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EVOLUTIONARY CONSERVATION OF GLUCOSE-DEPENDENT INSULINOTROPIC POLYPEPTIDE (GIP) GENE REGULATION AND THE ENTEROINSULAR AXIS

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Abstract

Glucose-dependent insulintropic polypeptide (GIP), an important component of the enteroinsular axis, is a potent stimulator of insulin secretion, functioning to maintain nutrient efficiency. Although well-characterized in mammals, little is known regarding GIP transcriptional regulation in *Danio rerio* (*Dr*). We previously demonstrated that *Dr*GIP is expressed in the intestine and the pancreas, and we therefore cloned the *Dr* promoter to compare GIP transcriptional regulation in *Dr* and mammals. Although no significant homology was identified between the highly conserved mammalian promoter and the *Dr*GIP promoter, 1072-bp of the *Dr*GIP promoter conferred tissue-specific expression in mammalian cell lines. Deletional analysis of the *Dr*GIP promoter identified two regions that, when deleted, reduced transcription by 75% and 95%, respectively. Mutational analysis of the upstream region suggested involvement of an Nkx binding site, although we were unable to identify the factor binding to this site. The *cis* element in the downstream region was found to be a GATA binding site. Lastly, overexpression and shRNA experiments identified PAX4 as a potential repressor of *Dr*GIP expression. These findings provide evidence that despite the identification of species-specific transcriptional regulators and differences in GIP expression patterns between *Danio rerio* and mammals, a moderate degree of regulatory conservation appears to exist.

Keywords

glucose-dependent insulintropic polypeptide; *Danio rerio*; regulation; GATA-4; PAX4

Introduction

Glucose-dependent insulintropic polypeptide (GIP) is a member of the secretin-vasoactive polypeptide family of gastrointestinal regulatory peptides [1]. In mammals, mature GIP is synthesized in endocrine K-cells located primarily in the small intestinal mucosa of the duodenum and proximal jejunum [1,2]. Size differences in mammalian GIP genes have been described (253-amino acid polypeptide in humans, 144-amino acid polypeptide in rodents), but the differences have been attributed principally to variations in intron length [3].

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Human and rat GIP genes have been isolated and their promoters partially characterized. The first 290-bp of the GIP promoter is highly conserved among mammals, correlating with the preservation of transcription factor binding sites, and suggesting similar regulation of mammalian GIP promoters. Due to the inability to adequately isolate and purify K-cells, information regarding the regulation of GIP expression has been obtained from studies using surrogate cell lines, such as STC-1 cells, a neuroendocrine cell line known to express multiple peptides, including glucagon, somatostatin, CCK, amylin, and GIP [4,5]. Distal and proximal promoters have been identified in the mammalian GIP gene. The proximal GIP promoter, located within the first intron, conferred only minimal promoter activity in β TC-3 cells and did not appear to include an enhancer core consensus sequence [6]. In contrast, deletional analysis of the more distal promoter demonstrated that the first 193-bp upstream of the transcription start site was sufficient to direct specific expression of GIP in STC-1 cells [5]. However, additional investigation has demonstrated that 2500-bp of the 5'-regulatory region of the rat GIP promoter was necessary to direct transgenic insulin expression specifically to K-cells [7]. Using a similar 3100-bp region of the rat GIP promoter containing regulatory elements found in GIP intron 1, a recent study demonstrated a high level of GIP expression in the stomach, suggesting that regulatory elements present in the first intron of the GIP gene might prevent high expression levels in the stomach [8].

Identification of the minimal essential GIP promoter allowed further studies to identify regulators of GIP expression. A binding motif located between base pairs -190 and -184 with respect to the transcription start site (+1), was found to bind a member of a family of DNA binding proteins, GATA-4 [9]. Members of the GATA family (4, 5, 6) are expressed in overlapping patterns along the longitudinal axis of the intestine [10]. Moreover, GATA-4 has been shown to play a role in regulating the transcription of numerous intestinal genes [11–14]. A second *cis*-regulatory region containing a binding site for the transcription factor ISL-1 was also identified. This site appears to act in conjunction with the GATA-4 site to regulate GIP gene expression [9]. ISL-1 has been shown to be expressed in pancreatic islets and has been implicated in the transcriptional regulation of other genes important for nutrient metabolism, such as insulin, proglucagon, somatostatin, and amylin [15–18]. A third transcription factor, PDX-1, has also been demonstrated to be involved in the regulation of GIP expression [19,20]. PDX-1 was originally identified as an activator of insulin and somatostatin, and has been shown to play an important role in pancreatic development [21–23]. In addition, PAX6, a known regulator of pancreatic development, may complex with PDX-1 to regulate GIP expression. Because GIP promoter activity has been found to be reduced in STC-1 cells following the inhibition of endogenous PAX6 [19] and because PAX6 is necessary for the expression of a second physiological incretin, GLP-1 [24,25], PAX6 may also be a potential regulator of GIP expression.

In this study, we investigated the regulation of the *Danio rerio* (zebrafish) GIP gene by isolating and characterizing its GIP promoter to identify sequence-specific *cis*-acting regulatory elements modulating expression. Studies in our laboratory have demonstrated that *Danio rerio* possess a unique GIP expression pattern [26], suggesting that both the functional significance and regulation of GIP expression may differ between mammals and zebrafish. Despite these differences in expression pattern, we established that the regulation of the GIP gene by GATA-4 was conserved between *Danio rerio* and mammals. Moreover, we also identified that the *Danio rerio* GIP promoter appears to share homology to insulin regulation by virtue of the presence of a potential Nkx binding site and, PAX4, a gene important in pancreatic development, was identified as a potential transcriptional repressor of *Danio rerio* GIP expression.

Methods

Cell culture

GTC-1 cells (mouse neuroendocrine), β TC-3 cells (mouse insulinoma) GH3 cells (rat pituitary), and NIH-3T3 cells (mouse fibroblasts) were grown in Dulbecco's Minimal Essential Media (DMEM, MediaTech, Manassas, VA) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT) at 37 °C in an atmosphere of 5% CO₂ in the presence of 100 U/mL penicillin G, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B (MediaTech).

Cloning of *Danio rerio* GIP promoter into pGL3 basic vector

A set of primers was generated (Invitrogen, Carlsbad, CA) to isolate the first 1072 bp of the zebrafish GIP promoter (NCBI accession number CR383677). The primers (promF and promR, Table 1) were designed with *Bgl*II and *Xho*I restriction sites to facilitate promoter cloning into the pGL3 basic reporter plasmid (Promega, Madison, WI). PCR reactions were carried out in a 50-µL volume containing 2 µL genomic DNA, 50 pmol primers, and 25 U Taq DNA polymerase and accompanying reagents (Roche, Manneheim, Germany). Inserts were sequenced (GeneCore, Boston University Core Facility) to confirm identity.

Construction of *Danio rerio* GIP promoter deletion and mutation constructs

A series of *Danio rerio* GIP promoter deletion constructs was created using PCR. The pGL3/1072 bp *Danio rerio* GIP promoter construct was linearized with *Xba*I (NEB) and used as a template for subsequent PCR reactions. The reverse primer (pGL3R, Table 1) corresponded to a sequence 128 bp downstream of the multiple cloning region of the pGL3 basic vector, and was used to generate all constructs. In addition, a series of forward primers containing a *Kpn*I site was generated (Table 1), corresponding to internal regions of the *Danio rerio* GIP promoter. PCR products were isolated and cloned into the pGL3 basic vector, and inserts were sequenced (GeneCore) to confirm identity.

A promoter construct containing a mutation in a selected predicted Nkx2.5 binding site (CTTAATTG), which spans base pairs −973 to −966 of the full-length *Danio rerio* GIP promoter, was generated using a two-step PCR reaction designed to introduce site-directed mutations. *Danio rerio* GIP promoter-specific primers containing base substitutions were synthesized (nkx1F and nkx1R, Table 1). The first step of the PCR reaction was initiated using one of these primers and either the pGL3R primer or a forward primer corresponding to the 5' end of the full-length promoter construct (zf 1072 F, Table 1). The products of the first PCR reaction (91-bp and 1136-bp) were resolved on an agarose gel and then combined and used as template for a second PCR reaction, in which the primers zf 1072 F and pGL3R were used. The resulting product (1227-bp) was then cloned into the vector pGL3 basic.

A second promoter construct containing a larger mutation in the predicted Nkx2.5 binding site was generated using the GeneTailor Site-Directed Mutagenesis kit from Invitrogen. Primers to be used with this kit were synthesized (nkx2F and nkx2R, Table 1). Briefly, plasmid DNA was methylated and amplified in a mutagenesis reaction containing the aforementioned set of overlapping primers. The mutagenesis mixture was then transformed into bacterial cells, and clones were sequenced to ensure that the proper mutation had been introduced into the *Danio rerio* GIP promoter sequence.

A promoter construct containing a mutation in the GATA binding site (ATCAATCAGC) which spanned base pairs −399 to −388 of the *Danio rerio* GIP promoter, was generated and PCR was used for site-directed mutagenesis. Specifically, a forward primer (gata-4 mutF, Table 1) was synthesized to mutate this region and was used in conjunction with the pGL3R primer to generate a promoter fragment spanning from −408 to +15 of the *Danio*

rerio GIP promoter containing the desired mutation. The mutated promoter fragment was then ligated into pGL3 basic and the mutation confirmed by sequencing.

Transient transfections

One day prior to transfection, cells (GTC-1, β TC3, GH3, or NIH-3T3) were plated on 6-well plates at a density of $\sim 1\text{--}2 \times 10^5$ cells per well. A mixture containing 1 μ g GIP reporter construct, 3 μ L Fugene (Roche), 20 ng pRL-CMV plasmid (control for transfection efficiency), and 100 μ L serum free medium was incubated at room temperature for 15 min, after which 100 μ L of the DNA/Fugene mixture were added to each well. At 24 h post-transfection, the medium was replaced with DMEM containing 10% FBS.

Luciferase Assays

Cells were washed twice with phosphate buffered saline (PBS), lysed in 250 μ L passive lysis buffer (Promega, Madison, WI), and subjected to two freeze/thaw cycles, using the manufacturer's instructions for the dual-luciferase reporter system. To measure luciferase and Renilla activities, 20 μ L of each sample were measured in duplicate using an Optocomp 1 luminometer (MJ Research Inc., Waltham, MA). Luciferase activity was expressed in light units and was corrected for Renilla activity to compensate for variations in transfection efficiency. All constructs were analyzed in triplicate in at least three separate experiments.

Short hairpin RNA (shRNA)

GATA-4 shRNA was purchased from Origene (Rockville, MD). The following sequence was targeted: pRS-GATA-4-18 (5'-GACTTCTCAGAAGGCAGAGAGTGTGTGCAA-3'). The plasmid pRS-shGFP (GFP), which contains a non-effective shGFP sequence cassette, and empty pRS vector were used as negative controls for these experiments. Cells were transfected as described above, with the exception that the transfection mixture contained 0.7 μ g of GATA-4 shRNA or control shRNA, 0.4 μ g of the zebrafish GIP/luciferase reporter plasmid, and 20 ng of Renilla (pRL-CMV), and then were analyzed using luciferase assays.

PAX4 overexpression studies

A PAX4 expression plasmid was obtained as a generous gift from the laboratory of Dr. Michael German (University of California San Francisco), and empty pBat12 vector was used as the negative control for these experiments. Cells were transfected as described above, with the exception that the transfection mixture contained 0.7 μ g of PAX4 expression plasmid or empty vector, 0.4 μ g of the zebrafish GIP/luciferase reporter plasmid, and 20 ng of Renilla (pRL-CMV), and then were analyzed using luciferase assays.

Statistical analysis

Data were analyzed using Student's t-test for unpaired samples, and statistical significance was assigned for $p < 0.05$.

Results

Danio rerio GIP promoter activity parallels rat GIP promoter activity

In mammals, the first 290-bp of the GIP promoter are highly conserved, with 64% homology among various species (mouse, rat and human). However, when the first 290-bp of the *Danio rerio* GIP promoter was aligned with mammalian GIP promoters, a far lesser degree of homology was detected (14%; data not shown). Because neither suitable *Danio rerio* cell lines nor an *in vivo* system are currently available, we examined whether the *Danio rerio* GIP promoter possessed the capacity to direct cell-specific expression in mammalian cell lines. Accordingly, we cloned the first 1072 bases of the *Danio rerio*

promoter (with respect to the transcription start site) into the luciferase reporter plasmid pGL3. Using luciferase reporter assays, we compared its activity to that of the minimal rat GIP promoter (193-bp) in various mammalian cell lines, including GTC-1, β TC3, NIH-3T3, and GH3 cells, in order to establish whether or not a mammalian cell system would enable an examination of DrGIP promoter activity. The latter is a rat pituitary adenoma cell line, which, while capable of processing and secreting peptide hormones, does not express endogenous GIP.

As depicted in Figure 1, the *Danio rerio* GIP promoter was capable of initiating expression in GTC-1 cells, a mouse neuroendocrine intestinal cell line that expresses endogenous GIP, and to a lesser extent, in β TC3 cells, a mouse insulinoma cell line that expresses a low level of endogenous GIP. In GTC-1 cells, the *Danio rerio* GIP promoter activity was increased 80-fold over control, while the *Rattus norvegicus* GIP promoter activity was increased 74-fold over control. In β TC3 cells, the activity of both the *Danio rerio* and *Rattus norvegicus* promoters were significantly lower, inducing only a 9-fold and 1.6-fold increase in luciferase activity, respectively, when compared to pGL3 basic vector. In the GH3 cell line, the *Rattus norvegicus* GIP promoter activity was not significantly different from control, while *Danio rerio* GIP promoter activity showed a modest 4-fold increase. However, in the mouse embryonic fibroblast cell line NIH-3T3, neither the *Rattus norvegicus* nor *Danio rerio* GIP promoter displayed increased expression over vector alone. These data demonstrate that in rodent cell lines, the expression pattern of the *Danio rerio* GIP promoter parallels that of the *Rattus norvegicus* GIP promoter. Thus, GTC-1 cells appear to represent a suitable *in vitro* model for analysis of the zebrafish GIP promoter.

Deletion analysis of the *Danio rerio* GIP promoter identified two regions, that when deleted, resulted in a decrease in promoter activity

To define DNA regions upstream of the GIP start site responsible for enhanced promoter activity a series of GIP promoter deletion constructs were generated. The GIP/luciferase constructs -158, -292, -408, -536, -886, -950, and -1017, with respect to the transcription start site, were transiently transfected into GTC-1 cells, and luciferase activity was measured. As demonstrated in Figure 2, two regions were identified that, when deleted, resulted in a significant reduction in promoter activity. The deletion of region 1 (between -1072 and -950) produced a 75% loss in promoter activity, while deletion of region 2 (between -408 and -292) resulted in an additional 10% loss in promoter activity. Although expression levels of the other promoter constructs varied slightly, the difference did not reach a level of statistical significance ($p < 0.05$).

To identify the transcription factors responsible for the difference in promoter activity between the -1072 and -950 constructs, TFSearch was used and identified a consensus binding motif for a candidate mammalian transcription factor, Nkx2.5. Nkx2.5 is a member of a homeodomain transcription factor family that has been demonstrated to act in synergy with GATA transcription factors [27], although they are principally regarded as regulators of pulmonary and cardiac development [28,29]. However, an additional family member with shared binding site homology, Nkx2.2, is known to be involved in the differentiation of pancreatic endocrine cell lineage (null mice fail to develop β -cells), and studies of the *Danio rerio* homologue Nkx2.2a demonstrated that a morpholino to this transcription factor resulted in a similar, but less severe, phenotype compared with mice [30].

Mutation of Nkx2.5 binding site decreases GIP promoter activity

To assess the involvement of the predicted Nkx2.5 binding site in the regulation of the zebrafish GIP promoter, two mutation constructs were generated in which the Nkx2.5 binding site (CTTAATTG), spanning the region from -973 to -966 of the zebrafish GIP

promoter with respect to the transcription start site, was mutated to CTCTGTTG or CTCCCCCG. The first mutation construct (mut 3) was selected because these three base pairs have been reported to be requisite for Nkx2.5 binding [31]. The second mutation construct (mut 5) was generated to disrupt the AT rich region of the predicted binding site to theoretically prevent binding of other Nkx family members. Figure 3 depicts the results of two independent luciferase assays in which the activity of these mutation constructs was compared to that of the full-length -1072 GIP/luciferase construct. As shown, the 3-bp mutation of the predicted Nkx2.5 binding site decreased reporter activity by approximately 80%. However, the larger 5-bp mutant only resulted in a modest reduction in promoter activity. These data suggest that two of the bases in the original wild-type binding site, -970 and -969 (AA), are essential for activator binding, although other nucleotides (CC) appear to be permissible. These observations suggest that although an activator appears to bind within the predicted Nkx2.5 site, in all likelihood slightly upstream or downstream of the identified site, it does not appear to be a member of the Nkx family.

PAX4 overexpression represses GIP promoter activity

Further examination of the region surrounding the predicted Nkx2.5 binding site revealed several potential PAX4 binding sites. Several PAX family members (PAX2 and PAX6) have been shown to be expressed in STC-1 cells, which are the parent cell line for the GTC-1 cells used in this study [19,32]. The PAX family of transcription factors contain two potential DNA-binding domains, a paired domain and a homeodomain [33]. In mammals, PAX4 acts as a transcriptional repressor via its paired domain, and it has been identified as a regulator of pancreatic endocrine development [34]. Mice containing a PAX4 null mutation possess a decreased number of pancreatic γ - and β -cells and an increased number of α -cells, although insulin expression is still detectable [35]. In addition, both PAX4 and PAX6 regulate insulin, glucagon, and somatostatin transcription [34].

Thus, we next sought to determine whether PAX4 was involved in the regulation of *Danio rerio* GIP expression by examining the effects of PAX4 protein overexpression on *Danio rerio* promoter activity in GTC-1 cells using co-transfection of a PAX4 expression plasmid with several *Danio rerio* GIP promoter constructs. As shown in Figure 4, overexpression of PAX4 decreased the activity of the full-length *Danio rerio* GIP promoter construct by approximately 70%. In addition, the effect of PAX4 overexpression on the activity of the region 1 mutation-bearing constructs was examined to determine whether PAX4 interacted with the transcription factor binding to the element in region 1. Overexpression of PAX4 resulted in a 70% decrease in promoter activity of mut 5 and a 10% decrease in promoter activity of mut 3, suggesting that PAX4 binding occurs independently of the element binding to region 1. These data suggest that although PAX4 appears to act as a transcriptional repressor of *Danio rerio* GIP expression, the precise region of binding could not be identified.

GATA-4 binds to region 2 of the GIP promoter

To determine whether GIP promoter regulation is conserved between mammals and *Danio rerio*, we examined three transcription factors known to direct cell-specific expression of GIP in mammals --PDX-1, ISL-1, and GATA-4. Analysis of the first 1072 bases of the *Danio rerio* GIP promoter for the presence of PDX-1 and ISL-1 consensus binding sites failed to detect potential binding sites. In contrast, six putative GATA binding sites were identified.

Using silencing RNA technology (shRNA), we examined whether a reduction in GATA-4 expression would attenuate GIP expression. As shown in Figure 5A, co-transfection of a GATA-4 specific shRNA construct (Origene) with the luciferase reporter plasmid

containing the full-length *Danio rerio* GIP promoter, which included all six potential GATA binding sites, decreased transcriptional activity by approximately 50%. In contrast, control shRNA plasmids containing either a non-effective shRNA cassette (pRS-shGFP) or vector alone (pRS) did not affect expression of the *Danio rerio* GIP promoter construct.

To identify the GATA binding site that might be responsible for the observed reduction in promoter activity, we compared the position of the consensus GATA binding motifs (Figure 5B) with the results from the deletional study. The GATA consensus motif located between base pairs 397 – 388 of the *Danio rerio* GIP promoter corresponded to the region 2. Therefore, a reporter construct was generated in which the original GATA binding site, SNNCATNNNN, was mutated between bases 393 – 389, producing a modified site, SNNCTCTCAN. As illustrated in Figure 6, abolishing this GATA site within the *Danio rerio* GIP promoter luciferase construct resulted in a 40% reduction of promoter activity, reducing the level of activity to that of the –292 construct. These data suggest that GATA-4 can activate the *Danio rerio* GIP promoter by binding to the *cis* regulatory region located between bases –397 and –388 of the *Danio rerio* GIP promoter.

Additional shRNA experiments were conducted using the smaller –292 and –408 *Danio rerio* promoter constructs. As demonstrated in Figure 7, attenuation of GATA-4 expression had no effect on the GATA-4 mutant construct. These results confirmed the interaction of GATA-4 with the previously identified binding region. These data suggest that GATA-4 can activate the *Danio rerio* GIP promoter by binding to the *cis* regulatory region located between bases –397 and –388 of the promoter.

Discussion

The majority of studies on incretin hormones and their regulation have been conducted in mammals, in which the enteroinsular axis has been thoroughly characterized. Analysis of the mammalian GIP promoter demonstrated that the first 290 bp are well conserved among rats, mice, and humans. Within this region, the binding sites for the transcription factors GATA-4, ISL-1, PDX-1, and PAX6 have been identified and shown to direct the cell-specific expression of GIP [9,19,20,36]. In addition, studies demonstrated that in mammals, GIP expression is restricted to the small intestine, distal antrum of the stomach, and the submandibular salivary gland [1–3]. In contrast to the expression pattern observed in mammals, studies in our laboratory have demonstrated that in the *Danio rerio*, GIP was expressed in endocrine cells of the pancreas, in addition to intestinal expression [26]. Furthermore, GIP and insulin co-expression was demonstrated in a subset of these pancreatic endocrine cells [26]. Consequently, we examined the regulation of the *Danio rerio* GIP gene, hypothesizing that differences in expression patterns observed for mammalian and *Danio rerio* GIP might preclude the conservation of transcriptional regulation. Owing to the lack of suitable *Danio rerio* cell lines, studies were conducted in the mouse neuroendocrine cell line GTC-1, a derivative of the original cell line in which mammalian GIP promoter studies were previously conducted [5].

Surprisingly, despite the absence of a high degree of sequence homology between the *Danio rerio* GIP promoter and that of mammals, we found that *Danio rerio* GIP expression paralleled mammalian GIP expression in multiple cell lines. In addition, our analysis of the *Danio rerio* GIP promoter identified an important *cis*-regulatory element that appears to bind the transcription factor GATA-4. Thus, similar to mammals, [9,36], GATA-4 appears to function as a transcriptional activator of the *Danio rerio* GIP promoter. Six GATA family members have been classified in vertebrates, and they have subsequently been divided into two subfamilies GATA-1, -2, -3 and GATA-4, -5, -6, based upon similarities in amino acid composition and patterns of expression. Members of the former family function in the

hematopoietic system [37], while members of the latter family are expressed in overlapping patterns in the developing cardiovascular system and in endoderm-derived tissues, including the liver, lungs, pancreas, and gut [38]. In mammals, GATA-4 has been shown to play a role in regulating the transcription of numerous intestinal genes [11–14], and GATA-4 was shown to be required for modulating the level of GIP expression [36].

Several researchers have examined the function of GATA family members during development in the *Danio rerio* through the use of morpholinos and *Danio rerio* mutants. The suppression of GATA-4 in the *Danio rerio* larvae had consequences in both the pancreas and the intestine. Although embryos retain the ability to form a gut tube, subsequent organogenesis failed, resulting in the absence of epithelial folds in the intestine, the liver, and the pancreas [39]. In addition, redundancy in the expression and function of GATA-4, -5, and -6 has been observed, and Hecklen-Klein *et al.* also reported GATA-6 expression in the *Danio rerio* intestine, liver, and pancreas [39]. Furthermore, suppression of GATA-6 resulted in a disruption of intestinal morphogenesis and decreased levels of Nkx2 family members involved in heart development [40]. Mutants for GATA-5 (*faust* mutants), in addition to having a *cardia bifid* phenotype, also disrupt anterior gut tube morphogenesis and in the pharyngeal endoderm due to the failure of cells to coalesce at the midline of the embryo [41].

In addition, consistent with our observations, other studies have linked GATA-4 to the regulation of pancreatic genes. In one study, GATA-4 and GATA-6 expression were demonstrated in the developing mouse endocrine pancreas [42]. In contrast, Ketola *et al.* reported that GATA-4 is a marker for exocrine pancreatic development in the mouse, while GATA-6 is responsible for endocrine pancreatic development [43], and that GATA transcription factors are important in the regulation of intestinal and pancreatic development. In the adult pancreas, GATA-4 expression was confined to the exocrine pancreas, but prenatally (E12.5), GATA-4 has been shown to be co-expressed with early glucagon- but not insulin-producing, cells [42]. Furthermore, GATA-4 has been reported to activate the glucagon gene in the fetal pancreas [42]. These studies all suggest that GATA-4 in the *Danio rerio* may be responsible for specifying GIP expression in pancreatic endocrine cells.

In the present study, examination of the second *cis*-regulatory region identified a potential Nkx2.5 binding motif. However, the results of our mutational analysis of this binding site are inconsistent with the binding of a Nkx family member to this element. Nevertheless, it is important to note that *Danio rerio* GIP promoter expression was examined in a mammalian system, which might create *in vitro* findings that may not correlate to actual *in vivo* regulation. However, it is interesting to note that in mammals, Nkx6.1 has been shown to be important for the regulation of islet development and may possibly be involved in insulin biosynthesis [44]. In addition, Nkx2.2 is known to be involved in the differentiation of the pancreatic endocrine lineage. Nkx2.2 null mice fail to develop β -cells. Moreover, studies of the zebrafish homologue Nkx2.2a have demonstrated that a morpholino to this transcription factor (antisense sequence that blocks access to specific region of DNA) resulted in a similar, but less severe, phenotype found in mice [30]. Similarities in the regulation of GIP and insulin would not be surprising. Previous studies have demonstrated that GIP and insulin expression involves the common transcription factors PDX-1 [20,45] and ISL-1 [9,46]. Furthermore, small intestinal K-cells and pancreatic β -cells share a common developmental lineage, providing further evidence that GIP and insulin may be regulated by similar transcription factors.

Although a specific Nkx family member was not identified as an activator of the *Danio rerio* GIP promoter, we detected several sequences corresponding to potential PAX4 motifs adjacent to the predicted Nkx2.5 binding motif. PAX4 overexpression studies demonstrated

a decrease in *Danio rerio* GIP promoter activity, suggesting that PAX4 may act as a transcriptional repressor of GIP expression by interacting in close proximity to the proposed Nkx2.5 binding motif. This hypothesis is supported by the identification of multiple members of the PAX family (PAX4, PAX5, PAX6) as transcriptional repressors of pancreatic development in mammals [34,35], and it has been hypothesized that this repression may be due to competition with transcriptional activators for DNA-binding sites [24]. Mice containing a PAX4 null mutation possess a decreased number of pancreatic γ - and β -cells and an increased number of α -cells, although insulin expression can still be detected [35]. Mice containing a PAX6 null mutation demonstrated decreased numbers of all pancreatic endocrine cell lineages, while double null mutants (PAX4 and PAX6) failed to produce any mature pancreatic endocrine cells [47]. Finally, both PAX4 and PAX6 also regulate insulin, glucagon, and somatostatin transcription [34]. Although a PAX4 homolog has not yet been identified, *Danio rerio* does possess PAX6A and PAX6B genes, which are paralogs that most likely resulted from genomic duplication in *Danio rerio* evolution [48,49]. In addition, expression studies have demonstrated that PAX6B, in addition to being expressed in the eye and nervous system, is also found in the endocrine cells of the pancreas, and PDX-1, a transcription factor important for mammalian regulation of GIP expression, appears to be necessary to activate expression of PAX6B in the *Danio rerio* pancreas [50].

In conclusion, despite the lack of a high degree of conservation between mammalian and *Danio rerio* GIP promoters, *Danio rerio* GIP possessed the capacity to direct cell-specific expression in mammalian cell lines. Two regions of the *Danio rerio* promoter were identified, that when deleted, resulted in a decrease in promoter activity. We have demonstrated an unknown activator binding to region 1 of the *Danio rerio* GIP promoter, which was originally believed to be an Nkx site. In addition, PAX4 was found to act as a transcriptional repressor of the *Danio rerio* GIP promoter, although the binding site was not identified. Furthermore, GATA-4 was identified as a transcriptional activator of the *Danio rerio* GIP promoter, demonstrating that although base pair homology is not maintained, the mammalian and *Danio rerio* GIP promoters still share homology sufficient to conserve transcriptional regulation of the GIP gene. These studies were conducted using an *in vitro* system and may thus not necessarily reflect functional *in vivo* promoter properties. However, it is interesting to note from an evolutionary perspective that while GIP expression patterns differ between zebrafish and mammals, their respective GIP promoters nevertheless appear to share GATA and PAX family members as conserved regulators of GIP expression. Additional studies will be needed to confirm the importance of GATA-4 and PAX4 in the *in vivo* regulation of *Danio rerio* GIP cell-specific expression. Furthermore, future studies should be aimed at identifying the unknown activator of GIP expression binding to the putative Nkx2.5 motif.

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Figure 1. Analysis of *Danio rerio* GIP promoter activity in various mammalian cell lines

Four different mammalian cell lines were transiently transfected with pGL3 basic, *Rattus norvegicus* GIP promoter construct, or *Danio rerio* GIP promoter construct. Cells were harvested 48 h after transfection, and cell lysates were analyzed for luciferase activity. Data were normalized to the activity of the promoterless pGL3 basic vector after correcting for differences in transfection efficiencies by the measurement of Renilla activity, and represent the mean activity. SE obtained from two separate transfections. P-values were calculated using Student's t-test, and significance was assigned for $p < 0.05$ (*).

Figure 2. Analysis of *Danio rerio* GIP promoter deletion constructs

GTC-1 cells were transiently transfected with the following GIP/luciferase promoter constructs: -158, -292, -408, -536, -886, -950, and -1072. Cells were harvested 48 h after transfection, and cell lysates were analyzed for luciferase activity. Data were normalized to the activity of the wildtype (-1072) promoter construct after correcting for differences in transfection efficiencies by the measurement of Renilla activity, and represent the mean activity. SE obtained from six separate transfections. P-values were calculated using Student's t-test, and significance was assigned for $p < 0.05$ (*). Region 1 refers to the reduction in promoter activity evident between -1072 and -950, and region 2 refers to the reduction in promoter activity detected between -408 and -292.

Figure 3. Analysis of the predicted NKx2.5 binding site

The effect of mutation of the Nkx2.5 binding site on the functional activity of the wildtype *Danio rerio* GIP promoter was assessed in GTC-1 cells. Cells were harvested 48 h after transfection, and cell lysates were analyzed for luciferase activity. Data were normalized to the activity of the wildtype (–1072) promoter construct after correcting for differences in transfection efficiencies by the measurement of Renilla activity, and represent the mean activity. SE obtained from two separate transfections. P-values were calculated using Student's t-test, and significance was assigned for $p < 0.05$ (*).

Figure 4. Effect of PAX4 expression on *Danio rerio* GIP promoter activity in GTC-1 cells

Co-transfections were performed using the wildtype *Danio rerio* GIP promoter construct or the promoter constructs with the NKx2.5 binding site mutations in conjunction with either the expression plasmid PAX4/pBat12 or the empty vector (pBat12). After 48 h, the cells were harvested and analyzed for luciferase and renilla activity. Data were normalized to the activity of the empty pBat12 vector after correcting for differences in transfection efficiencies by the measurement of Renilla activity, and represent the mean activity. SE obtained from two separate transfections. P-values were calculated using Student's t-test, and significance was assigned for $p < 0.05$ (*).

Figure 5. Analysis of the effects of inhibition of GATA-4 on *Danio rerio* GIP promoter activity in GTC-1 cells

A: The effect of GATA-4 inhibition on the functional activity of the *Danio rerio* GIP promoter construct was assessed in GTC-1 cells. Co-transfections were performed using the wildtype-1072 *Danio rerio* GIP promoter and the shRNA plasmid pRS (vector control), GFP (non-specific shRNA) or G18 GATA-4-shRNA plasmid. After 48 h, the cells were harvested and analyzed for luciferase and Renilla activity. Data were normalized to the activity of the wildtype promoter construct after correcting for differences in transfection efficiencies by the measurement of Renilla activity, and represent the mean activity. SE obtained from two separate transfections. P-values were calculated using Student's t-test, and significance was assigned for $p < 0.05$ (*). B: Location of GATA consensus binding motifs in the wildtype *Danio rerio* GIP promoter. Site positions are expressed relative to the transcription start site.

Figure 6. Analysis of a GATA-4 binding site mutation on *Danio rerio* GIP promoter activity in GTC-1 cells

Transfections were performed using either the -408 *Danio rerio* GIP promoter or the promoter with the mutant GATA-4 binding site. After 48 h, the cells were harvested and analyzed for luciferase and Renilla activity. Data were normalized to the activity of the -408 promoter construct after correcting for differences in transfection efficiencies by the measurement of Renilla activity, and represent the mean activity. SE obtained from two separate transfections. P-values were calculated using Student's t-test, and significance was assigned for $p < 0.05$ (*).

Figure 7. Analysis of the effects of inhibition of GATA-4 on the *Danio rerio* -408 promoter construct activity in GTC-1 cells

The effect of GATA-4 inhibition on the functional activity of the -408 GIP promoter construct was assessed in GTC-1 cells. Co-transfections were performed using either the -408 or -292 GIP promoter construct and the shRNA plasmid GFP or G18 GATA-4 shRNA plasmid. After 48 h, the cells were harvested and analyzed for luciferase and Renilla activity. Data were normalized to the activity of the -408 GIP promoter construct after correcting for differences in transfection efficiencies by the measurement of Renilla activity, and represent the mean activity. SE obtained from two separate transfections. P-values were calculated using Student's t-test, and significance was assigned for $p < 0.05$ (*). ns = not significant

Table 1

Primer list.

Gene	F primer	R primer	T _a	Cycle number
prom	CATAAAATTCGCAGCCTCGAGG	GAGAGATCTTAACTGGTGTATGACTCTCCAG	60	30
pGL3R		GAACCAGGGCGTATCTCTTCAT	60	30
158F	AAGGTACCTGACACCTAAGGCCTGTAAGAAA		60	30
292F	AAGGTACCACCAACCATTATACTGCTCAG		60	30
408F	ATGGTACCAATAACTGCTAATCAATCAGCACTA		60	30
536F	AAGGTACCAATTTGGCATGGCACCAAAG		60	30
886F	AAGGTACCACTTAAGGCAACAACGTGTGA		60	30
950F	AAGGTACCGCCAACAAAGTTATGATTTGAAAGT		60	30
nkx1	CATGTTTTTTAATTCTTTAACTCTGTTT	AAACAGAGTTAAAAGAATTAAAAAACATG	65	30
nkx2	TTAATTCTTTAACTCCCCCTTTAAAAGTTG	AGTTAAAAGAATTAAAAAACATGATTAAGTT	55	20
zf1072F	ATGGTACCCTCGAGGTAAAATATTGTATGATC		60	30
GATA-4 mutF	ATGGTACCAATAACTGCTAATTGTCTCACACTA		60	30