1*, a novel testis-specific gene. *Spef1* has evolutionary orthologues in a wide range of species including mammals, other vertebrates, *Drosophila*, and protozoans with motile cilia or flagella. A second homologue of the gene, *Spef2*, is also present in several species, suggesting that these genes form part of a novel gene family. The Spef1 protein has two conserved domains, one of which is more strongly conserved in both homologues of the gene. Expression analysis of *Spef1* in mice shows that it is expressed predominantly in adult testis, suggesting a role in spermatogenesis. Using an antibody generated to recombinant Spef1, we demonstrate a specific pattern of Spef1 localisation in the seminiferous epithelium of adult mouse testis. Further immunohistochemical analysis using electron microscopy shows Spef1 to be present in the tails of developing and epididymal sperm, internal to the fibrous sheath and around the outer dense fibres of the sperm flagellum.

Keywords: Reproduction; Spermatogenesis; Protozoa

1. Introduction

Mammalian spermatogenesis is a complex and tightly regulated process, involving the terminal differentiation of stem cells into mature flagellated spermatozoa (Hecht, 1998). This in turn requires the invocation of numerous and varied cellular processes, including control of cell division, regulation of the meiotic and mitotic pathways, apoptosis, chromatin remodelling, transcriptional and post-transcriptional regulation of gene expression, and cytoskeletal assembly. For these events to occur correctly, a large number of genes must be accurately expressed and regulated (reviewed in Wolgemuth and Watrin, 1991; Hecht, 1995; Wolgemuth et al., 1995; Eddy, 1998; Eddy and O'Brien, 1998). Many of the genes involved in spermatogenesis remain to be identified and characterised.

During spermiogenesis, the characteristic morphology of the spermatozoon is acquired through the assembly of cytoskeletal components to form the various necessary structures present in mature spermatozoa (Bearer and Friend, 1990; Fouquet and Kann, 1994; Yoshinaga and Toshimori, 2003). These include the perinuclear theca, the acrosome and the flagellum. The perinuclear theca, or perinuclear matrix, surrounds the nucleus with a condensed layer of cytoplasmic elements (Longo et al., 1987); while the acrosome, located at the apex of the spermatozoon, contains proteins that function in the interaction of the sperm with the oocyte (Toshimori, 1998; Olson et al., 2003; Yoshinaga and Toshimori, 2003).

Abbreviations: ODF, outer dense fibres; FS, fibrous sheath; cAMP, cyclic adenosine monophosphate; ATP, adenosine triphosphate; kb, kilobase; BLAST, basic local alignment search tool; EST, expressed sequence tag; cDNA, complementary DNA; BAC, bacterial artificial chromosome; ES, embryonic stem; NCBI, National Center for Biotechnology Information; RT-PCR, reverse transcription polymerase chain reaction; bp, base pair; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; MTN, multiple tissue northern; FITC, fluoroscein isothiocyanate; kDa, kilodalton; dpc, days post coitus; UTR, untranslated region; BSA, bovine serum albumin; mRNA, messenger RNA; CH, calponin homology.

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Extensive chromatin remodelling also occurs, involving the replacement of histones with protamines and allowing condensation of the sperm nucleus to about 5% of its previous volume (Sassone-Corsi, 2002).

The sperm flagellum is organised into three regions, the middle piece, principal piece and end piece, each with a different structural composition. The internal structure of the sperm flagellum bears much similarity to that of flagella and cilia found in other cell types and organisms. The central axoneme consists of a pair of microtubules (the central apparatus) surrounded by nine microtubule doublets, in a pattern that appears to be conserved across evolution (Toshimori, 1998). This axonemal structure contributes to flagellar motility. In the sperm flagellum, the axoneme is surrounded by structures termed the outer dense fibres (ODF) and the fibrous sheath (FS) (Eddy et al., 2003). The middle piece contains a mitochondrial sheath surrounding nine outer dense fibres associated with the nine microtubule doublets of the axoneme, while in the principal piece, the fibrous sheath is organised into two longitudinal columns with bridging semicircular ribs, and surrounds the ODF and axoneme structures, with two ODF being incorporated into the columns of the FS and the remainder surrounding the axoneme (Oko, 1998).

The FS and ODF are thought to play a role in flagellar motility, through controlling the flexibility of the flagella in a mechanical manner (Fawcett, 1975). In addition, however, the ODF and FS contain numerous other proteins (reviewed in Miranda-Vizuete et al., 2003), many of which remain uncharacterised. Several of these components have been shown to have other functions such as enzymatic activity (reviewed in Eddy et al., 2003). This suggests that they may also be involved in regulating flagellar motility on a molecular level, for example through kinase and phosphatase enzymes and cAMP signalling (see for example Carrera et al., 1996, and reviewed in Tash and Bracho, 1994), and production of ATP via glycolysis.

In this paper, we describe the identification and characterisation of a novel testis-expressed gene, *Spef1*, and demonstrate its involvement in the spermatogenic process. In mammals, expression of *Spef1* is primarily detected in the adult testis. However, *Spef1* is highly conserved across a broad range of species, including a number of ciliate and flagellate protozoans, thus defining a new class of proteins with a conserved domain. In the testis, *Spef1* is localised to developing spermatids, appears to be involved in flagellar formation, and is shown to be incorporated into the flagellum of mature spermatozoa, associated with the FS and ODF.

2. Materials and methods

2.1. Identification and cloning of Spef1

A region of approximately 6 kb from mouse chromosome 2 containing the mouse *Cenpb* gene was analysed by searching against a mouse EST database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) to find putative genes within the region. A number of ESTs derived from mouse testis were initially identified. Further BLAST searching identified a cDNA clone corresponding to one of the ESTs from a RIKEN mouse testis library.

The sequence obtained from the database was used to design primers for screening a cDNA library from 10- to 12-week-old mouse testis (Lamda Uni-ZAP XR library, Stratagene, containing mouse testis cDNA clones prepared in pBluescriptKS). Positive clones obtained from the screen were sequenced using the Big Dye Terminator kit (Applied Biosystems).

To clone the genomic sequence of *Spef1*, a BAC encompassing the *Spef1* genomic region (185L24 from Genome Systems, Library CITB from mouse ES cell line CJ7, strain 129SrES) was obtained and Southern blotting analysis was performed using restriction digest, agarose gel electrophoresis and transfer to nylon membrane (Hybond N^+, Amersham Biosciences). A probe for Southern hybridisation was generated by labelling the cDNA clone of *Spef1* with [α-^{32}P]dCTP (Roche Random Prime labelling kit) and hybridised at 65°C overnight in Church buffer. This showed that the complete sequence spanning the *Spef1* cDNA was contained in a 9 kb *Sac*I fragment of BAC 185L24. This fragment was subcloned into the plasmid vector pAlter (Promega) and sequenced.

2.2. Sequence analysis

Further sequence analysis of the *Spef1* cDNA was carried out using BLAST searching against various NCBI databases to identify *Spef1* paralogues and orthologues in other species. Where the full open reading frame was not available, the EST clones were obtained from relevant sources and sequenced or a consensus sequence was constructed from several ESTs (see Fig. 1). Comparison of mouse Spef1 and related sequences obtained from the databases and sequencing was performed using T-COFFEE (Notredame et al., 2000) to generate a multiple sequence alignment. This data was used to construct a phylogenetic tree for Spef1, using protein distance calculations by "protdist" (Dayhoff–PAM matrix) and the neighbour joining method. The tree was drawn using the "drawtree" method from the PHYLIP phylogeny inference program. Protein domain prediction analysis was performed using the PROSITE database at www.expasy.org.

2.3. RNA isolation and Northern blot analysis

RNA was isolated from mouse tissues and cell lines using Trizol reagent (Invitrogen) according to product directions. To detect the expression of *Spef1* in different tissues of adult mouse, a mouse multiple tissue Northern (MTN) blot containing poly(A)$^+$ RNA (Clontech) was used. For analysis