Functional characterization of the trout insulin promoter: implications for fish as a favorable model of pancreas development

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Abstract The complex anatomy of the mammalian pancreas, in which the endocrine cells are grouped in islets dispersed among the predominant exocrine component, has hampered study of the molecular events governing the development of pancreatic cell lineages. To investigate whether fish may provide relevant, complementary models of pancreas development, we characterized the trout insulin (tINS) promoter and its molecular interactions with PDX1, a key transcriptional and developmental factor of the mammalian pancreas. Transfection of a luciferase reporter plasmid containing the 280 bp 5′-flanking region of the tINS gene resulted in strong activity in mammalian pancreatic β cells but not in CHO or pituitary cells. Footprinting assays and cotransfection experiments indicated that mammalian PDX1 binds to and activates the tINS promoter. By microinjecting plasmids to fertilized zebrafish eggs, we showed that the expression of mouse PDX1 is capable of activating the co-injected tINS promoter plasmid in most cell types of the 24-h zebrafish embryo. The conserved role of PDX1 in vertebrate insulin gene regulation opens the possibility to exploit fish models in the study of pancreas development.

Key words: Rainbow trout insulin promoter; PDX1; Transcription; Hamster β cell; Zebrafish embryo

1. Introduction

The mammalian pancreas develops as an outpocketing of the primitive embryonic foregut to eventually produce a heterogeneous organ containing an exocrine and an endocrine component [1]. The endocrine cells are arranged in clusters or 'islets' of cells containing four distinct cell types: α (A), β (B), δ (D), and PP, which are responsible for production of the specific hormones glucagon, insulin, somatostatin and pancreatic polypeptide, respectively [2]. In part because of its complexity, relatively little information is available concerning the molecular events underlying formation and development of the mammalian pancreas.

The β cell-specific expression of mammalian insulin genes is controlled at the transcriptional level, through several well defined *cis* elements located in the proximal 5′-flanking region [3–6]. Several of the transcription factors involved in cell-specific transcription have been defined: they include PDX1 (also called STF1, IPF1 or IDX1) [7–9], a variant homeodomain protein which recognizes A/T-rich sites in the DNA, and several basic helix-loop-helix (bHLH) proteins including BETA2 (NeuroD) [10,11] and E2A [12–14]. Although neither PDX1 nor BETA2 is exclusively expressed in β cells (PDX1 is also found in intestinal epithelial cells [7] and BETA2 in brain cells [10]), PDX1, BETA2 and E2A interact synergistically on the insulin promoter to ensure a high level of β cell-specific transcription [10,15].

Implicit in the search for pancreatic gene transcription factors has been the expectation that such proteins would themselves be involved in early development of pancreatic cell lineages or would aid in isolating genes controlling early pancreatic development. Indeed, targeted inactivation of the PDX1 gene in mice leads to failure of pancreas development [16], clearly showing that, in addition to its role as an insulin gene transcription factor, PDX1 plays an essential role in early pancreatic development.

Teleost fish species display striking diversity in the relationship of endocrine to exocrine pancreas cells, ranging from species where the two cell types are intermingled, as in the other vertebrates, to species where they are anatomically separated. In the latter species, such as rainbow trout (*Oncorhynchus mykiss*), the endocrine cells are located in Brockmann bodies, comprising mostly endocrine tissue [17]. The separation of endocrine and exocrine populations in certain fish species offers the prospect of simpler analysis of pancreatic cell development. Towards this end, we have begun a study of the transcriptional control mechanisms underlying expression of the trout insulin (tINS) gene. As a first step, we have isolated and characterized the promoter of the gene. Although the proximal 5′-flanking region shows no striking sequence similarities to that of mammalian insulin genes, it functions in β cell-specific fashion upon transfection to mammalian cells. Based on a combination of in vitro and in vivo analyses, we showed that this preferential activity was due, at least in part, to the action of PDX1. We also showed that PDX1 is capable of activating the tINS promoter in a wide range of cell types of the developing zebrafish.

2. Materials and methods

2.1. Plasmids and mutagenesis

The p0-Luc reporter plasmid encoding the firefly luciferase gene [18] is described elsewhere [19]. The pCMV-βgal plasmid [20] encodes the *E. coli* β-galactosidase reporter gene under the control of the cytomegalovirus (CMV) promoter/enhancer region. pCMV-mPDX1 was prepared by cloning the mouse PDX1 (mPDX1) coding sequence [8] into the pcDNA-3 expression vector (Invitrogen). The 5′-flanking region (−280/+4) of the tINS promoter (280INS) was amplified from trout genomic DNA by PCR, directly sequenced and cloned in pGEM [21] yielding the pIN vector. The *Sal*I/*Bam*HI fragment of 280INS from pIN was cloned into the same sites of p0-Luc yielding the p280INS-Luc reporter plasmid. Two truncated derivatives (p160INS-Luc and p105INS-Luc) containing the −160/+4 and −105/+4 regions of the tINS gene, respectively, were prepared by PCR amplification from p280INS-Luc and cloning the resulting fragments in p0-Luc. The plasmid p468GH-Luc, containing the −468/+24 region of the trout growth hormone (GH) gene, has been described elsewhere [19].

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2.2. Cell culture and transfections

GC (rat pituitary tumor cells), HIT T15 M2.2.2 (hamster pancreatic β cells) and CHO (Chinese hamster ovary cells) were grown and transfected as described previously [19,22]. Briefly, the cells were cultured in Ham's F12 medium (GC and CHO) or DMEM (HIT), supplemented with 10% fetal calf serum and penicillin/streptomycin. Transfections were performed by electroporation (GC: 300 V, 1500 μF) or calcium phosphate precipitation (CHO and HIT) using the indicated amounts of plasmid and cells. Each transfection included 2 μg of pCMV-βgal to normalize the data to transfection efficiency. 48 h after transfection, the cells were washed and collected in PBS, and resuspended in 200 μl of 20 mM Tricine (pH 7.8), 2 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100 and 15% glycerol. The suspension was centrifuged for 1 min and the supernatant solution was processed for assay of luciferase and β-galactosidase activities as described [19].

2.3. DNase I footprinting

Nuclear extracts were prepared from HIT cells as described [23]. For the preparation of recombinant mPDX1, a 1060 bp fragment of the mouse PDX1 gene [8] encoding amino acids 6–284 was subcloned to the vector pRSET-B (Invitrogen) and introduced into *E. coli* JM109 cells. Bacteria were incubated in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside and the His-tagged PDX1 protein purified with nickel-chelating resin according to the manufacturer's instructions (Invitrogen). DNA fragments were end-labelled (8 ng, 2×10^4 cpm) and incubated with the indicated amounts of HIT nuclear extracts or purified mPDX1 protein, and then partially digested with DNase I according to Galas and Schmitz [24].

2.4. Microinjection

Zebrafish were raised and bred according to standard methods [25]. Microinjection was performed as described previously [22]. Briefly, fertilized zebrafish eggs were collected, placed in 1.2% agarose gel grooves, injected using glass capillaries containing the appropriate DNA at 40 μg/ml and incubated at 28.5°C for 24 h. In situ assay to reveal β-galactosidase-positive cells was performed as described [22,26]. Embryos were examined using Nomarski optics and blue cells were classified into five major groups (skin, sclerotome, myotome, notochord and nervous system), according to the criteria of cell location and morphology adopted by Westerfield et al. [26].

3. Results

3.1. The trout insulin promoter shows preferential activity in hamster pancreatic β cells

The promoters of the trout GH and prolactin genes confer pituitary-specific expression to a reporter gene in mammalian cells [19,27]. As a first step to study the regulation of the tINS gene, we therefore investigated whether its promoter could likewise drive the expression of a reporter gene in a cell-specific manner in mammalian cells. The sequence of the tINS promoter amplified from trout genomic DNA is shown in Fig. 1A. Computer comparisons with mammalian insulin promoters failed to reveal extensive regions of homology. The transcriptional activity of p280INS-Luc, a plasmid bearing the 5′-flanking region (−280/+4) of the tINS gene fused to the luciferase reporter gene, was tested in various mammalian cell lines. As shown in Fig. 2, p280INS-Luc could efficiently drive luciferase activity in hamster pancreatic β cells (HIT), which produce insulin, but not in hamster CHO cells or rat pituitary GC cells, neither of which expresses this hormone. Conversely, the p468GH-Luc plasmid, containing the promoter of the trout GH gene linked to the luciferase gene, was effectively activated (because of endogenous expression of the transcription factor Pit-1) in GH-producing GC cells, as shown previously [19], but not in HIT or CHO cells, which express neither Pit-1 nor GH (Fig. 2). Thus, the −280/+4 region of the tINS gene confers β cell-specific expression to a reporter gene in mammalian cells.

A

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-280 gtcgaccatacgcaggtcatcaatccctggaatcgtctgtatatc

-235 tcagacacaggtcacatcagttatgtgcgattcgctggccctctg

                                              |-160
-190 cttggtccaCTAATGTCACTTAGTAAGACAAacatgTCTAACGAG

                                                 |-105
-145 gcgaacaactgaatTGCCACTTACCATTTGATTTCATtgagagac

-100 ttctttttccgctgacactggcaaagaccacaggcccttgttcc

-55  gagacccgtctgctccaggggtataaatgggggaggacctggtg

-10  ttctgcctcaagct +4
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B

C/T	T	A	A	T	G	A1
C	T	A	A	T	G	TSE1
C	T	A	A	T	G	tINS (-181/-176)
C	T	A	A	c	G	tINS (-153/-148)
C	a	A	A	T	G	tINS (-116/-121)

Fig. 1. A: Sequence of the 5′-flanking region (−280/+4) of the tINS promoter. The three regions protected from DNase I digestion by recombinant mouse PDX1 (see Fig. 3) are in capital letters. Within each footprint, the CTAATG sequence and its closely related motifs are in bold characters. The TATA box is underlined and the transcription start site is indicated by an arrowhead. The 5′ end of the two deletion mutants used in this investigation (−160 and −105) are indicated. B: Comparison of the three potential PDX1 binding sites in the tINS promoter with characterized PDX1 binding sites in mammalian genes. The A1 [6] and TSE1 [7] PDX1 binding sites are from the insulin (human, mouse, rat I and II, guinea pig) and somatostatin (rat) promoters, respectively. Lower case letters indicate deviation from the consensus sequence. The sequence at −116/−121 is located on the lower strand.

3.2. Mammalian PDX1 binds to the promoter of the trout insulin gene

Since the above experiment implied an interaction of HIT cell nuclear proteins with the tINS promoter, we performed a DNase I protection assay to identify the relevant binding sites. Two adjacent A/T-rich sequences of the tINS promoter that contain CTAATG or closely related motifs (Fig. 1B) were protected from DNase I digestion by nuclear extracts of HIT cells (Fig. 3, lanes 1–3; see also Fig. 1). In mammals, the CTAATG motif occurs in the binding sites for PDX1, a pancreas-specific transcription factor involved in the activation of the insulin and somatostatin promoters [6,7] (Fig. 1B). Thus, the fact that PDX1 is expressed in HIT cells suggested that the footprints produced by HIT cell extracts could have been generated by this protein. This idea was confirmed by a footprinting assay performed with bacterially expressed mPDX1. In fact, recombinant mPDX1 produced three adjacent footprints extending over the same region of the promoter protected by the HIT extracts (Fig. 3, lanes 5–7). Protection of the two proximal sites required a higher concentration of mPDX1 than that required to protect the distal site. This suggests that this latter site, which bears a CTAATG motif, has a higher affinity for mPDX1 than the two proximal ones, which contain a CTAACG and a CAAATG sequence, respectively.

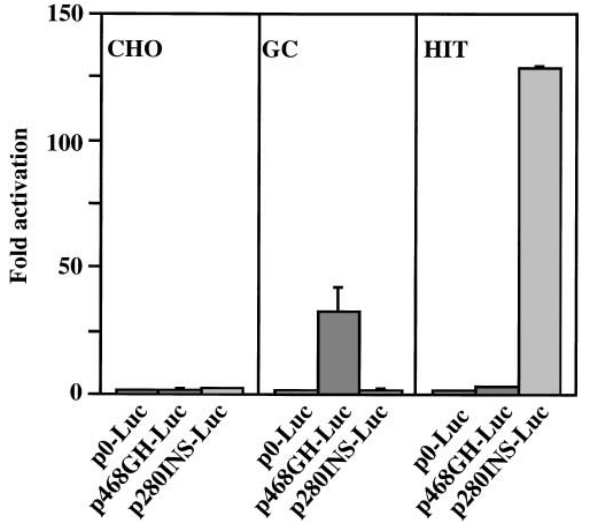

Fig. 2. The tINS promoter is specifically activated in pancreatic β cells. Hamster CHO (5×10^5), rat pituitary GC (2×10^6) and hamster β-pancreatic HIT (10^6) cells were transfected with 7 μg of the indicated plasmids. Data, expressed as fold activation relative to the promoterless (p0-Luc) plasmid, are the mean ± S.E.M. of at least three independent transfections.

3.3. Mouse PDX1 activates the trout insulin promoter

To determine whether the activation of the tINS promoter in HIT cells could be accounted for by the action of PDX1, we performed a cotransfection experiment in CHO cells using a cytomegalovirus enhancer-driven expression plasmid (pCMV-mPDX1) encoding mPDX1. Cotransfection of CHO cells with p280INS-Luc in the presence of increasing amounts of pCMV-mPDX1 led to a dose-dependent and saturable increase of luciferase activity (Fig. 4A), showing that mPDX1 can activate the tINS promoter in non-pancreatic mammalian cells. On the other hand, in HIT cells, the activity of p160INS-Luc, which lacks the distal high affinity PDX1 site, was reduced to 10% of that of p280INS-Luc, while p105INS-Luc, which lacks the whole PDX1 binding region, showed only minimal luciferase activity (Fig. 4B). These results indicate that the PDX1 binding sequences, in particular the distal PDX1 binding site, are required for optimal activation of the tINS promoter in HIT cells.

3.4. Mouse PDX1 activates transcription of the trout insulin promoter in all the main tissues of 24-h zebrafish embryos

To investigate whether PDX1 is able to transactivate the tINS promoter in fish cells, we used the zebrafish transient expression system [26]. In this model, reporter plasmids are microinjected into fertilized zebrafish eggs and the distribution of reporter gene expression in the different tissues of 24-h embryos is revealed histochemically. Microinjection of 1–2 cell zebrafish embryos with pCMV-βgal, a reporter plasmid bearing the β-galactosidase reporter gene under the control of the CMV enhancer, resulted in a mosaic expression of the reporter in all the main tissues of 24-h embryos (Fig. 5). This result, which is in accord with previous findings [22,26], is due to the random distribution of injected DNA within the embryo and to the capacity of the CMV enhancer to activate transcription ubiquitously. In embryos coinjected with pCMV-mPDX1 and p280INS-βgal, a reporter plasmid containing the β-galactosidase gene under the control of the tINS promoter, the expression of the reporter gene was also detected in virtually all the major tissues (Fig. 5). Conversely, stained cells were not observed in embryos coinjected with pcDNA-3 and p280INS-βgal (not shown), consistent with the fact that pancreatic exocrine and endocrine cells do not appear until around day 3 of development [28] and indicating that activation of the tINS promoter was due to mPDX1 produced by the expression vector. Hence, we conclude that mPDX1 is sufficient to transactivate the tINS promoter in all the main tissues of 24-h zebrafish embryos.

4. Discussion

In the present investigation we show that the role of PDX1 in regulating the transcription of the insulin gene is conserved in vertebrates. This opens the possibility of exploiting the peculiarity of fish pancreas anatomy in the study of the molecular events driving its organogenesis. From an evolutionary viewpoint, insulin-expressing cells appear to have progressed from endocrine cells of the open type scattered in the gut, as observed in protochordates (tunicates and *Amphioxus*) to endocrine cells of the closed type either clustered in a paraintestinal islet organ, as in cyclostomes, or finally grouped into islets within the exocrine pancreas, as in gnathostomes. The trend towards cellular aggregation away from the gut is max-

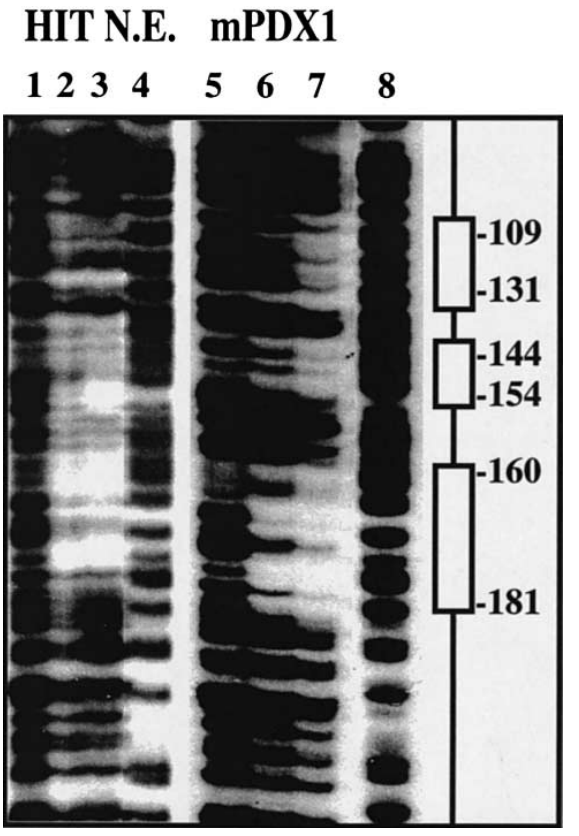

Fig. 3. DNase I footprinting analysis performed with nuclear extracts of HIT cells (left) or recombinant mouse PDX1 protein (right). The labelled tINS promoter was incubated with: 0 (lane 1), 80 (lane 2) or 200 (lane 3) μg of HIT nuclear extracts, or with: 0 (lane 5) 200 (lane 6) or 400 (lane 7) ng of recombinant mouse PDX1. Lanes 4 and 8: Maxam-Gilbert G+A sequencing ladder. DNase I digestions were performed with the following concentrations (μg/ml) of enzyme: 1.2 (lanes 1, 5, 6 and 7), 3.6 (lane 2) and 12 (lane 3).

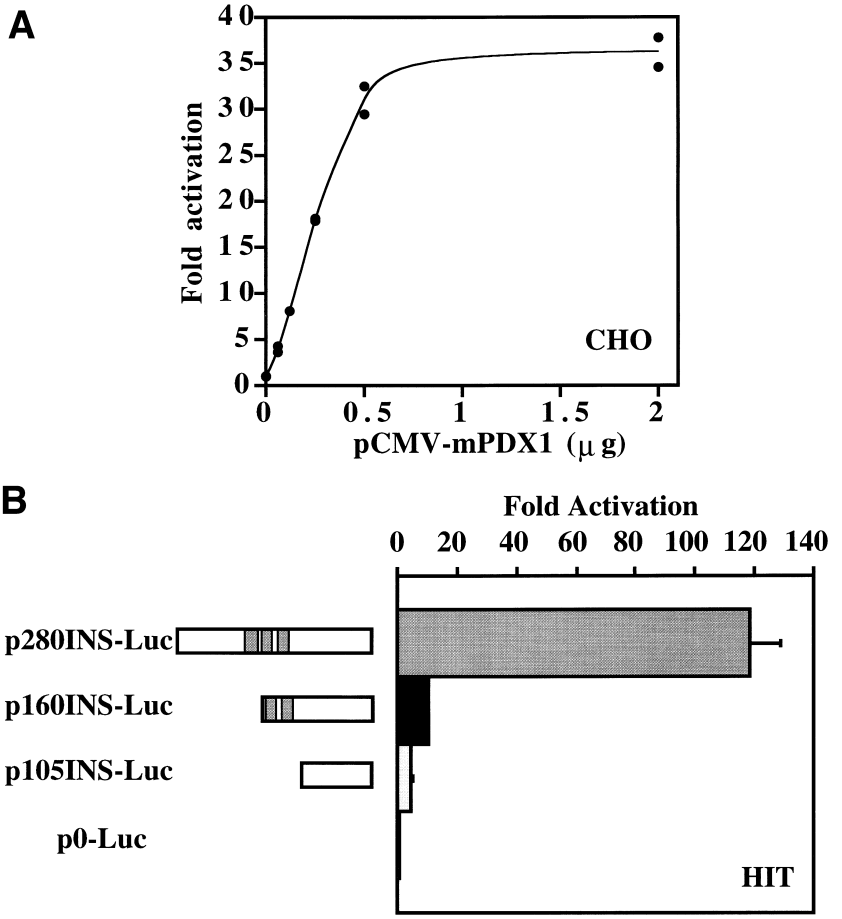

Fig. 4. A: Mouse PDX1 activates the tINS promoter. CHO cells (5×10^5) were cotransfected with p280INS-Luc (5 μg) and increasing amounts (0, 0.06, 0.12, 0.25, 0.5 and 2 μg) of pCMV-mPDX1 (encoding mouse PDX1); the total amount of transfected DNA was kept constant by addition of empty pCMV vector. Values from two independent transfections are shown. B: Deletion analysis of the tINS promoter in β cells. HIT β cells (10^6) were transfected with 7 μg of the indicated reporter plasmids. Data are the mean ± S.E.M. of three independent transfections. The presence and position of the three footprints obtained with recombinant mouse PDX1 (see Figs. 1 and 3) are schematically depicted on the left.

imally expressed in several higher teleosts in which islet cells are concentrated exclusively into one or a few Brockmann bodies composed mainly of β cells and receiving only minor contributions from the exocrine pancreas [17]. Thus, tissue heterogeneity is minimized in higher teleosts, a fact which may reflect more straightforward mechanisms for islet formation. This would make teleosts an attractive experimental model for developmental studies on the endocrine pancreas. Furthermore, in zebrafish, large-scale genetic screens have been initiated which have the potential to lead to identification of developmental control genes [29]. On the other hand, the diffuse nature of the acinous component in bony fish as opposed to the compact organ found in tetrapods would make the latter a better alternative to investigate the morphogenesis of the exocrine pancreas.

There has been controversy concerning the embryological origin and lineage relationships of the cells comprising the mammalian endocrine pancreas. Since pancreatic islet cells express a number of proteins characteristic of nervous tissue, it has been suggested that these cells originate from the neural crest, in contrast to the exocrine cells which are clearly derived from the gut endoderm [1,30]. Several studies are inconsistent with this neural crest hypothesis and it is currently believed that both endocrine and exocrine cells derive from the endoderm [30]. However, the lineage relationships among the endocrine cells remain unclear. On the basis of co-expression of islet hormones early in development, it has been proposed that all endocrine cells derive from a pluripotent stem cell: progressive differentiation of these cells would lead to a gradual restriction of the phenotype culminating in the mature cell which is capable of producing only its characteristic hormone [31,32]. On the other hand, extensive co-expression has not been observed by other workers [33,34] and, furthermore, results of cell ablation experiments using diphtheria toxin transgenes do not support the common endocrine stem cell idea [35]. Interestingly, four zebrafish mutants were recently identified, which showed almost complete loss of pancreatic exocrine cells but little or no effect on islet cells [28].

In recent years, much effort has been devoted to understanding the molecular mechanisms regulating the cell-specific expression of pancreatic genes in mammals. In particular, a number of transcription factors which play a key role in directing β cell-specific transcription of the insulin gene have been described. These include the homeodomain protein, PDX1 [7–9], and the bHLH proteins BETA2 [10,11] and E2A [13,14]. Our experiments strongly indicate that PDX1 plays an equally important role in fish insulin gene expression: not only does mouse PDX1 bind to regions of the promoter

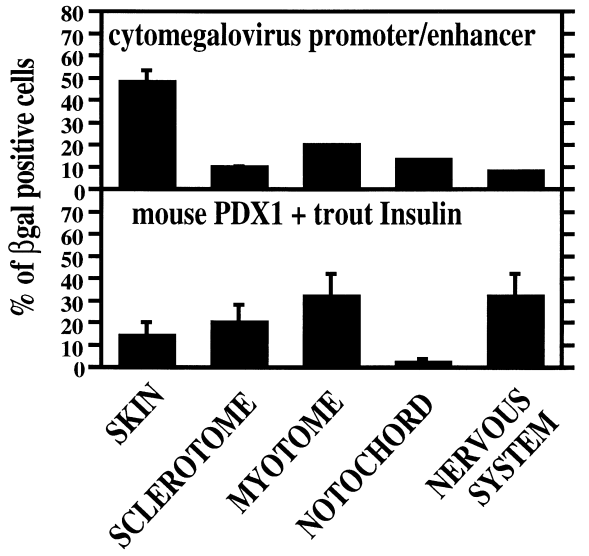

Fig. 5. Distribution of stained cells in embryos injected with pCMV-βgal (top) or a combination of pCMV-mPDX1 expression vector and p280INS-βgal reporter plasmid (bottom). Fertilized zebrafish eggs (1–2-cell stage) were injected with the indicated plasmids, and after 24 h the embryos without obvious malformations were processed to visualize β-galactosidase-positive cells. For each positive embryo, stained cells were counted and assigned to the five major groups according to the criteria established by Westerfield et al. [26], and the percentage of cells of each group was calculated. Results from a representative experiment are shown. Number of injected/surviving without malformations/positive embryos were: 58/29/23 (pCMV-βgal) and 57/25/11 (pCMV-mPDX1+p280INS-βgal). Data obtained from 864 cells in the 23 positive embryos injected with pCMV-βgal and 64 cells in the 11 positive embryos injected with pCMV-mPDX1+p280INS-βgal are the mean ± S.E.M. for each cell category.

important for expression in mammalian β cells, but ectopic expression of PDX1 in mammalian or fish cells leads to activation of a co-transfected tINS promoter plasmid. Under the conditions of our experiments, in the absence of PDX1, tINS shows no activity in 24-h embryos, consistent with the absence of differentiated pancreatic cells at this stage [28]. Further experiments will be required, focusing on later time points, to demonstrate activity in developing pancreatic cells. Because of the mosaic expression patterns of injected DNA as well as its dilution and degradation, larger numbers of embryos will be required for this. Alternatively, stably transformed lines of zebrafish may be used [36].

Despite the lack of extensive sequence similarity between rodent and trout insulin promoters, our results demonstrate that they share common regulatory elements with similar functional properties, a fact reminiscent of similar observations on mammalian and piscine GH and prolactin promoters [19,27]. This indicates that there may well exist many functional parallelisms in β cell development between fish and mammals, and thus information from the fish system may assist in dissecting the developmental programs operating in mammals. This is especially true since embryos of the most important fish models are completely transparent and suitable for analysis of whole-mount preparations. On the other hand, some differences are also evident: for example, our results to date show no evidence for an involvement of bHLH factors in tINS promoter activity (data not shown). It remains to be seen whether functional E box *cis* elements are located elsewhere in the tINS gene. Further studies on fish insulin gene expression and the relevant transcription factors, including PDX1, may significantly increase our understanding of pancreas development.

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