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Identification, Gene Structure, and Expression of Human Frizzled-3 (*FZD3*)

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We report the identification, genomic structure, chromosomal localization, and expression analysis of human frizzled-3 (*FZD3*), a 7-transmembrane receptor belonging to the frizzled family. The cDNA obtained from adult human brain shows 91% identity at the nucleotide level and 98% at the amino acid level to mouse frizzled-3 (*fzd3*). The *FZD3* locus is located on chromosome 8p21, spans 48 Kb and its coding sequence is distributed in 6 exons intercalated by 5 introns. *FZD3* is expressed in all analyzed human tissues, with quantitatively higher expression in the CNS and in urogenital structures. © 2000 Academic Press

Wnt factors represent a large class of secreted glycoproteins involved in the control of cellular proliferation, differentiation, apoptosis and fate [1, 2]. They act through interaction with a family of transmembrane receptors homologous to Drosophila *frizzled*, a 7-transmembrane (7TM) protein [3]. The downstream signals originating from the interaction of a Wnt ligand with a frizzled receptor appears to depend on the identity of the *frizzled* receptor [4]. For instance, activation of certain *frizzled* receptors (e.g. rat frizzled-2 and mouse *fzd*7 and *fzd*8) leads to the stimulation of a "canonical" downstream signalling pathway which parallels that discovered in the fruitfly, with activation of proteins homologous to Drosophila *dishevelled*, inhibition of glycogen synthase kinase (GSK3) activity, accumulation of β-catenin in the nucleus and consequent activation of target gene expression [1, 2]. Instead, *fzd*3, 4 and 6 appear to act as G-protein coupled receptors and their activation by Wnt signal results in the stimulation of inositol signalling and protein kinase C (PKC) activation [4]. An increasing body of evidence implicates Wnt signalling in the modulation of neuronal development and plasticity [e.g. 5–8], in the control of behaviour [9], and in development of specific CNS structures [10]. The finding that the mood stabilizing agents lithium and valproate negatively influence GSK3β activity, and that activated *frizzled* receptors can stimulate inositol signalling (a signal transduction pathway implicated in mood control; see for example 11) raises the possibility that *frizzled* proteins may themselves be implicated in aspects of behaviour and/or mood control. To date, at least 10 distinct frizzled receptor genes have been identified in the mouse [3, 12]. The human homologues of many of these have been cloned [3, 12–16], but cDNA sequences for *FZD3* and *FZD8* are not yet available. Here we present the identification and initial characterization of the *FZD*3 locus and cDNA, and the quantitative analysis of FZD3 expression in human tissues.

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MATERIALS AND METHODS

Sequence analysis and database searches. Genomic human sequences containing the complete *FZD3* gene were retrieved by BLAST 2.0 [17]. The mouse amino acid sequence (SwissProt Accession No. Q61086) was compared to genomic sequences (GenBank-Pri and GenBank-humHTG subdivisions) using the TBLASTN program. The pairwise alignments between the mouse protein and the human genomic sequences dynamically translated in all six frames were used to deduce the intron/exon positions on the genomic sequences. The splice site consensus sequence prediction was carried out using the WWW interface of Neural Network Splice Site Prediction Tool (NNSPLICE0.9, http://www.fruitfly.org/seq_tools/splice.html).

The multiple alignment program CLUSTALW [18] was used to compare the mouse and human deduced amino acid sequences.

The electronic PCR (e-PCR, http://www.ncbi.nlm.nih.gov/STS) tool was used for the refinement of human *FZD3* gene map.

PCR amplification employing a proofreading thermostable DNA polymerase, and automated DNA sequencing. PCR amplification was carried out employing the GeneAmp XL PCR kit (PE Biosystems, Branchburg, NJ), using human fetal brain cDNA (see below) as a template. Reaction conditions (for primers, dNTP and enzyme) were according to manufacturer's protocol, with a final $Mg(OAc)_2$ concentration of 0.8 mM. Primer sequences were as follows: 5′ primer 5′-GGAAGGATGGCTATGACTTGG-3′; 3′ primer: 5′-AGTTACAGCTATCAGTCATGC-3′. Reaction details were as follows: 94°C/3′; 45× (94°C/30″; 55°C/30″; 72°C/5′); 72°C/20′. PCR products were analyzed by electrophoresis on a 1% agarose gel poured and run in 1× TAE buffer [18]. The product was purified on a QUIAEX PCR chromatography column (QUIAGEN, GmbH) and subjected to auto-

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mated DNA sequencing by standard protocols using an ABI377 machine (PE Biosystems, Branchburg, NJ).

cDNA synthesis and RT-PCR studies. Human polyadenylated RNA from various human tissues was purchased from Clontech (Palo Alto, CA). 1 μg polyadenylated RNA from each tissue was converted to cDNA employing Superscript II reverse transcriptase (Life Tech. Inc., MD) and oligodT and random hexamer oligonucleotide (250 ng each) in a final volume of 20 μl, according to manufacturer's instructions. Following first strand cDNA synthesis, the reaction volume was increased to 100 μl and 1 μl of this was used for each PCR reaction. Assuming a 50% efficiency in the reverse transcription reaction, approximately 5 ng of cDNA were employed in each RT-PCR and TaqMan (see below) reaction. Conditions were as follows: forward primer: 5′-TGAATAGGCCTGATCATCTGAAT-3′ and reverse primer: 5′-ATAGGAGCGTAGAGTGCACAAAG-3′. PCR conditions were: 94°C/3′; 45 cycles of (94°C/30″; 56°C/30″; 72°C/30″); 72°C/10′. For β-actin amplification, PCR conditions were the same except that primers were 5′-TGAACCCTAAGGCCAACCGTG-3′ and 5′-GCTCATAGCTCTTCTCCAGGG-3′.

For the analysis of expression of *FZD* genes in the same panel of human tissue and cell line cDNAs, PCR conditions in all cases were the same as above, except that primers used were as follows: *FZD1,* forward primer 5′-CTCGAGGTTTCCTCACTAGACAA-3′ and reverse primer 5′-AATGGTTAAACCGCCCTAAATAA-3′; *FZD2,* forward primer 5′-GTTCCATGTTCTTCTCACAGGAG-3′ and reverse primer 5′-CATGCTGAAGAAGTAGAGCATCA-3′; *FZD4,* forward primer 5′-CCTGGAAGCATTCAACTCAGC-3′ and reverse primer 5′-TGTGGTTGTGGTCGTTCTGTG-3′; *FZD6,* forward primer 5′-CGCTACTTTGTACTCTTGCCACT-3′ and reverse primer 5′-ACATGGGATATGGTACTGACGAC-3′; *FZD7,* forward primer 5′-GCGAGGCGCTCATGAACAAGT-3′ and reverse primer 5′-CACGGCCACCATGAAGTAGCA-3′; *FZD9,* forward primer 5′-CTGCACGCTGGTCTTCCTACT-3′ and reverse primer 5′-CCGATCTTGACCATGAGCTTC-3′; *FZD10,* forward primer 5′-CGGCCAGCTCTATGTCATCCA-3′ and reverse primer 5′-ATGAAGGACGTGCCGATGACC-3′. Sequences employed for primer design have Accession numbers AB017363 (*FZD1*), AB017364 (*FZD2*), NM 012193 (*FZD4*), NM 003468 (*FZD5*), AB012911 (*FZD6*), AB017365 (*FZD7*), HSU82169 (*FZD9*), BAA84093 (*FZD10*). In all cases no RT controls were also carried out to rule out the detection of possible genomic DNA contamination (not shown).

Real time quantitative PCR (TaqMan). Real time quantitative PCR analysis of *FZD3* and β-actin expression was carried out with the aid of an ABI7700 machine (PE Biosystems, Branchburg, NJ). A 2× stock cocktail of reagents comprising all necessary TaqMan PCR components except primers and probe was purchased from PE Biosystems and employed according to manufacturer's instructions. TaqMan *FZD3* primers were as follows: 5′ primer: 5′-GGCTGTGTCAGCGGGCT3′; 3′ primer: 5′-TCTTCAGGCCAAGGAACACC-3′. The TaqMan *FZD3* probe was 5′-ACAGTGAGTGTTCGAAGCTCATGGAGATGTT-3′, and was FAM labelled at its 5′ end. Final primers and probe concentrations were 300 nM each primer and 200 nM, respectively. Reaction parameters were 50°C/2′; 95°C/10′; 35× (95°C/15″; 53°C/1′). Three measurements per sample were carried out in each of two independent experiments. Results were analyzed with the ABI Sequence Detector software version 1.6.3 (PE Biosystems, Branchburg, NJ). Quantitation was carried out relative to a standard curve of *FZD3* cDNA. For β-actin quantitation, a β-actin detection kit was purchased (PE Biosystems, Branchburg, NJ) and employed according to manufacturer's instructions.

Cell culture, RNA isolation and Northern blotting. NBOK1 human neuroblastoma cells were cultured in RPMI medium (Gibco BRL, MD) containing 200 mM Glutamine, 10% fetal calf serum, and 200 μg/ml penicillin in a 5% CO_2 humidified atmosphere. HepG2 cells were cultured in Eagle's Minimum essential medium with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, in the presence of 10% fetal bovine serum. Polyadenylated RNA was extracted from subconfluent monolayers employing a modified oligodT-cellulose binding protocol [19], and subjected to Northern blot analysis according to standard procedures [19, 20]. Blots were exposed on Kodak XAR-5 films at −80°C with intensifying screens for 10 days.

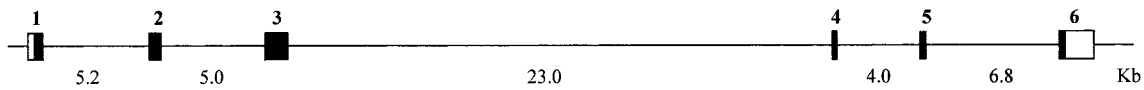
RESULTS

Although a significant body of information is available in the literature regarding frizzled-like receptor expression in several animal model systems, less is known of the pattern of expression of human frizzled receptor genes, with most studies concentrating on evaluating the tissue distribution of cloned genes by Northern blotting or RNase protection analysis [3, 12–16]. Based on an analysis carried out in the mouse [3], *fzd*3 appeared to show a relatively specific pattern of expression, with transcripts being mainly concentrated in CNS structures. We therefore sought out to identify and clone the human homologue of *fzd*3 and to quantitatively evaluate its pattern of expression in adult human tissues and in specific adult brain areas.

In silico characterisation of the human FZD3 gene. In order to identify human sequences homologous to mouse frizzled-3 (*fzd*3), GenBank (ESThum and HTGhum subdivisions) was searched using the mouse protein as a query. Along with several ESTs covering the entire coding sequence, two genomic sequences (GenBank Accession Nos. AC011132 and AC012411, respectively) appeared to comprise the entire coding exon complement of the *FZD3* locus. Based on homology with the available *fzd*3 cDNA sequence, the genomic structure of the *FZD3* locus was elucidated (Fig. 1). The human gene appears to be 48 Kb in length and its coding sequence is distributed in 6 exons intercalated by 5 introns. Exons vary from 151 bp to 1647 bp in size, whereas intron sizes range from 4 Kb to 23 Kb. The intron/exon splice junctions predicted and reported in Fig. 1 are in conformity with the AG/GT consensus rules. A pairwise alignment of the deduced amino acid sequence obtained from the reconstructed cDNA showed a 98% identity (654/666 aa) with the mouse protein (Fig. 2), whilst at the nucleotide level the two cDNAs are 91% identical (not shown).

The 3 prime untranslated region (3′-UTR) was also characterized. The mouse cDNA (GenBank Accession No. U43205) has 311 nt of 3′-UTR which shows 86% identity with genomic sequence AC011132 downstream the stop codon (TAA). In order to identify the putative polyadenylation signal and the end of the transcript, 4 Kb of genomic sequence around the stop codon were compared with GenBank-ESThum database subdivision. A cluster of 13 ESTs presenting 100% identity with the query sequence, were retrieved and aligned along 1431 bp. All ESTs ended close to a

A



B

	acceptor site	Score	donor site	Score
	intron exon		exon intron	
Exon 1			aatggag**GT**aagact	1.00
Exon 2	tcaattttaacttttccct**AG**ccattccaccctatggtgaa	0.75	gcagtag**GT**gcgaaa	0.15
Exon 3	tcatcttgttttgttttttt**AG**gttcccagattgtgatgagc	1.00	atatcag**GT**aaggga	1.00
Exon 4	cttttctttctgttcttcc**AG**gttactcaaatgagtcgtcc	1.00	aaaaaga**GT**aagttg	0.99
Exon 5	acttactattttgttgtct**AG**gatagtgaatgagagccgac	0.95	atggcag**GT**gagttt	0.98
Exon 6	caaaataaactctcatgac**AG**gtacacgccctgcagttaca	0.35		

FIG. 1. (A) Exon/intron gene reconstruction of a 48 Kb region of AC011132 genomic sequence. The coding sequence is organized in 6 exons and 5 introns. White boxes indicate the 5′ and 3′ UTRs, while filled boxes represent the CDS. (B) The intron/exon splice junctions are indicated together with their scores as calculated with the Neural Network Splice Site Prediction tool (see text).

stretch of 23 A, which in turn is located 17 nt downstream a polyadenylation signal (AATAAG, nucleotide positions 3747–3752 of the cDNA). The polyA stretch can thus be considered as a putative end of transcription.

The chromosomal localization of the *FZD3* locus was determined and refined in silico. The two genomic sequences are annotated as chromosome 8 clones, with no information on specific markers or chromosomal bands. Comparing the human *FZD3* cDNA sequence with the Sequence Tagged Sites (STS) database, the human STS WI-31127 could be detected in the cDNA, allowing its map location to be determined. The STS locus is in the interval between D8S1820 and D8S1609 markers on human chromosome 8p21, 54.2 cM from top of chomosome 8 linkage group (Genethon Integrated Map [21, 22]).

The mouse gene is located on chromosome 14 between the *Gja3* and *Nf1* genes [3]. The human/mouse homology map resource maintained at NCBI (http://www.ncbi.nlm.gov/Homology) shows a region of syntheny between mouse chromosome 14 and human 8p21. More precisely, mouse *Nfl* locus corresponds to the human NFL (Neurofilament protein) gene on 8p21 (OMIM number 162280), which in turn is proximal to D8S1820. Thus the human *FZD3* locus was mapped distal to *NFL* gene in the synthenic region of mouse chromosome 14.

Amplification and analysis of a FZD3 cDNA. In order to confirm the predicted *FZD3* coding sequence, a *FZD3* cDNA was amplified by PCR technology and subjected to DNA sequencing. Thus a set of nested PCR primers were designed around the predicted initiation (ATG) and STOP codons in the predicted *FZD3* sequence (Accession No. AJ272427). These codons were selected based on homology with the deposited mouse cDNA sequence. RT-PCR was therefore carried out on adult human brain cDNA (Clontech, CA), using a proofreading polymerase. A 2 Kb PCR product was obtained (not shown). Double pass DNA sequencing of the resulting PCR product confirmed the nucleotide sequence predicted from genomic DNA analysis and was deposited to the EMBL nucleotide sequence database under Accession No. AJ272427.

Qualitative and quantitative analysis of FZD3 expression. We next sought to analyze the pattern of expression of FZD3 in adult human tissues by RT-PCR (Fig. 3A) and by quantitative TaqMan RT-PCR analysis. In one report [3], RNase protection analysis on total RNA from a limited panel of adult mouse tissues revealed significant *fzd*3 expression in brain, with lower expression level in lung, eye and testis. We sought to examine *FZD3* expression in polyadenylated RNA from a panel of human tissues by means of RT-PCR. The results (Fig. 3A) indicate that *FZD3* is expressed at relatively higher levels in CNS areas, including regions of the limbic system which are thought to be involved in mood disorders [23]. Significant levels of expression are also observed in testis, kidney, and uterus, with other tissues displaying lower levels of expression. These data are consistent with the reported pattern of expression of the mouse homologue *fzd*3 [3] and the report on the developmental pattern of expression of the Xenopus homologue Xfzd3 [24], whose expression is detected predominantly in the CNS and in urogenital structures. In order to quantify the expression of *FZD3* in different human tissues, we employed real-time quantitative PCR [25] carried out with the aid of a Perkin Elmer ABI7700 machine. The results (Fig. 3B), indicate that *FZD3* is expressed in all

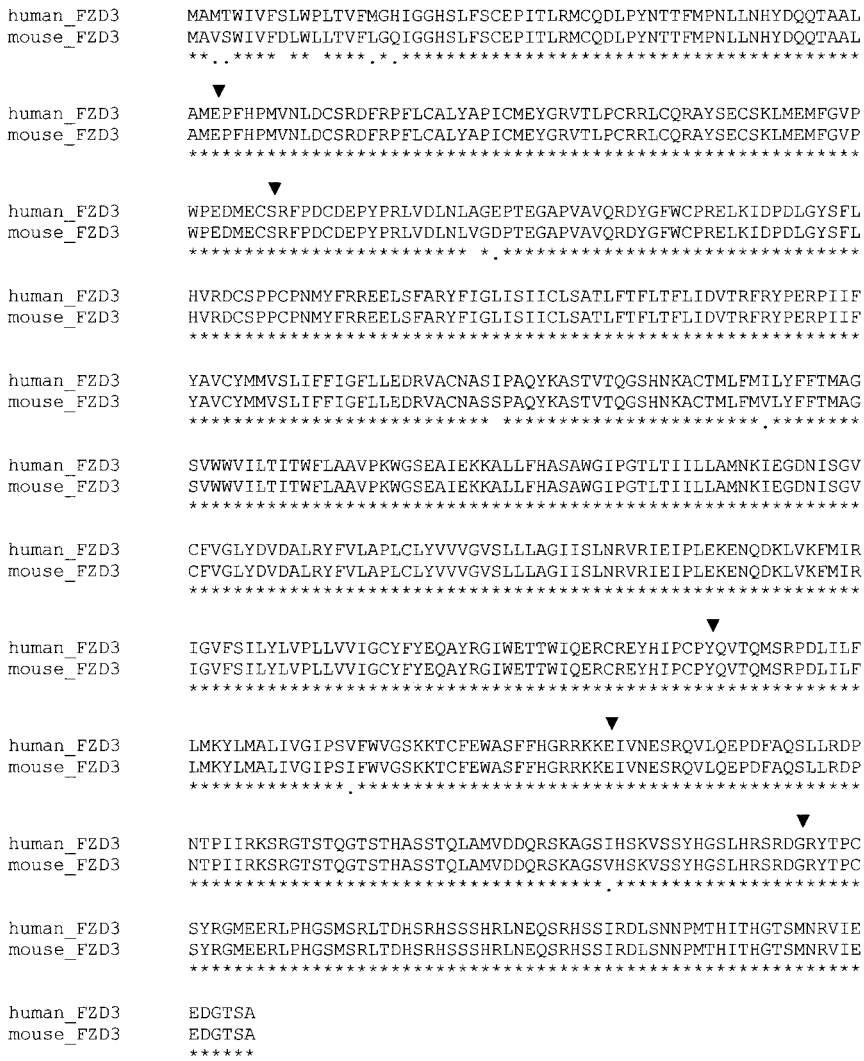


FIG. 2. Amino acid sequence comparison of the *fzd*3 protein to *FZD3* protein. Amino acid identities between the two proteins are indicated by asterisks, conserved amino acid substitutions are marked by dots. Arrowheads indicate the intron/exon boundaries determined for the *FZD3* cDNA.

tissues examined, but quantitative differences are consistent with relatively higher *FZD3* expression in brain areas, and in selected peripheral organs such as kidney and testis (in agreement with the RT-PCR data), pancreas and skeletal muscle. Discrepancies between the RT-PCR data and the quantitative TaqMan analysis (e.g. expression levels in the uterus) are most likely due to the non-quantitative nature of the standard RT-PCR technology. The low levels of *FZD*3 target cDNA sequence detected by the quantitative analysis (approx. 0.001–0.002% of polyadenylated RNA-derived cDNA, see Materials and Methods) suggests that *FZD*3 is expressed as a very low abundance transcript [19] and are consistent with the long exposure times required to obtained a signal on Northern blots of polyadenylated RNA (see Materials and Methods). For comparison, β-actin levels were quantitated in the panel of human tissue cDNAs at around 0.5–1% of the polyadenylated RNA-derived cDNA input (not shown), consistent with its classification as a medium-high abundance transcript [19].

In order to compare the pattern of expression of *FZD3* with that displayed by other known human frizzled genes, we investigated the pattern of expression of *FZD1, 2, 4, 5, 6, 7, 9,* and *10* in the available human tissue cDNA panel. A series of RT-PCR studies was carried out employing primers specific for each human frizzled gene (with the exception of the human homologue of *fzd8,* for which sequences are not yet available). The results are presented in Fig. 4. Though obtained through a non-quantitative methodology, our results suggest that while some frizzled receptor genes are expressed rather ubiquitously in man (e.g. *FZD1, 2* and *6*), the expression of *FZD4, 7, 9* and *10* appears to be restricted to specific tissues. Of these, only *FZD10* appears to show some specificity for CNS regions. We

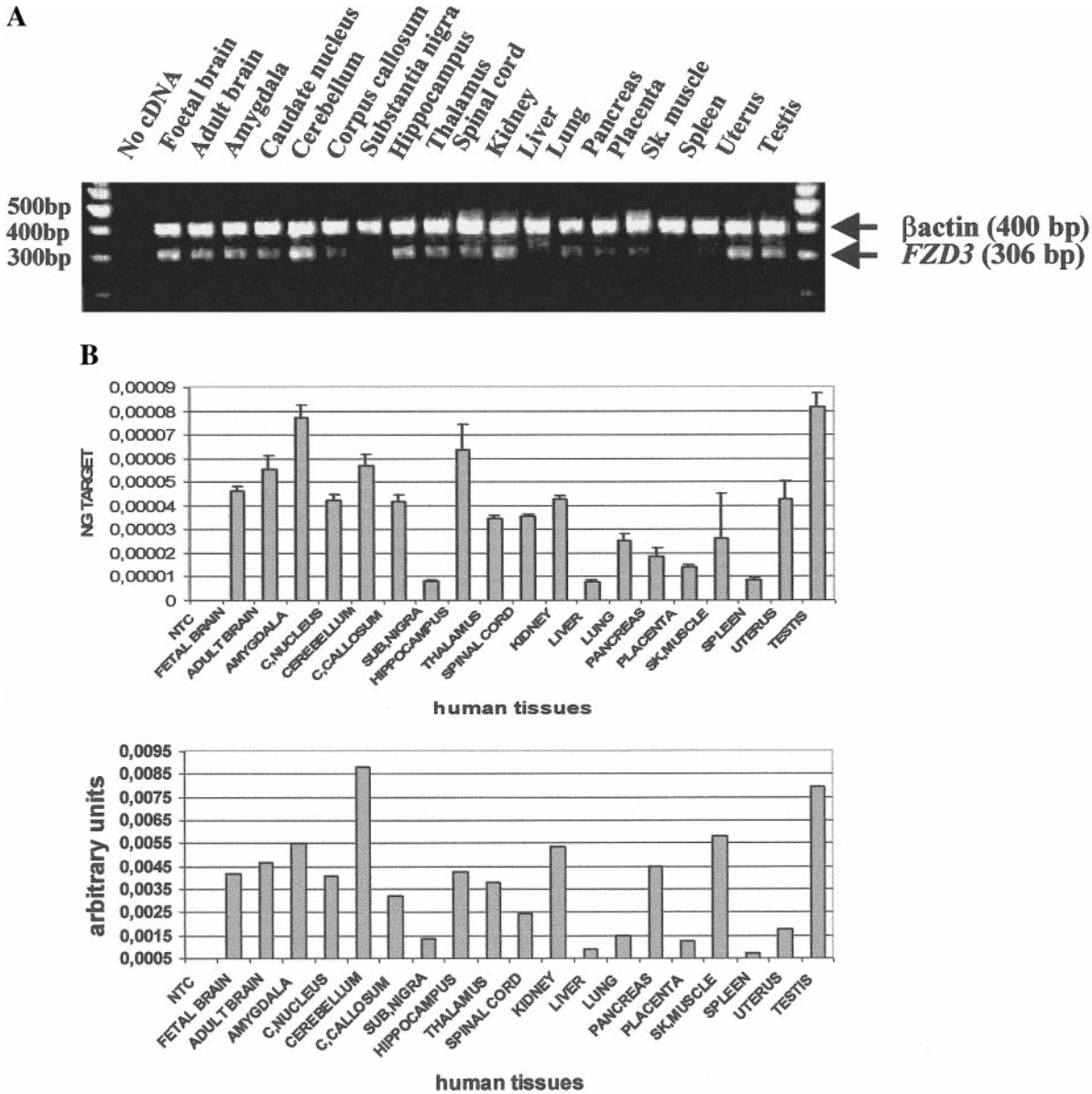


FIG. 3. (A) RT-PCR analysis of *FZD3* expression in a panel of human tissue cDNAs. Analysis of β-actin expression in the same samples was also carried out as a control for cDNA quality. Samples were loaded onto a 2% agarose gel alongside a 100 bp marker (Fermentas AB, Lithuania). No RT controls were also carried out to rule out the detection of possible genomic DNA contamination (not shown). (B) Real-time quantitative PCR (TaqMan) analysis of *FZD*3 expression in different human tissues. The upper panel represents the absolute quantitation of *FZD*3 expression in the various samples. Results shown are the averages and standard errors of three measurements per tissue. The lower panel illustrates the expression of *FZD*3 normalized with respect to β-actin levels in the same samples. Please refer to the Materials and Methods section for primer sequences.

were unable to detect expression of *FZD5* sequences under the conditions tested. Our data are generally consistent with the relatively wide pattern of expression analyzed by Northern blotting and reported for *FZD1*,2 and 6 [13, 15], and the more specific patterns of expression reported for *FZD4*, *7*, *9* and *10* [14, 13, 12, 16].

RT-PCR analysis revealed that *FZD3* is expressed in the human neuroblastoma cell line NBOK1 (data not shown). In order to determine the size of transcripts generated from the *FZD3* locus, we investigated the expression of *FZD3* in NBOK1 cells by Northern blotting of polyadenylated RNA. Lithium, which is often used as a therapeutic agent for affective disorders and schizophrenia [26], was tested for a potential effect on *FZD*3 expression in NBOK1 cells. The results (Fig. 5) indicate that NBOK1 cells express at least two *FZD3* transcripts, the predominant of which is about 3.3 Kb in length. When 10 mM LiCl was applied for 24 h, no significant effect could be observed on the expression of *FZD3* (Fig. 5), a result which was subsequently confirmed by quantitative RT-PCR employing TaqMan technology (data not shown).

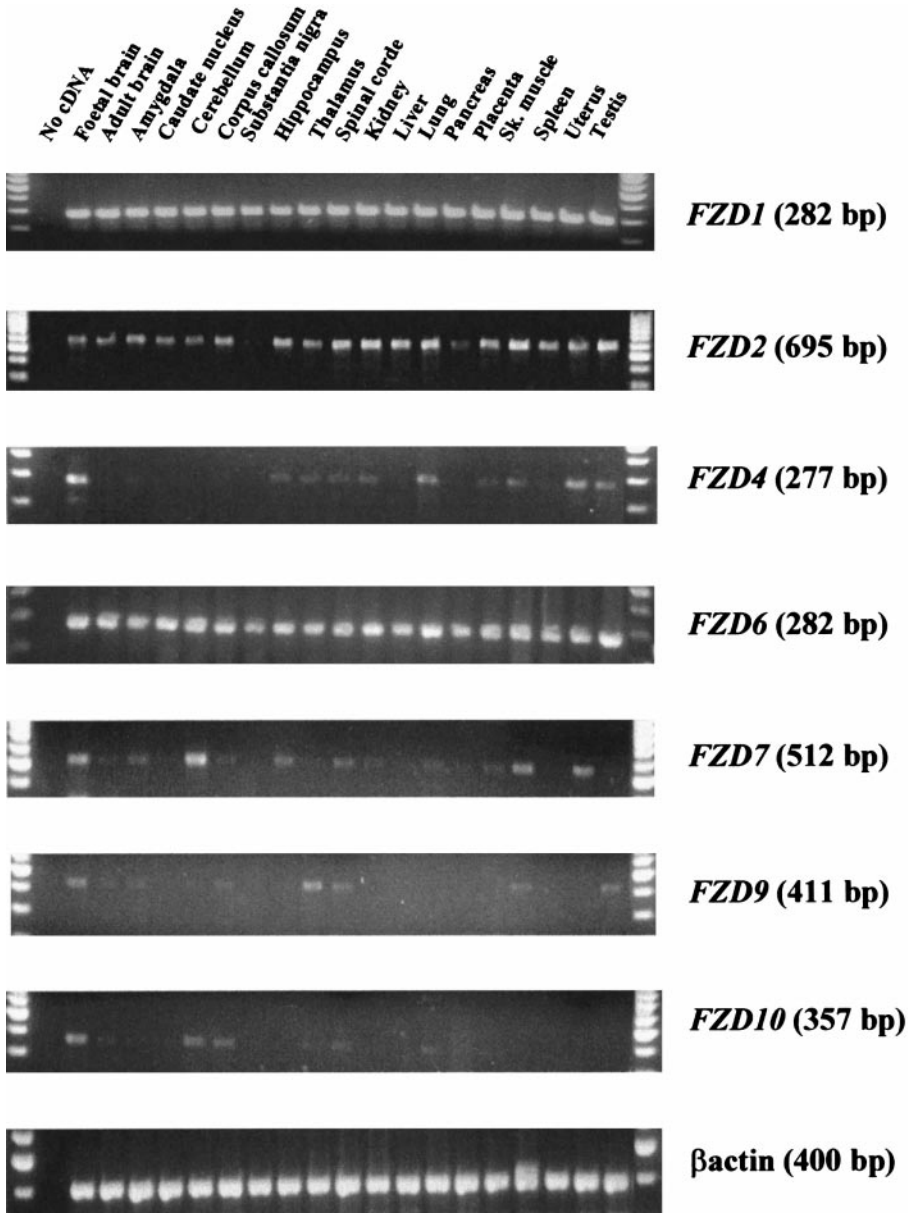


FIG. 4. Analysis of expression of *FZD* genes in the panel of human tissue cDNAs. PCR conditions in all cases were the same as in Fig. 3A. Please refer to the Materials and Methods section for primer sequences.

DISCUSSION

In this paper we report the gene structure analysis and expression distribution of the human *FZD3* gene. The identification of the complete gene was done in silico first, and then its tissue distribution was determined by RT-PCR and TaqMan analysis and compared to that of other known *FZD* genes. We find the gene is located on two genomic BAC clones from chromosome 18. Its physical mapping was refined in 8p21 between D8S1820 and D8S1609 markers by our in silico analysis. Our data are in broad agreement with another report on the cloning and characterization of *FZD*3 sequences (cDNA sequence, genomic structure, chromosomal location) which appeared following submission of our manuscript [27], but important differences regarding transcript sizes and expression patterns are apparent.

Based on comparison between mouse cDNA and human genomic sequences we could determine that the human *FZD3* coding sequence consists of 6 exons. The two additional exons in the 5 prime UTR, described by Kirikoshi *et al.* [27], are present on the genomic sequence AC011132 and positioned 2.7 Kb and 8.3 Kb upstream the first translated exon, respectively. Based on our Northern blot data, the major transcript sizes in

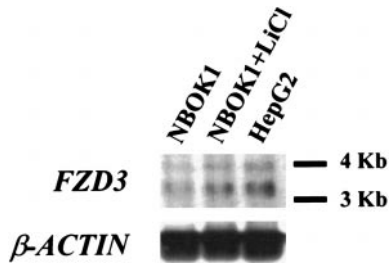


FIG. 5. Northern blot of polyadenylated RNA (3 μg/lane) from human cell lines probed for *FZD3* expression. RNA ladder size markers are indicated. A β-actin probing is provided as a loading control.

the human neuroblastoma cell line NBOK1 are about 3.3 Kb and ca. 4 kb (see results), in broad agreement with the *FZD*3 cDNA sequences of 3375 bp reported in [27] and in the present manuscript (3791 bp). We do not observe a truncated 1.8 kb transcript as described in [27], though this might be due to sample differences in *FZD*3 transcript expression (cell lines vs human tissues). Despite finding several public ESTs corresponding to *FZD*3 sequences, we found no evidence of EST sequences with an in-frame stop codon that could correspond to the truncated 1.8 kb transcript, which we might expect to find given the fact that this transcript appears to be the predominant one in the Northern blots reported in [27]. In [27], a *FZD*3 cDNA sequence of 3375 bp is reported despite the absence of an obvious band corresponding to this size in the Northern blots. This might be due to low levels of expression of the 3.3 kb transcript (which we observe in NBOK1 cells) in the panel of Northern blots analyzed in [27]. Quantitative analysis of *FZD*3 expression (this report) using a probe which would recognize all three transcripts (1.8 kb, 3.3 kb and 4 kb) indicates relatively higher expression in adult human brain areas (especially the cerebellum and the amygdala) with significant expression in a subset of other tissues including urogenital structures (kidney and testis), pancreas and skeletal muscle. Our quantitation of *FZD*3 expression in human adult tissues is in contrast with the pattern of *FZD*3 expression reported by [27], where *FZD*3 expression appears to be specifically confined to the pancreas, with much lower expression detectable in kidney and skeletal muscle. Our findings indicate quantitatively comparable *FZD*3 expression levels in the adult brain, kidney, pancreas, skeletal muscle and testis.

The effectiveness of lithium as a therapeutic agent for diseases such as schizophrenia and bipolar disorders is well established. Recent evidence on the molecular mechanisms of action of lithium in cell and developmental biology model systems indicates that members of the Wnt signalling pathway could represent important mediators of lithium effects [26]. At least one report has mapped components of the Wnt signalling pathway within chromosomal regions thought to harbour genes predisposing to mood and behavioural disorders [28]. Among the various components of the pathway, frizzled receptors represent particularly attractive candidates from the pharmacological viewpoint, although an involvement of *FZD* genes in the etiopathology of psychiatric diseases is presently speculative. Of the known *FZD* genes, *FZD3* is perhaps the most interesting for a number of reasons. The comparative RT-PCR data reported here suggest that its pattern of expression is relatively more specific for CNS areas than other *FZD* genes, in agreement with previous reports on the expression of mouse frizzled genes [3]. Furthermore it is expressed in areas thought to be involved in the pathology of schizophrenia and mood disorders, and its location on 8p12-21 is suggestive in the light of the findings of a probable locus predisposing to schizophrenia on 8p21 [30]. The mouse homologue of *FZD3* is among the frizzled proteins capable of signalling via a G protein-coupled pathway, activating PKC and thus influencing inositol levels in the cell. The finding that abnormal Wnt signalling has been reported in schizophrenic patients [31–32], that Wnt proteins can exert profound effects on the growth and development of neurons [5–8] and that ablation of some Wnt signalling pathway components in knockout mouse models results in phenotypes with a clear psychiatric and behavioural component [9] underscores the potential importance of this pathway for neurological disorders.

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