A Possible Role for the High Mobility Group Box Transcription Factor Tcf-4 in Vertebrate Gut Epithelial Cell Differentiation*

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The Wingless (Wg)/Wnt signaling pathway mediates essential aspects of early development and has been elucidated through a combination of genetic and biochemical studies in several species (1). One critical component of this signaling pathway is the cytoplasmic protein Armadillo/β-catenin, which is maintained at a low concentration in the free form in the cytoplasm. Wg/Wnt signaling raises the free β-catenin concentration to permit association with High Mobility Group (HMG)-box proteins of the T-cell Factor (Tcf)/Lymphoid Enhancer Factor (LEF) subfamily and mediates diverse functions in development, possibly including endoderm and gut differentiation. Determinants of tissue specificity in the response to Wg/Wnt signaling remain unknown. We have identified Tcf-4 as the predominant Tcf/LEF factor in the developing mouse gut. During fetal development, Tcf-4 mRNA expression is restricted to gut epithelium and specific regions of the brain, the thalamus and roof of the midbrain. In adults, expression is widespread, with highest levels observed in the liver, an endodermally derived organ, and persists in the gastrointestinal tract. Murine Tcf-4 has multiple RNA splice variants with consequentially significant heterogeneity in sequences 3′ to the HMG box. Microinjection of mRNA or plasmid DNA encoding Tcf-4 into Xenopus embryos results in ectopic expression of molecular markers of endoderm and differentiated gut epithelium in isolated animal cap explants. Taken together, these findings point to a potentially important function for Tcf-4 in development of the vertebrate gastrointestinal tract.

The Wingless (Wg)1/Wnt signaling pathway mediates essential aspects of early development and has been elucidated through a combination of genetic and biochemical studies in several species (1). One critical component of this signaling pathway is the cytoplasmic protein Armadillo/β-catenin, which is maintained at a low concentration in the free form in the cytoplasm. Wg/Wnt signaling raises the free β-catenin concentration to permit association with High Mobility Group (HMG)-box proteins of the T-cell Factor (Tcf)/Lymphoid Enhancer Factor (LEF) subfamily and translocation to the nucleus (2, 3), where the complex is presumed to effect a Wg/Wnt-responsive transcription program of gene expression (4). Transcriptional targets of this signaling pathway include the developmentally regulated genes engrailed, sianois, labial, and ultrabithorax (5–7), although many other genes are undoubtedly controlled through Wg/Wnt signaling in diverse cell types.

Besides the established role of Wg/Wnt signaling in vertebrate mesodermal differentiation and axis formation and in development of the larval cuticle in Drosophila, several lines of evidence point to a role for this pathway in the differentiation of endodermal derivatives. First, genetic and biochemical studies in Drosophila suggest that larval midgut development depends on the Wg signal (8, 9). Second, recent genetic evidence implicates homologs of Wg/Wnt signaling pathway components in gut development in Caenorhabditis elegans (10, 11). Finally, latency of cytoplasmic β-catenin may be maintained in part through the function of the product of the adenomatous polyposis coli (APC) gene (12, 13), a frequent target of mutation in human colorectal and other gastrointestinal epithelial malignancies (14). This potential role of APC in the Wg/Wnt signaling cascade likely reflects a critical function in maintaining gastrointestinal epithelial cell homeostasis. Indeed, a fraction of colorectal tumors with intact APC harbor activating mutations in the β-catenin gene (15), and at least one Tcf/LEF protein, human (h) Tcf-4, is commonly expressed in colon cancer cell lines and mediates transcriptional activation therein (16). The sum of these observations strongly implicates β-catenin and Tcf/LEF family proteins in normal gut development and in the pathogenesis of gastrointestinal tumors.

The important question of how Wg/Wnt signaling achieves lineage-specific outcomes in diverse cell types remains unresolved and relies in part on a better understanding of the transcriptional effectors of the signaling pathway. In Drosophila, mutations in dTCF (also known as pangolin) result in phenotypes that are identical to those seen in wg mutants (9, 17), implying that Pangolin functions exclusively within this pathway. The correspondence may, however, be more complicated in vertebrates, which have multiple Tcf/LEF-related proteins with varying patterns of expression in embryos and adults. Both Tcf-1 and LEF-1 were originally identified through studies in lymphocytes, where their expression is restricted in adult mice (18–20); during fetal development, their expression is wide and largely overlapping (21, 22). Mice lacking Tcf-1 develop normally (23), whereas LEF-1−/− mice manifest developmental abnormalities consistent with a role for LEF-1 in inductive interactions between mesenchymal and epithelial cells (22). Development of the gut has long been recognized to depend upon such inductive interactions but Tcf-1 and LEF-1 are not expressed in this organ and absence of either gene does not lead to obvious gut anomalies. Characterization of other members of this HMG-box protein subfamily has been less detailed, and the full extent of the subfamily is unknown.

We sought to identify Tcf/LEF proteins that are expressed in the developing vertebrate gut and to examine their function in

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AF107298 and AF107299.

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§ The abbreviations used are: Wg, Wingless; HMG, High Mobility Group; Tcf, T-cell factor; LEF, Lymphoid Enhancer Factor; IFABP, intestinal fatty acid binding protein; PCR, polymerase chain reaction; ED, embryonic day; UTR, untranslated region; BCIP, 5-bromo-4-chloro-3-indolyl phosphate.
differentiation of the epithelium. Using degenerate polymerase chain reaction (PCR) cloning, we isolated a single Tcf/LEF family member as the dominant protein of this class in the developing mouse gut. The mRNA and predicted amino acid sequence of this clone are most closely related to those of hTcf-4, previously identified through near uniform expression in colon cancer cell lines (16); during preparation of this report, Korinek et al. also reported the cloning of murine (m) Tcf-4 (24).

In mouse embryos, expression of Tcf-4 mRNA is restricted to the gut epithelium and specific regions of the developing brain; in adults, expression is widespread, with highest levels observed in the liver, an embryonic midgut derivative. mTcf-4 mRNA possesses multiple alternative splice forms, the significance of which is presently unclear. Ectopic expression of one of these mTcf-4 mRNA isoforms in Xenopus embryos induces expression of gastrointestinal epithelial markers in isolated animal cap explants. These observations point to a possibly important function for Tcf-4 in differentiation of the gastrointestinal tract and vertebrate gut development.

EXPERIMENTAL PROCEDURES

Construction of Mouse Embryonic Gut cDNA Library—Poly(A)+ RNA isolated from the fore- and mid-guts of 250 ICR strain mouse fetuses at post-coital day 13.5 was reverse transcribed, size selected over 1 kb by column chromatography, and cloned directly into a modified pC52 vector (pC5105; kindly provided by Dr. R. Harland, Berkeley, CA) using the SuperScript Plasmid System (Life Technologies, Inc., Bethesda, MD). The library contained $>5 \times 10^6$ transformants, from which 105 clones were arrayed robotically onto high density membrane filters (Genome Systems, St. Louis, MO).

PCR Cloning—Total RNA from the fore- and midguts of embryonic day (ED) 14.5 mouse fetuses was reverse transcribed and PCR amplified using degenerate oligonucleotide primers spanning nucleotides 405–421 of Tcf-3 (19). Reaction conditions were 30–32 cycles for Xlhbox8 (5°C/60°C annealing temperature) and 5°C/70°C; the latter transcript is expressed at varying levels in many parts of the developing mouse embryo but has not been detected previously in the gut, and its amplification in this experiment is of unclear significance. In contrast, hTcf-4 is known to be expressed in the epithelial lining of the adult colon and in many colon cancer cell lines (16).

In Situ Hybridization—C57BL/6 mouse embryos were harvested at various stages of gestation, fixed in 4% paraformaldehyde overnight at 4°C, dehydrated in ethanol, and embedded in paraffin. Serial 5-µm sagittal and parasagittal sections were mounted on Superfrost Plus glass slides (Fisher). mTcf-4B plasmid DNA was linearized, and sense and antisense digoxigenin-UTP-labeled RNA probes were synthesized using T7 RNA polymerase (Boehringer Mannheim). The slides were deparaffinized, rehydrated, treated with proteinase K, rehydrated, and used to probe the above high density membrane filters to obtain full-length cDNA clones. For cloning splice variants, PCR primers were 5'-AGGCCACATATAAAGACCCCT-3' and 5'-GCAGAACAGAAACAGAAGAAGAAAGA-3'.

RESULTS

Isolation of a Tcf/LEF Subfamily Member Expressed in the Developing Mouse Gut—The epithelial lining of the murine gastrointestinal tract begins to differentiate in mid-gestation. To study the early development of this epithelium, we prepared cDNA from foregut and midgut tissues harvested from fetuses at ED13.5. Using degenerate oligonucleotides complementary to the ends of the highly conserved HMG box of Tcf/LEF proteins, we performed PCR with this cDNA as the template and cloned the amplified products. DNA sequencing of ten PCR clones revealed nine identical inserts with sequence most closely related to hTcf-4 and a single clone encoding the HMG box of mTcf-1. The latter transcript is expressed at varying levels in many parts of the developing mouse embryo but has not been detected previously in the gut, and its amplification in this experiment is of unclear significance. In contrast, hTcf-4 is known to be expressed in the epithelial lining of the adult colon and in many colon cancer cell lines (16).

We used the PCR-amplified HMG-box of mTcf-4 as a probe to screen 106 plasmid clones from an embryonic gut cDNA library and recovered a single strongly hybridizing clone whose nucleotide sequence is >80% homologous to that of hTcf-4 and whose predicted amino acid sequence bears >95% identity with hTcf-4 up to and including the HMG box. The sequence of this clone through amino acid position 405 is identical to that reported recently (24), except that the space between amino acids designated 268 and 269 has the residues SPLSS; this difference increases the homology with Tcf-3 (3). Additional PCR on >5 \times 10^5 clones using mTcf-4-specific primers corresponding to sequences in the 3' end of the gene resulted in isolation of three additional clones encoding two distinct C termini (Fig. 1); one of these (here designated mTcf-4(K)) is identical to the recently reported cDNA sequence of mTcf-4 (24). Thus, Tcf-4 appears to be the predominant Tcf/LEF subfamily HMG-box protein expressed in the gut of the mid-gestation mouse embryo.

mTcf-4 is closely related to other members of the Tcf/LEF subfamily and particularly to Tcf-1 in the extensive alternative mRNA splicing that leads to a heterogeneous pool of mRNAs differing at their 3' ends; presumptive intron-exon junctions toward the 3' terminus also appear to be conserved between both mouse and man (Fig. 1). In addition, there is a remarkable 70% identity of nucleotide sequences within the 5'-untranslated regions (UTRs) of the human and mouse genes (Fig. 2), raising the possibility that important regulatory information is encoded by this sequence.

Determination of Tcf-4 mRNA Expression During Development—The expression profiles of the two best characterized Tcf/LEF proteins follow a strikingly similar theme. Both Tcf-1 and Lef-1 are expressed only in lymphocytes in adult mice but exhibit wide and partially overlapping domains of expression in the fetus (21, 22). Whereas mice lacking Tcf-1 have an isolated T-cell defect (23), Lef-1−/− mice die as a result of multiple
developmental abnormalities that correlate with dominant sites of fetal expression (22). To acquire insight into the potential functions of Tcf-4, we examined its pattern of expression. mTcf-4 mRNA is first detected between ED11.5 and 13.5, when it is localized to the thalamus in the developing central nervous system (Fig. 3). Extension of the staining into the roof of the midbrain is detected as early as ED13.5; expression in both the di- and mesencephalon becomes more prominent by ED15.5 (Fig. 3), when it assumes its characteristic pattern in the posterior portion of the mesencephalic roof, spatially separated from predominant expression within the thalamus. This pattern of central nervous system expression persists at least until ED18 (Fig. 3) and probably beyond (see below). Although brain expression dominates the in situ hybridization studies, lower levels of Tcf-4 mRNA are clearly detected in the embryonic gut by Northern analysis (Fig. 4A); by in situ hybridization, this expression is confined to the epithelial lining of the developing gastrointestinal tract (Fig. 4B). This is consistent both with our original isolation of mTcf-4 from ED13.5 gut cDNA and with the apparently low (≤1/105 clones) representation of mTcf-4 mRNA within this source. Notably, mTcf-4 is expressed both in the developing stomach (Fig. 4C) and intestine (Fig. 4, B and D) and in many sections displays a patchy distribution among epithelial cells, with some areas staining more prominently than others (Fig. 4, C and D). However, the stratified and incompletely differentiated epithelium of the mouse fetal gut precludes better characterization of the strongly positive cells. We do not detect mTcf-4 mRNA expres-
sion outside of the central nervous system and gut during fetal development, and particularly note its absence in other major endodermal derivatives, the liver and bronchial epithelium.

**mTcf-4 Expression in the Adult**—Unlike Tcf-1 and LEF-1, whose expression becomes remarkably restricted after birth, mTcf-4 is expressed widely in adult tissues. Although the brain remains a major site of expression, high levels of Tcf-4 mRNA are also observed in the liver; lower but detectable levels are present in the heart, lungs, kidneys, and testis, with lowest levels in muscle and spleen (Fig. 5A). This expression pattern is distinct from that reported by Korinek et al. (24) who failed to detect appreciable mTcf-4 levels outside of the adult brain. In our hands also, mTcf-4 is difficult to detect on Northern blots with total RNA and requires a high specific activity probe against 1–2 μg of poly(A)+ RNA to generate a signal. Furthermore, Northern analysis consistently reveals two distinct RNA species in embryos as well as adults, corresponding to transcript lengths of 4.7 and 4.0 kilobases; the smaller species is expressed principally in the fetal and adult brain, whereas the larger species predominates in all other sites, including the liver. Both species react with full-length mTcf-4 as well as with a probe encompassing ~400 nucleotides in the 3′-UTR at high stringency (Fig. 5A). This finding virtually excludes the possibility of a cross-reacting transcript and suggests that the distinct mRNA species reflect heterogeneity outside the 3′-UTR. As predicted, mTcf-4 is also expressed in the adult gastrointestinal tract. Korinek et al. have reported a small increase in mRNA levels along the rostro-caudal axis of the intestine (24). Here we show that expression also extends rostrally to the stomach (Fig. 5B), the most proximal portion of the gut that is lined by a glandular epithelium, but at a much lower level than in the brain.

**Function of mTcf-4 in Endodermal Differentiation**—Protein functions in cell differentiation are best assessed in experimental systems that approximate normal tissue development. The animal cap of Xenopus blastulas is normally fated to develop...
into ectoderm but retains the capacity for both mesodermal and endodermal differentiation in the presence of the TGF-β-related ligand activin or selected ectopically expressed genes (25–28). To examine the potential role of Tcf-4 in the differentiation of endoderm and its tissue derivatives, we expressed mTcf-4B mRNA in one-cell stage embryos and followed the expression of three molecular markers in animal cap explants: endodermin, a pan-endodermal marker (29), Xlhbox8 (also known as Pdx1, IPF1, or STF-1), a marker of foregut derivatives including the duodenum and pancreas (30), and the IFABP (31). Expression of these markers is weakly but reproducibly induced by full-length mTcf-4B mRNA and by the known inducer chordin but not by a 5'-truncated and frameshifted mTcf-4 control (Fig. 6A). Notably, however, the magnitude of induction is considerably smaller than that by activin (Fig. 6A) or by the recently described homeobox gene Mixer (28), a potent early activator of endoderm differentiation in Xenopus embryos (data not shown).

Injection of mRNA into Xenopus embryos results in potentially rapid gene expression that persists through many cell divisions; in contrast, injected plasmid DNA undergoes zygotic transcription only after the mid-blastula transition. Injection of mTcf-4B plasmid DNA also induced the gastrointestinal markers in explanted Xenopus animal caps (Fig. 6B), suggesting that the ectopic expression can influence endodermal differentiation relatively late. Injection of plasmid DNA encoding hLEF-1 had a similar effect (Fig. 6C), suggesting that other Tcf/LEF subfamily proteins can substitute for this function. We conclude that Tcf-4 participates in a biochemical pathway of differentiation that leads to gastrointestinal derivatives of the endoderm.

**DISCUSSION**

The molecular mechanisms by which the endoderm-derived epithelial cells lining the aerodigestive tract differentiate are largely unknown. Several lines of evidence point to a role for Wg/Wnt signaling in differentiation of the gastrointestinal epithelium, including genetic studies in invertebrates (6, 10) and biochemical analysis of colon carcinomas (16). This raises the intriguing possibility that there are gut-specific components or modifiers of the Wnt signaling pathway that mediate tissue-specific responses. However, many of the components of this signaling pathway in gut epithelial cells are largely presumed on the basis of biochemical studies in other developmental systems. We have, therefore, focused on characterizing gut epithelium-specific aspects of Wnt signaling. Here we report that the predominant Tcf/LEF subfamily member present in the developing vertebrate gut is Tcf-4, which is only expressed here and in selected regions of the central nervous system during fetal development.

**mTcf-4 Structure and Expression—Mouse and human Tcf-4**

**FIG. 5. Expression of mTcf-4 in adult tissues.** Northern analysis of mRNA isolated from various tissues (panel A, commercial blot) or brain and stomach (panel B) from 8–12-week adult mice and probed sequentially with DNA probes corresponding to full-length mTcf-4, mouse β-Actin, and a 400-base pair region in the 3'-UTR of mTcf-4. Autoradiographic exposure times: mTcf-4, 2 days; Actin, 6 h; 3'-UTR, 12 h.

**FIG. 6. Endodermal differentiation in Xenopus animal cap explants following embryonic microinjection of mTcf-4B mRNA (A) or plasmid DNA (B) or human LEF-1 plasmid DNA (C).** RT-PCR detects expression of the pan-endodermal marker endodermin (Edd), foregut-specific marker Xlhbox8, small intestine-specific marker IFABP, and loading control EF1α. Marker induction is compared with embryos injected with chordin mRNA (A) and with uninjected caps cultured in 50 ng/ml recombinant activin A (A, B) and with plasmid DNA (B) corresponding to an out-of-frame 5' truncation of the first 76 nucleotides of the mTcf-4B coding sequence. These results are representative of three independent experiments with DNA and RNA injections, respectively.

are virtually identical at the amino acid level, at least in sequences N-terminal to the HMG box. The relationship of Tcf-4 to Tcf-1 extends to a remarkable similarity in splice variants and of a second reading frame in one of the terminal exons that encodes the isoform designated Tcf-4B. This potentially leads to considerable heterogeneity in the C termini of both proteins (32), and its evolutionary conservation suggests that this may be of functional importance. Although the various C termini of Tcf-1 do not reveal detectable differences in transactivation properties in transient transfection assays (32), the Tcf-4 isoforms may well harbor functional heterogeneity that is relevant in vivo. Tcf-1 is also heterogeneous at the N terminus, in part reflecting use of alternate promoters (32). Although we have not observed the same structure in mTcf-4 cDNAs isolated from the fetal gut, the major mRNA isoform present in the brain is distinct from that in other tissues (Fig. 5A) and probably reflects 5' heterogeneity; the precedent with Tcf-1 suggests that this also may represent dual promoter usage. The roughly equal frequency with which we isolated alternatively spliced clones suggests, but does not prove, that these splice variants are expressed in low but equal proportions in the fetal gut; our data do not address the existence or relative abundance of mTcf-4 splice variants in the brain.

Recently, Korinek et al. (24) reported cloning the mouse Tcf-4 gene, and our in situ hybridization studies confirm localization...
of fetal expression to the gut epithelium and di/mesencephalon. Whereas these investigators failed to detect appreciable mTcf-4 RNA levels in most adult tissues, however, we note that the gene is widely expressed postnatally, with highest levels in the liver, an endoderm-derived organ, and brain. The same pattern is seen in Northern analysis with either a full-length cDNA probe or one corresponding to a fragment of the 3′-UTR, which argues strongly in favor of specificity over cross-reactivity with related species. This apparent discrepancy is best explained by our use of high specific activity probes against Northern blots of poly(A)+ rather than total RNA. Indeed, Korinek et al. (24) readily detected mTcf-4 transcripts in poly(A)+ RNA isolated from various segments of the adult intestine, and we were also repeatedly frustrated in efforts to demonstrate expression outside the brain using total RNA. Thus, the expression pattern of Tcf-4 deparnts significantly from that of either Tcf-1 or LEF-1, both of which are expressed broadly during fetal development but restricted to lymphocytes in adult mice.

The restricted fetal expression of Tcf-4 might suggest that it mediates essential aspects of signaling by Wnts or related molecules during development of the gut epithelium and, especially, of the diencephalon (thalampus), where expression levels are highest. Notably, at least 7 of the 16 known mammalian Wnt genes are expressed in various regions of the central nervous system and at least one of these, Wnt-1, is required for mid- and hind-brain development (33, 34). The lack of brain abnormalities in mouse embryos lacking Tcf-1 or LEF-1 further hints at a possible requirement for Tcf-4 in central nervous system development or function.

**Tcf-4 Function**—Our most important finding pertains to the potential role of Tcf-4 in differentiation of endodermally derived tissues. Injection of mTcf-4 in the early Xenopus embryo leads to ectopic expression of endodermal and gut markers in animal cap explants; at sibling tadpole stages beyond 35–36, endoderm specifically marks endodermal derivatives in the gut (29), whereas XlHbox8 and IFABP are specific markers of the duodenum and pancreas (30) and small intestine (31), respectively. This implicates Tcf-4 as functioning within a biochemical pathway that promotes gastrointestinal epithelial cell differentiation.

Several aspects of this finding merit further discussion. First, the assay does not directly address the extent of cytodifferentiation promoted by Tcf-4; indeed, complete differentiation of endodermal derivatives in *in vivo* is highly dependent on inductive interactions with mesenchyme (35) and probably does not occur in isolated animal caps (26). However, induction of XlHbox8 mRNA, a specific marker of differentiated foregut derivatives (30), suggests that Tcf-4 might promote relatively advanced differentiation and may be particularly relevant to development of the pancreas. Second, the mechanism of cellular changes induced by Tcf-4 in the animal cap remains uncertain. Untreated animal caps differentiate into atypical, ciliated epidermis in isolation but retain considerable developmental plasticity early on. Ectopic expression of gastrointestinal markers in this tissue may reflect selective expansion of rare progenitor cells with intrinsic endodermal potential, realization of such potential in naive embryonic cells, or perhaps some combination of these possibilities; this consideration applies to all experiments with *Xenopus* animal caps. Finally, loss-of-function studies, including gene targeting in mice, are necessary to establish a functional requirement for Tcf-4 in development of the endoderm and its derivatives in *in vivo*. During the review of this manuscript, Korinek *et al.* reported that Tcf-4 knockout mice die at birth and their only detectable histopathologic abnormality is a reduced number of cells in small intestinal crypts, suggestive of a depleted stem cell compartment (36). Together with the restricted fetal expression in mice, this observation and our gain-of-function studies in *Xenopus* implicate Tcf-4 as a regulator of the vertebrate gut epithelium. The similar results with either mRNA or DNA injection in *Xenopus* embryos further suggest that Tcf-4 can function in this capacity relatively late, i.e. after mid-blastula transition, in embryonic development.

Although genetic experiments in *Drosophila* suggest that dTcf/Pangolin exclusively subserves Wg signaling (9, 17), it is entirely possible that in the vertebrate gut ligands other than Wnt signal through Tcf-4 to influence epithelial cell differentiation. The full complement of Wnt-related proteins expressed in the developing gut remains unknown, and several proteins without a known connection to Wnt signaling have also been implicated in endodermal and gut epithelial differentiation (37–39). The required inductive effect of adjacent mesenchyme for complete maturation of the gut epithelium (35) raises the further possibility that Tcf-4 is an effector for signals delivered by cell surface ligands. The interactions between the biochemical pathways involved in these processes and in gastrointestinal tumorigenesis remain to be determined. Identification of Tcf-4 as the predominant Tcf/LEF protein expressed in the developing and adult gut epithelium and its potential role in cytodifferentiation should facilitate molecular approaches to these questions.

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