The shorter zinc finger protein ZNF230 gene message is transcribed in fertile male testes and may be related to human spermatogenesis

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The zinc finger gene family represents one of the largest in the mammalian genome, with several of these genes reported to be involved in spermatogenesis. A newly discovered gene has been identified that is expressed abundantly in the testicular tissue of fertile men as determined by mRNA differential display. The gene encodes a C_3HC_4-type zinc finger protein motif (ring finger motif) consistent with a role in pre-meiotic or post-meiotic sperm development. The gene was named ZNF230 and mapped to the short arm of chromosome 11 (11p15). ZNF230 has two transcripts, of 1 kb and 4.4 kb in length. The shorter 1 kb transcript was only detected in testicular tissue whereas the longer 4.4 kb transcript was not detected in testis but was found in several other tissues. The lack of detectable ZNF230 expression in azoospermic patients by reverse transcriptase-mediated PCR analysis is interpreted to mean that this gene is involved in maintaining normal human male fertility.

Key words: male infertility, mRNA differential display, RACE, rapid amplification of cDNA ends, zinc finger gene.

INTRODUCTION

Spermatogenesis is a complex process of cell development and differentiation during which extensive changes in cell morphology and intracellular organization occur. This process requires the highly regulated expression of a network of genes located on both the autosomes and the sex chromosomes [1–4]. In humans, about 20% of cases of male infertility display a severe or complete loss of mature spermatozoa, called oligozoospermia and azoospermia respectively. Some of these cases have been linked to deletions of genes on chromosome Yq11 that encode an azoospermia factor (AZF) [5]. Extensive molecular-deletion analysis of Yq11 confirmed that three non-overlapping regions in band Yq11, called AZFa, AZFb and AZFc, are essential for human sperm production [6]. Several AZF candidate genes have been identified in these regions with a putative function in human spermatogenesis [4]. Furthermore, the sex-determining region (SRY) on the short arm of chromosome Y has been confirmed to be required for expression of the male phenotype in humans.

In order to further our understanding of normal and abnormal spermatogenesis we identified novel genes expressed during spermatogenesis by mRNA differential display (mRNA-DD). Thus the mRNA transcription pool of the testicular tissues from two healthy fertile adults was compared with that of one patient with a post-meiotic spermatogenic disruption at the early spermatid phase. This comparison identified a series of known and novel transcription products expressed only in normal testicular tissues that were categorized by searching for homologous sequences in GenBank. One transcription product was part of the previously undiscovered zinc finger gene, ZNF230, expressed only in testicular tissues.

The zinc finger gene family, one of the largest gene families in mammals, is defined by a conserved cysteine- and histidine-rich domain essential for the binding of zinc ions [7,8]. This gene family may be divided into many subfamilies, including C_2H_2, glucocorticoid receptor, ring finger, GATA-1, GAL4 and LIM family genes [9–11]. More than 20 different zinc finger genes located on sex chromosomes or autosomes have been proposed to play a regulatory role in mammalian spermatogenesis [12,13]. Among them, *ZFY* and *ZFX* are C_2H_2-type zinc finger genes located on the Y and X chromosome respectively [14,15]. Autosomal C_2H_2-type zinc finger genes include *Zfp-35*, *Zfp-29* and *Zfp-37* expressed in pachytene spermatocytes (*Zfp-35* [16]) or in round spermatids (*Zfp-29* [17]). *Zfp-37* is expressed most abundantly near the end of meiosis, pointing to a role in post-meiotic spermiogenesis [18]. Two ring finger genes (sperizin, a $C_3H_2C_3$ type, and XYbp, a C_3HC_4 type) are expressed in haploid germ cells (sperizin [19]) or in the XY bivalent of spermatocytes (the XY body [20]). This suggests that the zinc finger genes in the male germ line function primarily during meiosis and early spermatid development.

The newly discovered zinc finger spermatogenesis gene described in this paper, ZNF230, belongs to the ring finger subfamily (C_3HC_4) and was mapped to chromosomal band

Abbreviations used: mRNA-DD, mRNA differential display; 5′-RACE, rapid amplification of 5′ cDNA ends; RT-PCR, reverse transcriptase-mediated PCR; FISH, fluorescence *in situ* hybridization; AZF, azoospermia factor.

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The nucleotide sequence for ZNF230 has been deposited in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AF214680.

11p15. Multiple-tissue Northern-blot and reverse transcriptase-mediated PCR (RT-PCR) analysis among testicular tissues of fertile and infertile men indicate a possible role for the 1 kb isoform of ZNF230 in human male fertility.

EXPERIMENTAL

Materials

Fresh testicular tissue and other tissues of normal human adults who had died in accidents, as well as fetal testicular tissue, were obtained from West China Hospital, Sichuan University, Sichuan, People's Republic of China, with the consent of their relatives. Testis biopsy material from a patient with idiopathic azoospermia was obtained from the Department of Urology, Sichuan College of Genital Health. The patient proved to have a post-meiotic spermatogenic disruption at the early spermatid phase. He had a normal karyotype and other conditions such as viral parotitis, varicocele, obstruction of the vasa deferens and undescended testes, which may have caused his infertility, were excluded. These conditions were also excluded in three other patients for expression studies.

Preparation of RNA

Total RNAs from human tissues were prepared by using the RNeasy Mini Kit (Qiagen) with DNase I (RNase-free) to eliminate DNA contamination. Pellets of the total RNA were dissolved in diethyl pyrocarbonate-treated water and stored at −80 °C prior to use.

mRNA-DD

The mRNA-DD experiment was performed by using the RNAimage Kit (Gene Hunter) according to the protocol of Liang and Pardee [21]. Briefly, 500 ng of total RNAs from two normal adults and one azoospermic patient were reverse transcribed by incubating at 65 °C for 5 min, then at 37 °C for 60 min and 75 °C for 5 min, with anchored oligo-dT primers.

The reverse-transcription products (2 μl) were amplified at 94 °C for 30 s, 40 °C for 2 min and 72 °C for 1 min, for each of 40 cycles followed by a 5 min extension at 72 °C with an anchored oligo-dT primer and an arbitrary forward primer (Gene Hunter). The amplified cDNAs were separated on a 6% polyacrylamide sequencing gel for differential analysis. Bands displayed in the two normal control lanes (N1 and N2 in Figure 1, see below) but not in that of the patient (P in Figure 1) were cut out from the gel and the DNA was recovered by NucleoTrap Gel Extraction Kit (Clontech) according to the manufacturer's instructions. The eluted DNAs were re-amplified with the same set of primers used in the mRNA-DD experiment. The re-amplified fragments were cloned into the pGEM-T Easy vector (Promega) as described by the manufacturer, and sequenced with Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech) using an ALFexpress automatic DNA sequencer (Pharmacia Biotech).

Rapid amplification of 5′ cDNA ends (5′-RACE)

5′-RACE experiments were performed using the SMART RACE cDNA amplification kit (Clontech) [22]. Briefly, 5′-RACE-Ready cDNA was obtained by reverse transcription using total RNA from testicular tissue of healthy fertile adults as a template. Universal primer mix provided in the kit and gene-specific primers based on the sequence of the fragment obtained from mRNA-DD were used for the 5′-RACE experiments. The PCR-amplification profile was 94 °C for 2 min, five cycles of 94 °C for 5 s and 72 °C for 3 min, five cycles of 94 °C for 5 s, 70 °C for 10 s and 72 °C for 3 min, 25 cycles of 94 °C for 5 s, 68 °C for 10 s and 72 °C for 3 min, and 72 °C for 5 min. The PCR products were recovered using the QIAEX II Gel Extraction Kit (Qiagen) and cloned as described above. Clones with inserts of the expected sizes were identified by *Eco*RI digestion and sequenced as described above.

Multiple-tissue Northern-blot analysis

Multiple Tissue Northern (MTN) blot membranes (Clontech, catalogue numbers 7759-1 and 7760-1) were used to determine the tissue-expression pattern of ZNF230. A 5′-RACE clone of ZNF230 and the primer pair R4-F1 (5′-ATGGGACAGCAAA-TTTCGGATCAGACAC-3′) and R4-R6 (5′-CCTGCCTCATC-AGCCATGTTAAGAAT-3′) were used to amplify a 673 bp probe for Northern-blot analysis. The PCR amplification protocol was 94 °C for 2 min, 94 °C for 30 s, 68 °C for 30 s and 72 °C for 30 s for 30 cycles. Then PCR products were recovered as mentioned above and labelled in the presence of [^{32}P]dCTP (DuPont) with the Prime-a-Gene Labelling System according to the manufacturer's protocol (Promega). Hybridization of the probe to the MTN-membrane-bound RNA was performed as described by the manufacturer of the membranes (Clontech) including hybridization with a human β-actin cDNA probe (Clontech, catalogue number 9800-1) as the control.

RT-PCR analysis of ZNF230

Total RNAs from normal human tissues including lung, liver, spleen, kidney, brain, testis, skeletal muscle and stomach were extracted for RT-PCR analysis. Total RNAs (2.5 μg) from each tissue were used as templates for reverse transcription using the cDNA First-Strand Synthesis System (Gibco BRL). Then, 1 μl of the reverse-transcription products was used as a template for the subsequent PCR experiments. The primers used to amplify a 282 bp fragment were 5′-AACATGTTACGTTGGTTCGAGA-GAG-3′ (forward) and 5′-CCTGCTGCTTGACTTGTGAT-ATCT-3′ (reverse). A 401 bp fragment of β-actin was co-amplified as an internal control. All primers used in RT-PCR were located in different exons. The PCR reaction profile was 94 °C for 2 min followed by 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s for 25 cycles, with a final extension stage at 72 °C for 5 min. The PCR products were then separated by 2% agarose-gel electrophoresis and analysed for RNA expression.

In addition, ZNF230 expression was compared using RNAs extracted from the testicular tissues of four normal fertile adults, three fetuses and four azoospermia patients, including the one used in the mRNA-DD experiment by RT-PCR under the same conditions.

Chromosome mapping of ZNF230 by fluorescence *in situ* hybridization (FISH) and PCR analysis

Mapping of the ZNF230 gene on human metaphase chromosomes was completed by FISH. A genomic fragment was amplified by the long PCR protocol of Wu and Zhang [23] and used as a hybridization probe. It was labelled with biotin-11-dUTP (Enzo Diagnosis) by nick-translation (Intergen) and hybridized to denatured metaphase chromosomes at a final probe concentration of 20 ng/ml in 50% formamide, 10% dextran sulphate, 2 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.2 mg/ml Cot-1 DNA (Gibco-BRL) with 2 mg/ml salmon sperm DNA and 2 mg/ml *Escherichia coli* tRNA that served as carriers. The hybridized signals were

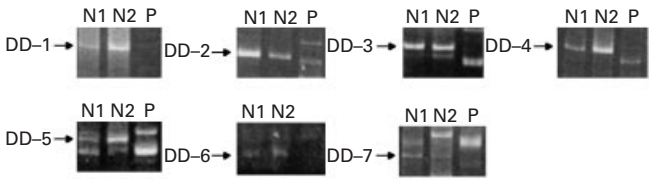

Figure 1 mRNA-DD of a patient with idiopathic azoospermia and two normal fertile adults

Arrows indicate the bands expressed only in the normal fertile adults (N1 and N2) but not in the patient (P). DD-1, DD-3 and DD-5 are novel ESTs, i.e. not found in GenBank, and DD-2, DD-4, DD-6 and DD-7 are found in the database as testicular ESTs but not as full-length cDNAs.

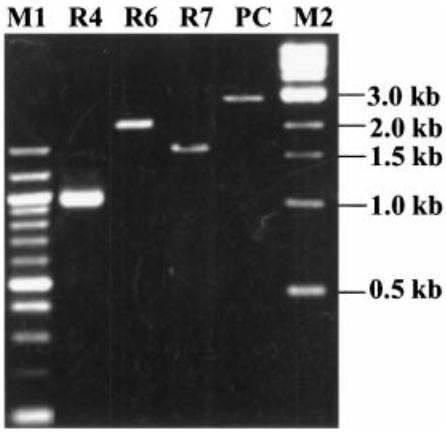

Figure 2 Results of 5′-RACE

R4, R6 and R7 are 900 bp, 1.9 kb and 1.5 kb RACE products, respectively, and PC is a 2.6 kb human transferrin receptor positive control. M1 and M2 are 100 bp and 1 kb DNA ladders respectively.

detected with FITC-avidin (Boehringer Mannheim). Metaphase cells were counterstained with DAPI II (4,6-diamidino-2-phenylindole). The slides were examined through a Zeiss fluorescent microscope with a integrating CCD camera (Photometrics, Tucson, AZ, U.S.A.) and the images were captured by using a Cyto Vision-Ultra workstation (Applied Imaging) with Probe Software.

The ZNF230 gene's chromosome location was confirmed by PCR amplification of DNAs extracted from a set of hamster–human hybrid cell lines containing overlapping sets of human chromosomes prepared by Karl-Heinz Grzeschik (University of Marburg, Marburg, Germany). The primer pair ZNF230for3 (5′-GTGATCGACACAGGAATTGCCC-3′) and ZNF230rev3 (5′-GGGGGACAAATGATCAGAATAGC-3′) were amplified under the following conditions: denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C, 57 °C and 65 °C for 1 min at each temperature, and a final extension step at 65 °C for 2 min. All PCR reactions were performed in a Biometra T96 PCR machine. The expected PCR amplification products are 294 bp for ZNF230. The PCR buffers recommended by the supplier of *Taq* polymerase (Gibco BRL) were modified by adding 0.5 % DMSO to improve the reaction specificities.

RESULTS AND DISCUSSION

Specific testicular transcription products displayed differentially in fertile and infertile men

By mRNA-DD with a pool of testicular transcription products of two healthy fertile men and one azoospermic patient, we identified 51 RT-PCR products expressed only in the testes of the fertile men and not in those of the infertile patient. Of these, 31 were cloned into the pGEM-T Easy vector for sequencing. BLAST analysis of nucleotide sequences in the GenBank Nucleotide Sequence Database revealed that these 31 ESTs could be grouped into five categories: novel ESTs, ESTs homologous with those in the testis library that were not full-length cDNAs, known gene sequences, ubiquitously expressed ESTs and mitochondrial DNAs. For example, EST DD-10 is identical with the 3′ region of a human transcription factor p38-interacting protein (accession number AF093250) and DD-23 is part of the mitochondrial gene NADH dehydrogenase III (accession number AF004342). Although our results suggest that these genes may also play a role in spermatogenesis, this remains to be proven.

For cloning of full-length cDNAs with a potential role in human spermatogenesis, four ESTs that shared homology with ESTs from the testis library that were not full-length cDNAs (DD-2, DD-4, DD-6 and DD-7) and three novel ESTs (DD-1, DD-3 and DD-5) were used as starting points to isolate the related full-length cDNA sequences by appropriate 5′-RACE experiments. The DD1–DD7 differential display products are displayed in Figure 1.

Isolation of a full-length cDNA for a novel C_3HC_4-type zinc finger gene, ZNF230

Rapid amplifications and sequence analyses of the cDNA 5′ ends of DD1–DD7 were performed with the SMART RACE cDNA amplification kit. As shown in Figure 2, 5′-RACE fragments were obtained for DD4 (R4, 900 bp), DD6 (R6, 1.9 kb) and DD7 (R7, 1.5 kb). These fragments were then cloned into the pGEM-T Easy vector and sequenced completely. The segments that overlapped with the corresponding mRNA-DD sequences, including the polyadenylated [poly(A)$^+$] tail, were assembled to obtain three full-length cDNAs.

The first cDNA (R4 + DD4) represents a novel C_3HC_4-type zinc finger gene (ring finger), tentatively designated ZNF230. The ZNF230 cDNA is 1005 bp in length and contains a 690 bp open reading frame (ORF) that starts at position 121 and ends at position 813. It encodes a polypeptide of 230 amino acid residues with the C_3HC_4 zinc finger domain in positions 155–196. The presumed translation-initiation codon ATG is preceded by four in-frame stop codons and the canonical polyadenylation signal AATAAA was found 15 bp upstream of the poly(A)$^+$ tails, as expected (Figure 3).

ZNF230 belongs to the ring finger subfamily of zinc finger genes. The ring finger is a specialized zinc finger structure, containing 40–60 amino acid residues, that binds two atoms of zinc with the consensus sequence $CX_2CX_{9-39}CX_{1-3}HX_{2-3}CX_2CX_{4-48}CX_2C$. The three-dimensional structure of the zinc-complex structure is unique to the ring domain and referred to as the 'cross-brace' motif [8–9,24]. Proteins with such structure generally are nuclear transcriptional factors and the motif is involved in both protein–DNA and protein–protein interactions [11,24,25]. Multiple sequence alignments of ZNF230's ring finger motif with those of other ring finger genes [26–32] indicate that this motif is highly conserved among mammalian species (Figure 4). When analysed with PSORT, a computer program for the prediction of protein-localization sites in cells, ZNF230 was proposed to be located in

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1     ACG CGG GAG GTC TGA GCT GTG GGC TGA GGC AGC GCA GCC GCT GCG
46    CCA GGG TGC GCG ATG CCT TGA ACC TGG GAA ACT ATG TGA AGC AAC
91    ACT CTG GAT TTT GAA AGA CAT CTT TTC ATC ATG GGA CAG CAA ATT
                                          Met Gly Gln Gln Ile    5
136   TCG GAT CAG ACA CAG TTG GTT ATT AAC AAG TTA CCA GAA AAA GTA
      Ser Asp Gln Thr Gln Leu Val Ile Asn Lys Leu Pro Glu Lys Val   20
181   GCA AAA CAT GTT ACG TTG GTT CGA GAG AGT GGC TCC TTA ACT TAT
      Ala Lys His Val Thr Leu Val Arg Glu Ser Gly Ser Leu Thr Tyr   35
226   GAA GAA TTT CTC GGG AGA GTA GCT GAG CTT AAT GAT GTA ACG GCT
      Glu Glu Phe Leu Gly Arg Val Ala Glu Leu Asn Asp Val Thr Ala   50
271   AAA GTG GCT TCT GGC CAG GAA AAA CAT CTT CTC TTT GAG GTA CAA
      Lys Val Ala Ser Gly Gln Glu Lys His Leu Leu Phe Glu Val Gln   65
316   CCT GGG TCT GAT TCC TCT GCT TTT TGG AAA GTG GTT GTA CGG GTG
      Pro Gly Ser Asp Ser Ser Ala Phe Trp Lys Val Val Val Arg Val   80
361   GTC TGT ACC AAG ATT AAC AAA AGC AGT GGC ATT GTG GAG GCA TCA
      Val Cys Thr Lys Ile Asn Lys Ser Ser Gly Ile Val Glu Ala Ser   95
406   CGG ATC ATG AAT TTA TAC CAG TTT ATT CAA CTT TAT AAA GAT ATC
      Arg Ile Met Asn Leu Tyr Gln Phe Ile Gln Leu Tyr Lys Asp Ile  110
451   ACA AGT CAA GCA GCA GGA GTA TCG GCA CAG AGC TCC ACC TCT GAA
      Thr Ser Gln Ala Ala Gly Val Ser Ala Gln Ser Ser Thr Ser Glu  125
496   GAA CCT GAT GAA AAC TCA TCC TCT GTA ACA TCT TGT CAG GCT AGT
      Glu Pro Asp Glu Asn Ser Ser Ser Val Thr Ser Cys Gln Ala Ser  140
541   CTT TGG ATG GGA AGG GTG AAG CAG CTG ACC GAT GAG GAG GAG TGT
      Leu Trp Met Gly Arg Val Lys Gln Leu Thr Asp Glu Glu Glu Cys  155
586   TGT ATC TGT ATG GAT GGG CGG GCT GAC CTC ATC CTG CCT TGT GCT
      Cys Ile Cys Met Asp Gly Arg Ala Asp Leu Ile Leu Pro Cys Ala  170
631   CAC AGC TTT TGT CAG AAG TGT ATT GAT AAA TGG AGT GAT CGA CAC
      His Ser Phe Cys Gln Lys Cys Ile Asp Lys Trp Ser Asp Arg His  185
676   AGG AAT TGC CCT ATT TGT CGC CTA CAG ATG ACT GGA GCA AAT GAA
      Arg Asn Cys Pro Ile Cys Arg Leu Gln Met Thr Gly Ala Asn Glu  200
721   TCT TGG GTG GTA TCA GAT GCA CCC ACT GAA GAT GAT ATG GCT AAC
      Ser Trp Val Val Ser Asp Ala Pro Thr Glu Asp Asp Met Ala Asn  215
766   TAT ATT CTT AAC ATG GCT GAT GAG GCA GGC CAG CCC CAC AGG CCA
      Tyr Ile Leu Asn Met Ala Asp Glu Ala Gly Gln Pro His Arg Pro  230
811   TGA CCT TGA AGT GAA AGT CTT CTG TTG CTA TTG TGG GCT CAA ATA
      End
856   TTT GGT CAT GGG GGA AGA ATG TAG GGT TGT GGC ACT GGC ACA GAC
901   ACA GGA AAA TCC ATT TTC CCC ACT CTT TTA TTT TTG CTA TTC TGA
946   TCA TTT GTC CCC CTT TTA AAA ATA AAC TTC CCA TGT CTT CCA AAA
991   AAA AAA AAA AAA AAA
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Figure 3 Nucleotide and predicated amino acid sequences of ZNF230

The in-frame stop codons are shown in bold and the tailing signal is underlined. The predicated C_3HC_4 zinc finger motif is boxed and the conserved cysteines and histidine are emboldened. The sequence numbers for nucleotides and amino acids are listed on the left and right respectively.

the nucleus, even though no nuclear consensus signal was found in its sequence. Spermatogenesis in the patient whose tissues were used in the mRNA-DD experiment is blocked at the early spermatid phase and other factors that can cause azoospermia have been excluded in this case (see the Experimental section). Thus we deduced that the ZNF230 gene probably functions as a germ-line-specific nuclear transcription factor involved in spermiogenesis, but this, of course, needs to be confirmed by further biochemical studies.

In addition to ZNF230, two other genes were also cloned: one was the GRTH (gonadotropin-regulated testicular RNA helicase) gene (R7+DD7), cloned independently by Tang et al. [33], and represented a novel member of the DEAD-box protein family expressed in rat, mouse and human testes. GRTH has ATPase and RNA helicase activities and increases translation *in vitro*. This gene is developmentally regulated in both somatic (Leydig cells) and germinal (meiotic spermatocytes and round haploid spermatids) cells. Its product is predominantly localized in the cytoplasm, where it may function as a translational activator in the male germ line [33]. This result is consistent with the status of our patient (see the Experimental section). Another new gene, ZNF463 (R6+DD6), was a KRAB-type (Krüppel-associated box-type) zinc finger gene. The 1.9 kb transcript was highly expressed in normal adult human testis, and two transcripts of approx. 4.7 kb and 5 kb were detected in normal thyroid tissue. It is also considered as a strong candidate target gene of the specific 19q aberrations in benign thyroid tumours [34,35]. These results suggest that our experimental approach for

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ZNF230_HUMAN   CCICMD-G----RADLILPCAHSFCQKCIDKWSDRHR-N------CPIC
RFP_HUMAN      CPVCLQYF---A-EPMMLDCGHNICCACLARCWGTA--ETNV--SCPQC
PML_HUMAN      CQQC-QAE---AKCPKLLPCLHTLCSGCLE---ASGM-Q------CPIC
XYbp_MOUSE     CPLCLLRHSKD-RFPDIMTCHHRSCVDCLRQYLRIEISESRVNISCPEC
RPT1_MOUSE     CPICLE-L---LKEPVSADCNHSFCRACITLNYESNR-NTDGKGNCPVC
ME18_MOUSE     CALCGGYF---IDATTIVECLHSFCKTCIVRYLETNK-Y------CPMC
MSL2_DROME     CVVCCQLLV-DPYSPKGKRCQHNVCRLCLRGKKHLFPSCTQ----CEGC
RA18_YEAST     CHICKD-F---LKVPVLTPCGHTFCSLCIRTHLNNQP-N------CPLC
ICP0_HSV11     CAVCTDEIAPHLRCDTF-PCMHRFCIPCMKTWMQL-R-NT-----CPLC
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Figure 4 Multiple sequence alignment of ring finger domain of ZNF230 with those of other members of the same family

The conserved cysteines and histidines are emboldened. Human RFP (Ret finger protein) is a developmentally regulated protein that may function in male germ-cell development; human PML (promyelocytic leukaemia gene) is a probable transcription factor; mouse RPT-1 is a *trans*-acting factor that regulates gene expression; mouse XYbp is expressed in the XY bivalent of spermatocytes; mouse ME18, which is expressed in a variety of tumour cells, is a transcriptional repressor; *Drosophila* male-specific MSL-2 protein is a DNA-binding protein that is involved in X chromosome dosage compensation and is essential for elevating transcription of the male single X chromosome; RA18 is a DNA-repair protein from *Saccharomyces cerevisiae*; Herpes virus *trans*-acting transcriptional protein ICP0/IE110 has been characterized in many different herpes viruses and is a *trans*-activator and/or -repressor of the expression of many viral and cellular promoters.

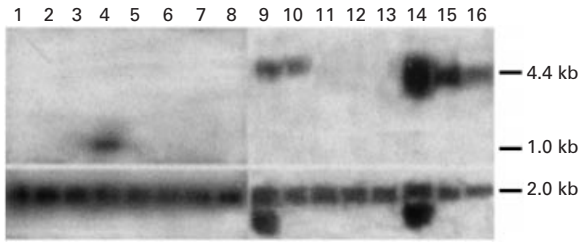

Figure 5 Expression analysis of ZNF230 in multiple human tissues by Northern blot

Lanes 1–16 contained RNAs from spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leucocyte, heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. There were two transcripts detected: the 1 kb transcript of ZNF230 was expressed only in testis and the other 4.4 kb isoform was detected in heart, brain, skeletal muscle, kidney and pancreas. The 2.0 kb band detected in all lanes was the human β-actin control.

the isolation of full-length cDNAs of novel human spermatogenesis genes by mRNA-DD was successful.

Testis-specific expression of the ZNF230 gene

In order to understand the expression profiles of the ZNF230 gene in various tissues, we examined further the expression of ZNF230 in 16 human tissues by Northern blot. As shown in Figure 5, an expected 1 kb transcript of the ZNF230 gene was expressed only in testis whereas another 4.4 kb transcript was detected in heart, brain, skeletal muscle, kidney and pancreas. The relative abundance, from the highest to the lowest, was skeletal muscle, kidney, pancreas, heart and then brain. The control 2 kb β-actin mRNA was present in all tissues.

Since Northern-blot analysis is sometimes not sufficiently sensitive to detect expression, multiple-tissue RT-PCR was also performed. The expected 282 bp PCR product of the ZNF230 gene was expressed exclusively in testis whereas the internal control, human β-actin, was expressed equally in all the tissues tested (Figure 6, top panel). The RNA samples used for this experiment had been treated with RNase-free DNase I and the primer pairs for the reaction were located in different exons, so

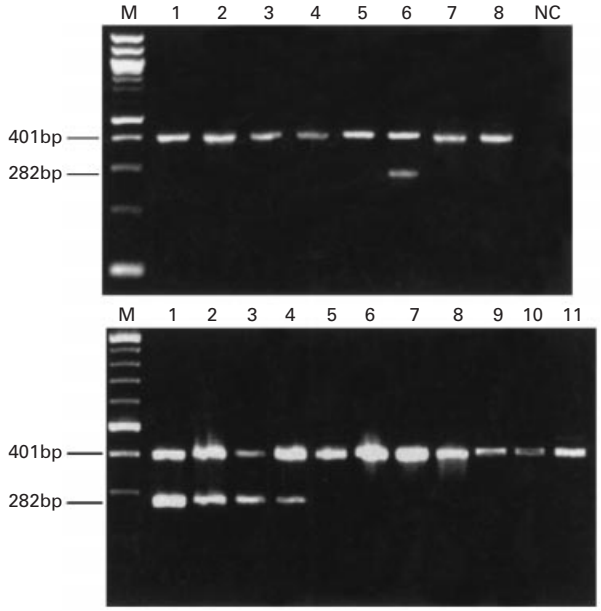

Figure 6 Expression analysis of ZNF230 by RT-PCR

Top panel: lanes 1–8 contained RNAs from lung, liver, spleen, kidney, brain, testis, skeletal muscle and stomach. NC was a negative control and lane M contained a 100 bp ladder. The expected 282 bp product was only detected in testis. Bottom panel: RNAs from normal adults (lanes 1–4), fetuses (lanes 5–7) and patients with azoospermia (lanes 8–11) were used in RT-PCR. The 282 bp products were detected in normal adults but not in fetuses or patients. In both panels human β-actin (401 bp) was co-amplified as an internal control.

that false positive amplification from minute DNA contaminations was not detected.

When the cDNA of the ZNF230 gene was used to search against the UniGene database, the UniGene cluster Hs.44685 was obtained. This cluster included 37 ESTs that came from testis, skeletal muscle, colon and kidney as well as some tissues not used in our Northern blot, such as breast, eye, foreskin, stomach, thyroid, uterus, adrenal gland and bone. This result was consistent with our Northern-blot and RT-PCR results except that

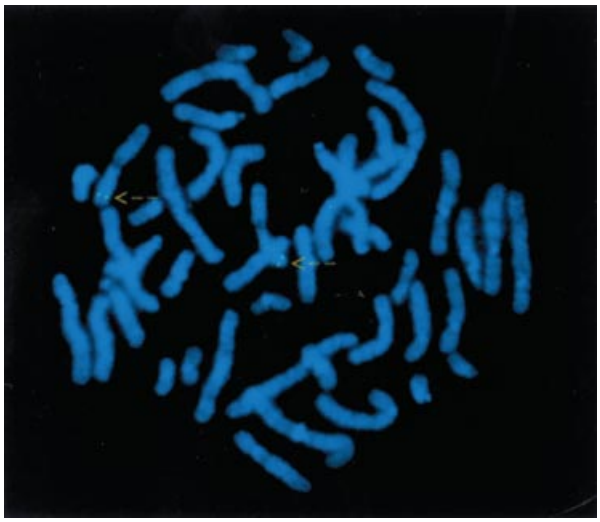

Figure 7 Chromosome localization of the ZNF230 gene by FISH

Arrows indicate that ZNF230 was mapped to chromosome 11p15.

neither the 1 kb nor the 4.4 kb transcript was detected in colon. The fact that RT-PCR did not detect any expression of the 4.4 kb transcript of the ZNF230 gene in heart, brain, skeletal muscle, kidney and pancreas suggests that in the amplification region the mRNA sequences of both transcripts were different. Based on these results, we believe that the 1 kb transcript of the ZNF230 gene is the testis-specific isoform. Interestingly, the 4.4 kb transcript is very abundant in skeletal muscle.

RT-PCR analysis of ZNF230 expression in fetus and azoospermic patients

To examine the expression of ZNF230 in the testis during development and to control its possible modification or absence in the testicular tissue of the azoospermic patient used in our mRNA-DD experiment, RT-PCR experiments were performed from testicular RNA samples of four fertile adults, three fetuses and four azoospermic patients. For ZNF230, the expected 282 bp RT-PCR products were found to be present in all four fertile adults, but not in the fetal RNA samples or in the azoospermic patients (Figure 6, bottom panel). Thus it can be inferred that expression of the 1 kb ZNF230 gene transcript is related to testis development and idiopathic azoospermia.

ZNF230 is localized in chromosome region 11p15

By FISH analysis we mapped ZNF230 to chromosome band 11p15 on DAPI-banded chromosomes (Figure 7). This result was confirmed further by the genomic PCR mapping experiment with a hamster–human hybrid cell DNA panel containing overlapping sets of human chromosomes (results not shown). Our results are also in accordance with mapping *in silico* performed with the UniGene database. Generally, zinc finger genes are aggregated on chromosomes in clusters. When GenBank was searched, several other ring finger-containing genes, such as RNF21, RNF22, SSA1 and TRAF6, were found in this same chromosome region. Comparing the ZNF230 gene with these four genes failed to identify homologous nucleic acid or amino acid sequences, further confirming that ZNF230 is unique.

Table 1 Exon–intron structure and splicing sites of ZNF230

The splice acceptor/donor columns show sequences that span the splicing junctions. Exonic sequences are shown in upper-case letters whereas intronic sequences are in lower-case. Invariant nucleotides (gt/ag) are in bold type.

Exon no.	Exon size (bp)	Splice acceptor	Splice donor
1	≈ 66		AACCTGGG**gt**gagctc
2	190	tttgac**ag**AAACTATG …	… AATGATGT**gt**aaggct
3	109	cttttc**ag**AACGGCTA …	… GTACCAAG**gt**gagact
4	182	attttt**ag**ATTAACAA …	… ATGGGAAG**gt**atataa
5	108	cattgc**ag**GGTGAAGC …	… GATAAATG**gt**aagtta
6	324	tctaat**ag**GAGTGATC …	

Genomic structure of the ZNF230 gene

We searched the human genome databases with the cDNA sequence of ZNF230 to identify the homologous genomic sequence. A BAC clone, RP11-68C8, from chromosome 11 (GenBank accession number AC021914) was found to have six regions that are highly homologous to ZNF230. According to the results of alignment 2 sequences, the intron/exon boundaries in the mature RNA were defined and all had standard splice-site sequences (Table 1). The significance of the differentially spliced 4.4 kb transcript in other tissues remains to be determined.

In summary, we have cloned a novel ring finger gene, ZNF230, and mapped it to chromosome band 11p15. Primary structure and expression analyses found two isoforms with different expression patterns. The 1 kb transcript is specifically expressed in normal adult testis but not in fetal testis or patients with azoospermia, suggesting that ZNF230 may function as a germ-cell-specific transcription factor in spermatogenesis.

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