Structure and expression of a novel *frizzled* gene isolated from the developing mouse gut

Talat H. MALIK*† and Ramesh A. SHIVDASANI*†[1]

*Departments of Adult Oncology and Cancer Biology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, U.S.A., and †Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, U.S.A.

The Wnt/APC (adenomatous polyposis coli)/β-catenin pathway plays a central role in the pathogenesis of colorectal cancer and probably also in normal development of the gastrointestinal tract. Frizzled proteins function as cell-surface receptors for the Wnt family of extracellular ligands. Many components of the Wnt signalling pathway are expressed widely, and determinants of tissue-specific functions are poorly understood. A better understanding of how Wnt signalling regulates tissue-specific development and gut epithelial homoeostasis requires characterization of the many components of this signalling pathway. We therefore wished to identify *frizzled* genes with limited tissue distribution of expression and isolated *Mfz10*, a novel member of the mouse family of *frizzled* genes, from the developing fetal gut. Highest levels of Mfz10 mRNA are detected throughout late embryonic development, in the brain, heart, lung and digestive tract. In adult mice Mfz10 mRNA is detected at highest levels in the heart, brain and lung. Expression in the adult gastrointestinal tract is much weaker, with higher levels in foregut derivatives (oesophagus and stomach) compared with regions derived from the fetal midgut and hindgut; particularly strong mRNA expression is observed in the squamous epithelium of the oesophagus. The amino acid sequence of Mfz10 is nearly identical to that of human FzE2 (also known as FzD2). Interestingly, mRNA levels of human FzD2 are reported to be up-regulated in oesophageal squamous cell carcinomas. These findings suggest a likely role for Mfz10 in the developing and adult foregut.

Key words: FzD2, Mfz10, oesophageal epithelium, Wnt receptor.

INTRODUCTION

The Wnt genes encode a large family of secreted proteins that play key roles in developmental processes and in oncogenesis. Wnt signalling regulates cell proliferation and differentiation and embryonic development in species as divergent as nematodes, flies, frogs and humans, and molecular mechanisms of Wnt signalling have been elucidated in considerable detail [1–3]. Wnt-family ligands bind to receptor molecules of the Frizzled (Fz) family [4] to initiate a signal-transduction cascade that sequentially involves the cytosolic protein Dishevelled and the serine/threonine kinase glycogen synthase kinase (GSK)-3β [5–7] and leads to stabilization of cytosolic β-catenin. This protein then translocates to the cell nucleus as part of a complex with high-mobility-group (HMG)-box transcription factors of the Tcf (T-cell factor)/LEF (lymphoid enhancer factor) sub-family and provides a transactivation domain to regulate gene expression. Stabilization of β-catenin thus activates expression of Wnt-responsive genes [8,9]. Modulators of this biochemical pathway include Axin/Fused, the GSK-3-binding protein GBP, and the product of the adenomatous polyposis coli (*APC*) gene.

At least 16 distinct Wnt genes have been identified to date in vertebrates, and are presumed to signal through interactions with receptors of the *frizzled* gene family. The Frizzled proteins constitute a large family of serpentine receptors with at least eight members in mammals and 11 in zebra fish [10,11]. All Frizzled proteins share the following structural features: a signal sequence at the N-terminus, a conserved region of 120 amino acids in the extracellular domain containing a motif of 10 invariantly spaced cysteine residues (the cysteine-rich domain or CRD), a seven-pass transmembrane region and a cytoplasmic tail [10,12,13]. With few exceptions, most mammalian Frizzled family members are expressed in a variety of tissues, with overlapping patterns. Although it is likely that each member fulfils some specific functions, the extent of the repertoire of the Wnt and Frizzled families in vertebrates raises important questions pertaining to specificity and function. The degree of promiscuity or specificity in binding between Wnt and Frizzled proteins remains unclear, as does the extent of cross-talk among signals originating from individual Wnt–Frizzled interactions in the same cell or tissue. Fully characterizing the repertoire of these families is necessary to achieve a complete understanding of the role of the Wnt pathway in development, cell differentiation and cancer.

At least two independent lines of evidence point to an important function for components of the Wnt signalling pathway in aspects of gut epithelial cell homoeostasis. First, inactivating mutations in the *APC* gene are virtually a prerequisite for development of human colorectal carcinoma [14] and possibly other gastrointestinal malignancies [15–19]. Second, targeted disruption of the mouse gene encoding the major Tcf-family protein expressed in the gut, Tcf-4, results in isolated failure of normal gut epithelial stem-cell differentiation [20]. Although the Wnt signalling pathway thus provides a strong link between development and oncogenesis in the gut, the roles of individual components of the pathway and their functional requirements in gut epithelial homoeostasis remain to be clarified. Here we report isolation of a *frizzled* gene from the developing mouse gut and examine its distribution of tissue expression with particular attention to regions of the fetal and adult gastrointestinal tract.

Abbreviations used: E, embryonic day; Tcf, T-cell factor.

[1] To whom correspondence should be addressed, at the Dana-Farber Cancer Institute (e-mail ramesh-shivdasani@dfci.harvard.edu).

The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Database under the accession numbers AF206321 and AF206322 for *Mfz10* and *Mfz10a*, respectively.

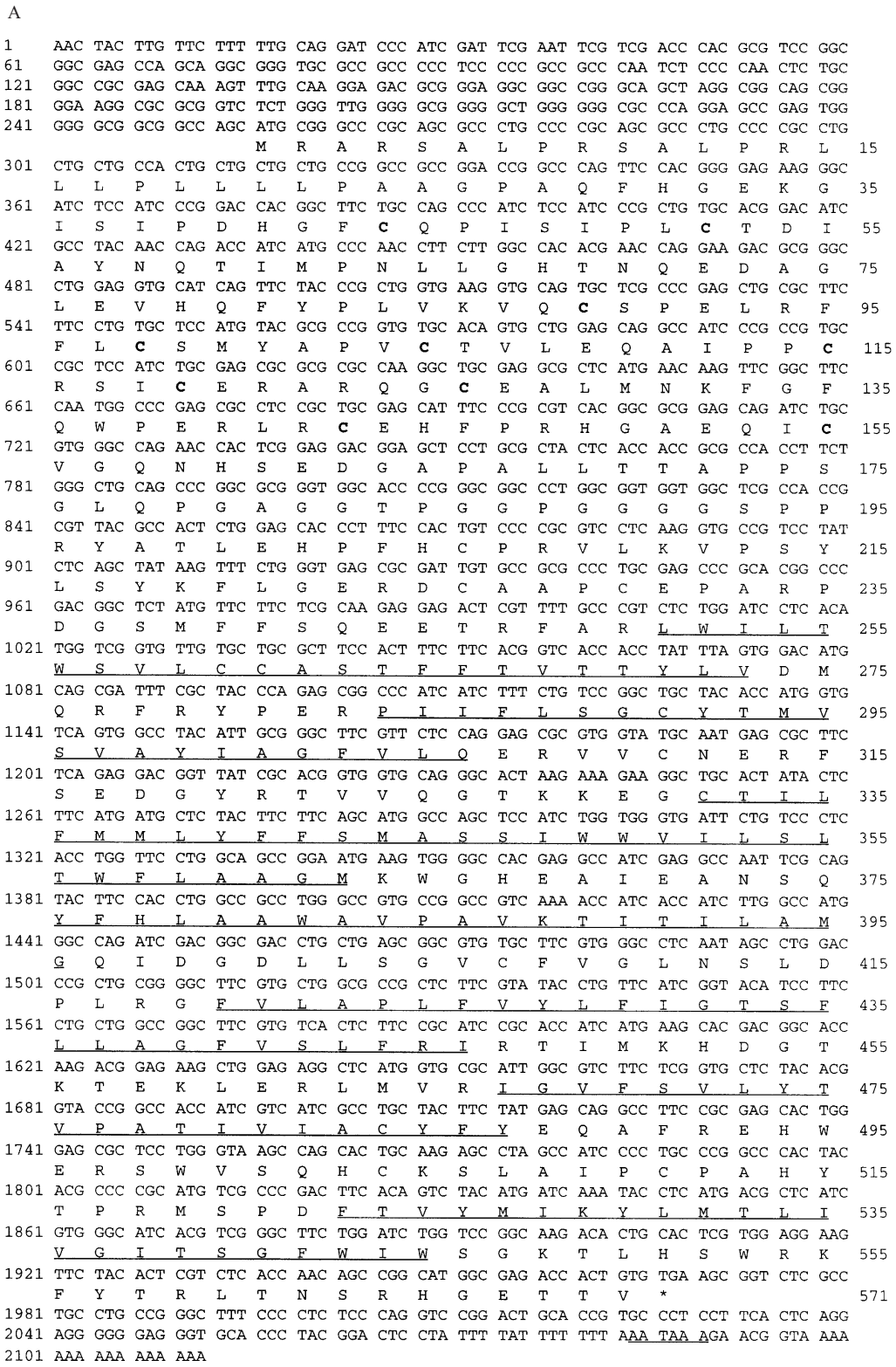

Figure 1 For legend see facing page.

EXPERIMENTAL

Cloning of mouse *frizzled* genes

Total RNA from the fore- and midguts of mouse fetuses from embryonic day (E) 14.5 was reverse-transcribed and amplified by PCR using degenerate oligonucleotide primers corresponding to conserved *frizzled* sequences: 5′-TAYCCNGARCGNCCNAT-YATYTT-3′ (which encodes YPERPIIF) and 5′-CCANGTNA-GNGTNAGDATNACCCACCA-3′ (encodes WWVILSLTW). The PCR conditions were 94 °C for 1 min, 48 °C for 1 min and

B

```
    YPERPIIFLSGCYTMVSVAYIAGFLL..ERVVCNERFSEDGYRTVAQGTKKEG.......  Consensus

280 YPERPIIFLSGCYTMVSVAYIAGFVL.QERVVCNERFSEDGYRTVVQGTKKEG.......  Mfz10
345 -------S------A-A-------L-.ED------KFAED-A---A---N---.......  Mfz1
233 --------YAV--M---LIFFI--L-E.D--A--...........ASSPAQYKASTVTQG  Mfz3
250 ----------M--NIY-I---VRLTVGR--IS-.............................  Mfz4
229 -------YY-V--SI--LM-FV--L-G.NSTA--...........KADEKLELGDTVVLG  Mfz6
282 ----------G--F--A--HV---L-.ED-AV-V----D------A-------.......  Mfz7
305 ----------A--LF---G-LVRL-AGH-K-A-SGGAPGA-G-GGAGGAAAAGAGAAGRG  Mfz8
275 --------------------------.----------------------------.......  Hfz2

    ............................CTILFMMLYFFGMASSIWWVILSLTWFLAAGM Consensus

332 ............................CTILFMMLYFFSMASSIWWVILSLTWFLAAGM MFz10
397 ............................-------------------------------- Mfz1
281 S.......................HNKA--M---V----T--G-V-----TI------VP Mfz3
283 ........DFEE..AAEPVLIQEGLKNTG-A-I-LLMYFFGMASSIWWVILTLTWFLAAGL Mfz4
277 S.......................KNKA-SVV--F----T--GTV-----TI-------R Mfz6
334 ..............................------V----G-------------------- Mfz7
365 ASSPGARGEYEELGAVEQHVRYETTGPAL--VV-LLV---G-------------------- Mfz8
327 ............................-------------------------------- Hfz2
```

Figure 1 Sequence of Mfz10

(**A**) Nucleotide and deduced amino-acid sequence of Mfz10. Nucleotides and amino acids are numbered on the left and right, respectively. The transmembrane domains (underlined), conserved cysteine residues in the N-terminal extracellular region (bold; amino acid residues 44–155), termination codon (asterisk) and polyadenylation signal (underlined) are indicated. (**B**) Comparison of the second and third transmembrane domains of Mfz10 with other members of the Frizzled family. Dashes represent amino acid identity, and dots designate spaces introduced to optimize alignment. The consensus sequence indicates residues present in at least three of the nine proteins compared.

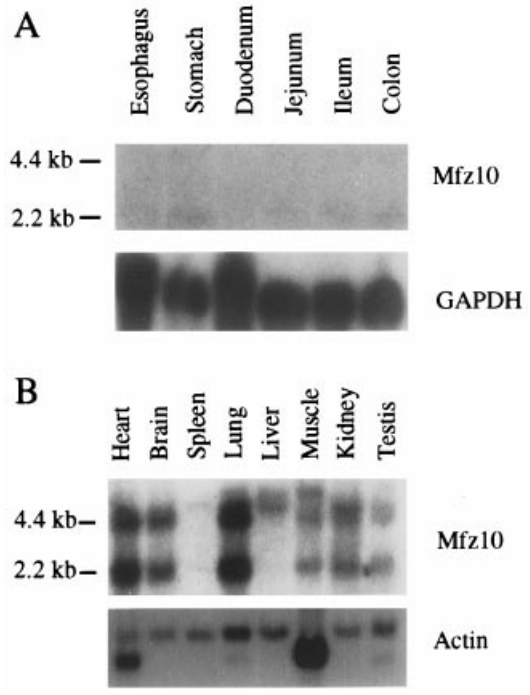

Figure 2 Northern analysis of Mfz10 on poly(A)$^+$ RNA isolated from (A) regions of the adult mouse gut and (B) various adult mouse tissues

To confirm equivalent RNA loading, the same blots were hybridized separately to control probes, β-actin or GAPDH. Transcript sizes are indicated on the left.

72 °C for 1 min for five cycles, followed by raising the annealing temperature to 55 °C for 30 cycles. An amplified fragment of $\approx$ 235 bp was cloned into the TA vector (Invitrogen). Out of 19 clones that were sequenced, 11 encoded the same novel mouse Frizzled protein. To obtain full-length cDNA clones, the PCR fragment was radiolabelled with [α-^{32}P]dCTP by the random hexamer method (Amersham, Piscataway, NJ, U.S.A.) and used to probe high-density membrane filters of a mouse embryonic gut cDNA plasmid library [21] at 55 °C in 2.5 M NaCl, 0.5 M Na_2HPO_4, 0.026 M EDTA, pH 7.0, and 1 % sarcosyl. Membranes were washed in 1 mM Tris/HCl, pH 8.0, and 1 % sarcosyl for 15 min followed by four washes for 15 min in 1 mM Tris/HCl, pH 8.0. The nucleotide sequences of *Mfz10* and *Mfz10a* have been deposited in the GenBank Nucleotide Sequence Database, with accession numbers AF206321 and AF206322, respectively.

Northern-blot hybridization

Total RNA was isolated from mouse embryonic tissues using Trizol reagent (Gibco-BRL, Rockville, MD, U.S.A.) and poly(A)$^+$ RNA from various regions of the adult mouse gut using Oligotex columns (Qiagen, Valencia, CA, U.S.A.), resolved on a 1 % formaldehyde/agarose gel, and transferred to Duralon-UV membranes (Stratagene, La Jolla, CA, U.S.A.). Alternatively, a commercial multiple-tissue Northern blot (Clontech, Palo Alto, CA, U.S.A.) was used to survey adult mouse tissues. A 1.2-kb cDNA probe from the 3′ end of *Mfz10* was radiolabelled with [α-^{32}P]dCTP using the random hexamer method (Amersham). Hybridization was carried out overnight at 68 °C in Church buffer (1 M sodium phosphate buffer, pH 7.0/0.25 M EDTA/7 % SDS/1 % BSA). Membranes were washed in a final solution of 0.1 × SSC/0.1 % SDS (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) and exposed for autoradiography.

In situ hybridization

Paraffin sections (5 μm) of mouse tissues were deparaffinized, fixed in 4 % paraformaldehyde, and treated with proteinase K (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.).

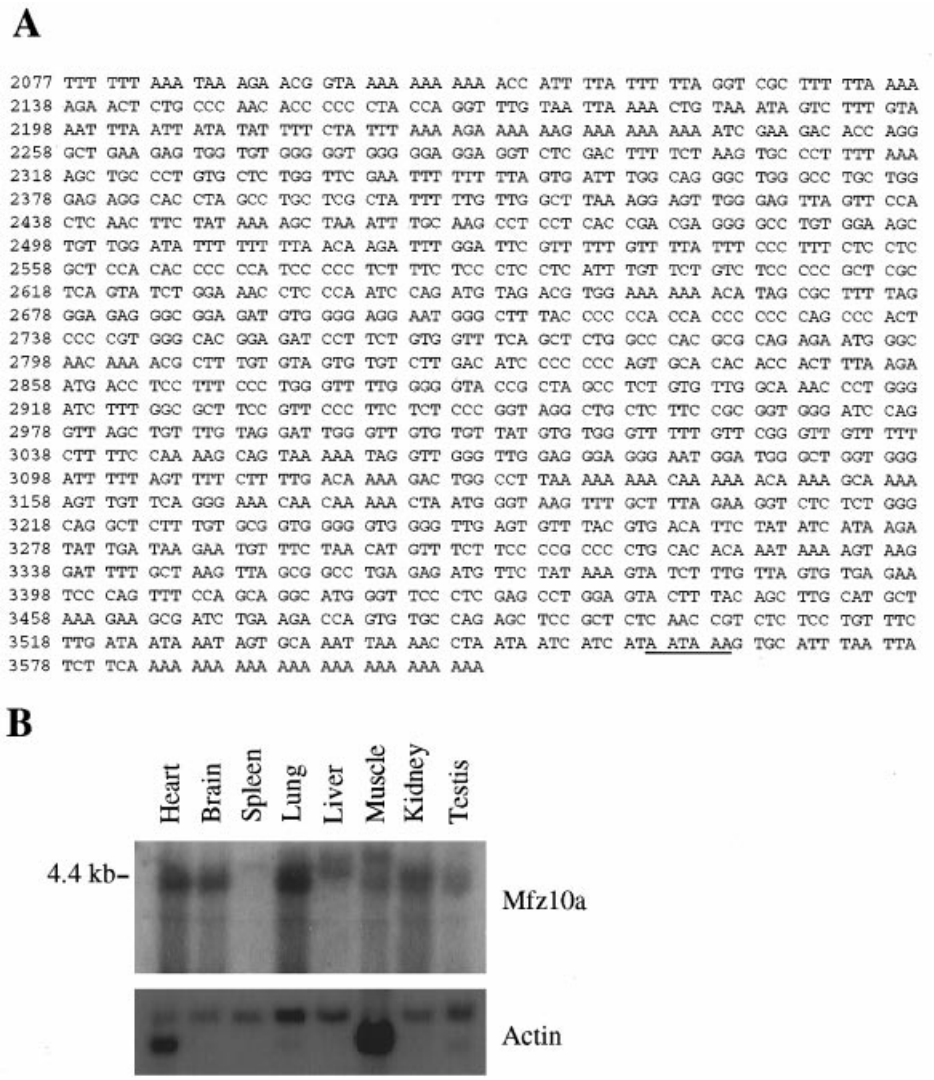

Figure 3 Splice variant of Mfz10

(**A**) Nucleotide sequence of the 3′-untranslated region of *Mfz10a*. Nucleotides are numbered according to the scheme established in Figure 1(A). (**B**) Northern analysis of Mfz10a on poly(A)$^+$ RNA isolated from adult mouse tissues, with transcript size indicated on the left.

After washing in 0.5 × SSC, the sections were covered with hybridization solution, prehybridized for 1–3 h at 55 °C, and hybridized overnight with sense or antisense ^{33}P-labelled riboprobe transcribed from the mouse cDNA. After hybridization, sections were washed at high stringency, dehydrated, dipped in photographic emulsion NTB_3 (nuclear track emulsion 3; Eastman Kodak Co., Rochester, NY, U.S.A.), stored at 4 °C for 14 days, developed, and counterstained with haematoxylin and eosin.

RESULTS AND DISCUSSION

Identification of a Frizzled family member expressed in the developing mouse gut

To characterize determinants of Wnt signalling in the developing gut, we used a degenerate PCR strategy to identify members of the *frizzled* gene family expressed in the mouse intestine at E14. All 19 PCR products sequenced encoded Frizzled proteins, attesting to the specificity of the PCR strategy; eleven of these encoded a fragment corresponding to a novel family member. We used this novel PCR product to probe 10^5 transformants from an embryonic gut cDNA plasmid library [21] and recovered five clones that permitted assembly of full-length cDNAs. Following the convention of serialized nomenclature, we designate this product Mfz10 (Figure 1A). The predicted sequence contains a putative signal peptide, a cysteine-rich extracellular domain (CRD) with 10 invariant cysteine residues, seven C-terminal hydrophobic regions consistent with transmembrane segments, and a 25-amino acid cytoplasmic tail. The sum of these features defines the *frizzled* gene family [10,12,13]. *Mfz10* encodes a protein of 571 amino acids that is most similar to Mfz1 and Mfz7, with 75 and 77 % overall identities, respectively (Figure 1B), and which shares less similarity with other mouse Frizzled proteins. The amino acid sequence is nearly identical to that of human FzE2 (also known as FzD2), which suggests that *Mfz10* may be the murine homologue of this gene [22,23]. Interestingly, mRNA levels of human FzD2 are up-regulated in oesophageal

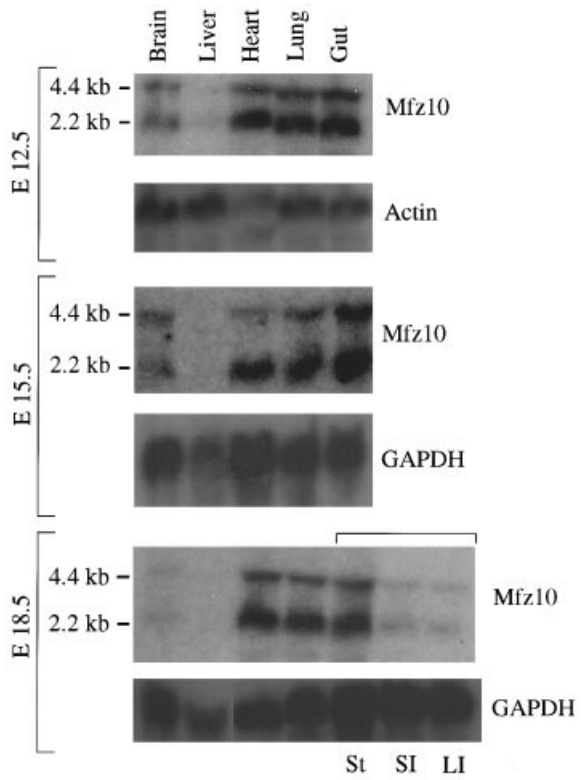

Figure 4 Northern analysis of Mfz10 on total RNA harvested from isolated organs at the indicated stages of mouse fetal development

At E18.5 the gut was further subdivided into the stomach (St) and small (SI) and large (LI) intestines. To confirm equivalent RNA loading, the same blots were hybridized separately to control probes, β-actin or GAPDH.

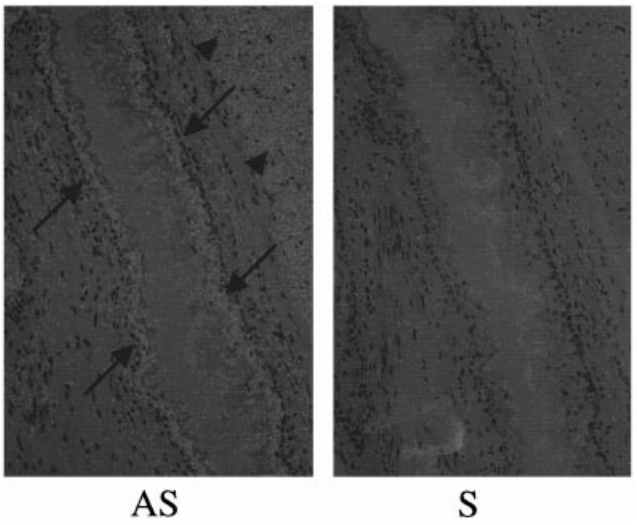

Figure 5 *In situ* hybridization showing the tissue distribution of Mfz10 mRNA in the squamous epithelium of the adult mouse oesophagus (arrows) and in para-oesophageal skeletal muscle (arrowheads)

Results with the antisense riboprobe (AS) are shown on the left with a control sense riboprobe (S) on the right.

squamous cell carcinomas compared with adjacent normal oesophageal tissue [24].

Expression analysis of *Mfz10*

Using a 1.2-kb cDNA fragment from the 3′ end of *Mfz10* for Northern analysis on adult mouse tissues, we detected principally two transcripts of 2.2 and $\approx$ 4.4 kb, with higher levels in heart, brain and lung than in skeletal muscle, kidney or testis (Figure 2B). Only a single message, slightly larger than 4.4 kb, is present in the liver at low levels, transcripts of slightly different size are noted in skeletal muscle, and Mfz10 is barely expressed in the spleen. Despite isolation of Mfz10 from fetal mouse gut, with a relative mRNA abundance of 5 in 10^5 transcripts, only weak expression is detected in the adult gastrointestinal tract (Figure 2A), with modestly higher levels in foregut derivatives (oesophagus and stomach) compared with the intestine. Interestingly, this gradient of mRNA expression along the rostro-caudal axis of the adult mouse gut is opposite to that of mTcf-4, whose mRNA levels increase steadily from the duodenum to the distal colon [25].

The 2.2-kb *Mfz10* transcript corresponds to four of the five cDNA isolates. The fifth clone represents a variant transcript (designated *Mfz10a*) with an identical coding sequence but an additional 1550 bp in the 3′-untranslated region (Figure 3A). A cDNA probe derived from this unique 3′-untranslated region hybridizes only with the 4.4-kb transcript in adult mouse tissues (Figure 3B). Thus the two Mfz10 mRNA species correspond to alternative isoforms that encode the same protein. These variants may serve in differential regulation of message stability, a possibility that is consistent with their differential tissue expression.

Expression of Mfz10 mRNA is higher during development than in adult tissues, as judged by the intensity of signals from the Northern blots. Mfz10 mRNA is present in the brain, heart, lung and whole gut at E12.5 (Figure 4), E14.5 (results not shown) and E15.5 (Figure 4), but there is weak or no expression in the fetal liver. Together with the virtual absence of Mfz10 mRNA in the adult spleen (Figure 2), this result indicates that Mfz10 probably plays no significant role in haematopoiesis. At E18.5 Mfz10 is expressed at highest levels in the heart and lung, and at a lower level in the brain (Figure 4), but remains undetectable in the liver. There is strong expression in the stomach, a foregut derivative, with weaker expression in the small and large intestines, which are derived from the primitive midgut and hindgut, respectively.

Using mRNA *in situ* hybridization in developing mouse fetuses at E13.5 and E14.5, we detected diffuse expression throughout the brain, heart, lung and gut, without discrete tissue localization (results not shown). Strong expression of Mfz10 mRNA is evident throughout the squamous epithelium of the adult oesophagus, with much weaker expression in submucosal tissue; strong expression is also detected in para-oesophageal (paravertebral) skeletal muscle (Figure 5). Consistent with the results of Northern analysis, Mfz10 mRNA expression is also detected, albeit at lower levels, in the mucosa of the stomach but not in that of the small or large intestines (results not shown). There is weak expression in sub-epithelial smooth muscle throughout the gastrointestinal tract.

Thus there are several notable aspects of Mfz10 mRNA expression in general and in the gut in particular. First, overall expression is considerably stronger during development than in adults. This suggests that Mfz10 probably functions primarily in responding to developmental signals. Second, there are two RNA isoforms that encode the same protein and

which are expressed in roughly equal proportions in most sites. Third, in the gastrointestinal tract there is stronger expression in the foregut than in the midgut or hindgut, during both fetal and adult life, although selected foregut derivatives such as the liver do not express Mfz10. Expression is substantially higher in the developing than in the adult gut, which probably reflects stage-specific functions. Tanaka et al. [24] recently reported frequent up-regulation of human FzD2 in oesophageal squamous cell carcinomas relative to adjacent normal tissue. This raises the possibility that Mfz10/human FzD2 is an oncofetal protein whose aberrant expression in epithelial cells contributes to the pathogenesis of oesophageal cancer. Alternatively, the *Mfz10/hFzD2* gene may aberrantly be re-activated as part of a programme of gene mis-expression during malignant transformation.

This work was supported in part by fellowships from the Dolphin Trust, Harcourt General Foundation and the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation.

REFERENCES

1 Cadigan, K. M. and Nusse, R. (1997) Wnt meeting 1996. Biochim. Biophys. Acta **1332**, R1–R5
2 Wodarz, A. and Nusse, R. (1998) Mechanisms of Wnt signaling in development. Annu. Rev. Cell. Dev. Biol. **14**, 59–88
3 Dierick, H. and Bejsovec, A. (1999) Cellular mechanisms of wingless/Wnt signal transduction. Curr. Top. Dev. Biol. **43**, 153–190
4 Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J. and Nusse, R. (1996) A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. Nature (London) **382**, 225–230
5 Klingensmith, J., Nusse, R. and Perrimon, N. (1994) The *Drosophila* segment polarity gene dishevelled encodes a novel protein required for response to the wingless signal. Genes Dev. **8**, 118–130
6 Noordermeer, J., Klingensmith, J., Perrimon, N. and Nusse, R. (1994) dishevelled and armadillo act in the wingless signalling pathway in *Drosophila*. Nature (London) **367**, 80–83
7 Peifer, M., Sweeton, D., Casey, M. and Wieschaus, E. (1994) wingless signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo. Development **120**, 369–380
8 Nusse, R. (1997) A versatile transcriptional effector of Wingless signaling. Cell **89**, 321–323
9 van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A. et al. (1997) Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. Cell **88**, 789–799
10 Wang, Y., Macke, J. P., Abella, B. S., Andreasson, K., Worley, P., Gilbert, D. J., Copeland, N. G., Jenkins, N. A. and Nathans, J. (1996) A large family of putative transmembrane receptors homologous to the product of the *Drosophila* tissue polarity gene frizzled. J. Biol. Chem. **271**, 4468–4476
11 Dale, T. C. (1998) Signal transduction by the Wnt family of ligands. Biochem. J. **329**, 209–223
12 Vinson, C. R. and Adler, P. N. (1987) Directional non-cell autonomy and the transmission of polarity information by the frizzled gene of *Drosophila*. Nature (London) **329**, 549–551
13 Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C. J. and Moon, R. T. (1996) A frizzled homolog functions in a vertebrate Wnt signaling pathway. Curr. Biol. **6**, 1302–1306
14 Kinzler, K. W. and Vogelstein, B. (1996) Lessons from hereditary colon cancer. Cell **87**, 159–170
15 Boynton, R. F., Blount, P. L., Yin, J., Brown, V. L., Huang, Y., Tong, Y., McDaniel, T., Newkirk, C., Resau, J. H., Raskind, W. H. et al. (1992) Loss of heterozygosity involving the APC and MCC genetic loci occurs in the majority of human esophageal cancers. Proc. Natl. Acad. Sci. U.S.A. **89**, 3385–3388
16 Tamura, G., Maesawa, C., Suzuki, Y., Tamada, H., Satoh, M., Ogasawara, S., Kashiwaba, M. and Satodate, R. (1994) Mutations of the APC gene occur during early stages of gastric adenoma development. Cancer Res. **54**, 1149–1151
17 Caca, K., Kolligs, F. T., Ji, X., Hayes, M., Qian, J., Yahanda, A., Rimm, D. L., Costa, J. and Fearon, E. R. (1999) Beta- and gamma-catenin mutations, but not E-cadherin inactivation, underlie T-cell factor/lymphoid enhancer factor transcriptional deregulation in gastric and pancreatic cancer. Cell Growth Differ. **10**, 369–376
18 Park, W. S., Oh, R. R., Park, J. Y., Lee, S. H., Shin, M. S., Kim, Y. S., Kim, S. Y., Lee, H. K., Kim, P. J., Oh, S. T., Yoo, N. J. and Lee, J. Y. (1999) Frequent somatic mutations of the beta-catenin gene in intestinal-type gastric cancer. Cancer Res. **59**, 4257–4260
19 Ozturk, M. (1999) Genetic aspects of hepatocellular carcinogenesis. Semin. Liver Dis. **19**, 235–242
20 Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. J. and Clevers, H. (1998) Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat. Genet. **19**, 379–383
21 Lee, Y. J., Swencki, B., Shoichet, S. and Shivdasani, R. A. (1999) A possible role for the high mobility group box transcription factor Tcf-4 in vertebrate gut epithelial cell differentiation. J. Biol. Chem. **274**, 1566–1572
22 Zhao, Z., Lee, C. C., Baldini, A. and Caskey, C. T. (1995) A human homologue of the *Drosophila* polarity gene frizzled has been identified and mapped to 17q21.1. Genomics **27**, 370–373
23 Sagara, N., Toda, G., Hirai, M., Terada, M. and Katoh, M. (1998) Molecular cloning, differential expression, and chromosomal localization of human frizzled-1, frizzled-2, and frizzled-7. Biochem. Biophys. Res. Commun. **252**, 117–122
24 Tanaka, S., Akiyoshi, T., Mori, M., Wands, J. R. and Sugimachi, K. (1998) A novel frizzled gene identified in human esophageal carcinoma mediates APC/beta-catenin signals. Proc. Natl. Acad. Sci. U.S.A. **95**, 10164–10169
25 Korinek, V., Barker, N., Willert, K., Molenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree, O. and Clevers, H. (1998) Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. Mol. Cell. Biol. **18**, 1248–1256

Received 16 March 2000/9 May 2000; accepted 1 June 2000