XIHbox 8, An Endoderm-Specific *Xenopus* Homeodomain Protein, Is Closely Related to a Mammalian Insulin Gene Transcription Factor

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The *cis*-acting sequences that mediate insulin gene expression exclusively in pancreatic islet β-cells are localized within the 5′-flanking region between nucleotides −340 and −91. We have identified an evolutionarily conserved, A+T-rich element at −201/−196 basepairs in the rat insulin II gene that is essential for efficient expression in β-cells. Affinity-purified antibody to the XIHbox 8 protein supershifted the major β-cell-activator factor complex binding to the −201/−196 element. XIHbox 8 is a *Xenopus* endoderm-specific homeodomain protein whose expression is restricted to the nucleus of endodermal cells of the duodenum and developing pancreas. Antibody to XIHbox 8 specifically interacts with a 47-kilodalton protein present in this DNA complex. Immunohistochemical studies revealed XIHbox 8-like proteins within the nucleus of almost all mouse islet β-cells and a subset of islet α- and δ-cells. These results are consistent with the proposal that an XIHbox 8-related homeoprotein of 47 kilodalton is required for expression of the mammalian insulin gene in β-cells. Experiments conducted with antiserum raised to somatostatin transcription factor-1 (STF-1), a recently isolated mammalian XIHbox 8-related homeoprotein, indicate that the STF-1 protein is the mammalian homolog of *Xenopus* XIHbox 8. (Molecular Endocrinology 8: 806–816, 1994)

INTRODUCTION

In adult mammals, the insulin gene is transcribed only in the β-cell of the pancreatic islet of Langerhans. The islet also contains three other phenotypically distinct cells, each of which expresses a unique pancreatic polypeptide hormone. The expression of the cell-specific products from islet α-cells (glucagon), β-cells (insulin), δ-cells (somatostatin), and pancreatic polypeptide cells is tightly regulated during organogenesis. Islet precursor cells are of endodermal origin. Each of these islet cell types appears to be derived from a common multipotent endodermal progenitor cell (1–4).

The factors that control cell determination and differentiation of pancreatic precursor cells into specific islet cell types during embryogenesis are unknown. In general, gene transcription is regulated by the binding of a specific set of positive and/or negative *trans*-acting protein factors to specific *cis*-acting control elements within the 5′-flanking region of the gene (5–7). Cell type-specific transcription of the insulin gene is principally regulated by factors interacting within the insulin enhancer region, which is located between nucleotides −340 and −91 relative to the transcription start site (8). There appear to be a number of distinct DNA sequence elements within the insulin enhancer that are important for expression in β-cells (9–11).

A key element of insulin enhancer-stimulated activity is the insulin control element (ICE) (10, 12, 13), whose core motif 5′-GCCATCTG-3′ is found within the transcription unit of all characterized insulin genes (14). Detailed characterization of the rat insulin-I gene indicates that transcriptional control mediated by this enhancer is also regulated by two mutationally sensitive A+T-rich elements (12, 15): Flat-F (−222 to −215) and Flat-E (−213 to −208). [Rats have two nonallelic insulin genes (termed rat I and rat II) which are almost equally transcribed in rat pancreas β-cells and are not expressed in the other pancreatic cell types (16).] However, only the sequences of the Flat-E element are conserved within the homologous region of other mammalian insulin genes (14). A third conserved A/T-rich element is found within the promoter region of the

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insulin gene, termed the insulin promoter factor-1 element [IPF-1; −81 to −77 basespairs (bp)] (17). The protein-DNA complexes formed with the Flat-E and IPF-1 elements in β-cell extracts have been shown by binding site competition analysis to interact with closely related factors (18).

To understand the role of the conserved Flat-E and IPF-1 elements in expression of the rat insulin-II gene, we have examined the effects of mutations within these elements on expression in pancreatic α- and β-cell lines. This analysis demonstrated that mutations within the Flat-E element greatly reduced the expression of various insulin enhancer/promoter constructs in β-cells. In contrast, mutations within the IPF-1 element had little effect on gene activity. A common β-cell-factor-DNA complex was detected in the gel mobility shift assay with the Flat-E and IPF-1 elements. Interestingly, we found that antibodies to the *Xenopus* XIHbox 8 homeoprotein supershifted both the Flat-E and IPF-1 protein-DNA complexes. Previous studies in *Xenopus laevis* indicated that the XIHbox 8 protein is selectively expressed in cells of the pancreas, duodenum, and anterior midgut, suggesting that it is involved in region-specific endodermal cell differentiation (19). The XIHbox 8 antibody specifically detects a 47-kilodalton (kDa) protein present in the mobility shift complex formed with both the Flat-E and IPF-1 elements. Nuclear staining with XIHbox 8 antibody was also detected within the vast majority of mouse islet β-cells and a fraction of the α- and δ-cells. Our results suggest that the mammalian 47-kDa XIHbox 8-related protein plays an important role in mediating pancreatic β-cell-specific transcription of the insulin gene. While this work was being prepared for publication, an XIHbox 8-related mammalian transcription factor was independently isolated in two laboratories from rats [somatostatin transcription factor-1 (STF-1)] (20) and mice (IPF-1) (21). The XIHbox 8 protein is strikingly similar to IPF-1/STF-1 in the N-terminal and homeodomain regions. Antiserum to the STF-1 protein specifically blocked binding of the 47-kDa XIHbox 8-related protein to Flat-E element sequences. We conclude that the mammalian STF-1/IPF-1 transcription factor is the XIHbox 8-related transcription factor required for transcription of the insulin gene in β-cells.

RESULTS

The Flat-E Element Is Required for Activation by the Rat Insulin-II Enhancer

To determine whether the Flat-E (−201 to −196 bp) and IPF-1 (−81 to −77 bp) elements in the 5′-flanking region of the rat insulin-II gene were important in transcriptional control, basepairs within each element were changed by transversion mutation to disrupt their A+T-rich composition. Two insulin-producing cell lines, HIT T15 2.2.2 (HIT) and βTC-3, and a noninsulin-producing islet α-cell line, αTC-6, were cotransfected with wild-type or mutant insulin enhancer expression plasmids and an internal control expression plasmid, pSVCAT. Mutations within the Flat-E element in either the −322 or −238 expression constructs reduced their activity relative to that of the wild-type plasmids in transfected β-cells (Fig. 1). The reduction in Flat-E-dependent activity was greater in transfected βTC-3 than HIT cells. Previously, Crowe and Tsai (10) had shown that a Flat-E mutation had only a modest effect on rat insulin-II enhancer-mediated expression in transfected HIT cells. It is unclear why βTC-3 cells respond so differently to this mutation, because the IPF-1 element mutation had only a small effect on expression in both of these β-cell lines. In addition, mutating these A/T-rich elements within either the −322 or −238 wild-type plasmid had very little effect on their activity in transfected αTC-6 cells.

The overall activity of these wild-type insulin enhancer constructs is much higher in transfected HIT and βTC-3 cells than in αTC-6 cells (Fig. 1). The reduced expression of these constructs in αTC-6 cells appears to result from the absence of key positive insulin gene transcription factors (22). These results indicate that the Flat-E element is a site of positive control within the rat insulin-II gene, whereas the IPF-1 element is much less important. Furthermore, they indicate that the activator of Flat-E element-mediated expression is distributed (or active) in β-cells, but not α-cells.

β-Cell Factors Bind to the Flat-E Element

The gel mobility shift assay was used to determine the distribution of the cellular factors interacting with the Flat-E element. Protein extracts were prepared from insulin-producing βTC-3 and HIT cells and the noninsulin-producing αTC-6 cell line. Binding reactions were conducted in the presence of extract and a ^{32}P-labeled Flat-E probe corresponding to rat insulin-II gene sequences from −213 to −192. Several protein-DNA complexes were detected in these extracts (Fig. 2A). The specificity of binding of these proteins to the Flat-E element probe was determined in competition assays in which wild-type and mutant alleles of the Flat-E element and the wild-type IPF-1 element were used as competitors (Fig. 2B). The same Flat-E mutant element that reduced insulin gene transcription *in vivo* was used. The pattern of competition with the various versions of these A+T-rich elements indicates that there are two specific protein-Flat-E element complexes, in that their formation was prevented by nonradioactive wild-type Flat-E sequences, but not by the mutant Flat-E competitor. These specific complexes are referred to as A and B in Fig. 2. Neither the A nor the B complex was detected in extracts prepared from αTC-6 cells (Fig. 2A, lane 1) or a number of nonislet cell lines, including HeLa, NIH 3T3, and At T20 (data not shown). Thus, our results indicated that the factors binding to these complexes may only be present in β-cells.

The B complex was the predominant specific protein-Flat E element complex formed in β-cell extracts (Fig.

Reporter Plasmid		βTC-3			HIT T-15			αTC-6		
		Activity	(n)	Relative Activity	Activity	(n)	Relative Activity	Activity	(n)	Relative Activity
-322 WT	LUC	102,857 +/- 25,714	(4)	1.00	92,638 +/- 13,896	(5)	1.00	39,233 +/- 12,162	(3)	1.00
-322 E-MT	LUC	7,437+/- 2,311	(4)	0.07	30,570 +/- 4,891	(5)	0.33	22,362 +/- 9,827	(3)	0.57
-322 I-MT	LUC	47,314 +/- 6,897	(4)	0.46	51,877 +/- 10,375	(5)	0.56	44,725 +/- 15,653	(3)	1.14
-238 WT	LUC	37,044 +/- 8,348	(4)	1.00	66,315 +/- 10,936	(6)	1.00	22,776 +/- 7,694	(3)	1.00
-238 E-MT	LUC	8,601 +/- 1,702	(4)	0.23	27,852+/- 4,556	(5)	0.42	27,787+/- 7,947	(3)	1.22
-238 I-MT	LUC	26,181 +/- 7,330	(3)	0.69	51,725 +/- 10,448	(3)	0.78	28,836 +/- 8,650	(3)	1.27

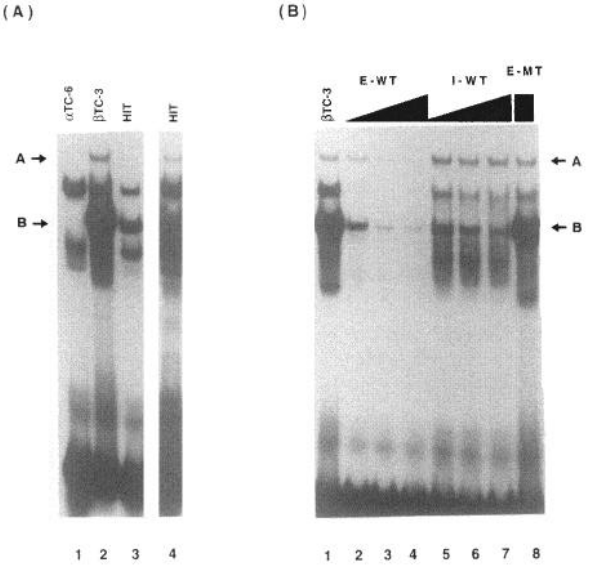

Fig. 2. Binding of the α- and β-Cell Nuclear Proteins to Flat-E Element Sequences

A, Equal concentrations of αTC-6 (lane 1), βTC-3 (lane 2), and HIT T15 (lanes 3 and 4) protein extract (10 μg) were analyzed for Flat-E binding. The gel in lanes 1–3 was exposed to film for 18 h; lane 4 represents a 72-h exposure of the lane 3 sample to make the presence of the A complex more apparent. The complex that runs between A and B is not specific, as its levels vary considerably between extract preparations. For example, note in Fig. 3B that this complex was not detected in this βTC-3 extract. B, Binding reactions were conducted with the Flat-E probe with βTC-3 extract either alone (lane 1) or in the presence of a molar excess of 50-fold (lanes 2 and 5), 150-fold (lanes 3 and 6), or 300-fold (lanes 4, 7, and 8) of the unlabeled competitor to probe. The wild-type Flat-E, wild-type IPF-1, and mutant Flat-E competitors are referred to as E-WT, I-WT, and E-MT, respectively. The positions of the A and B complexes discussed in the text are labeled.

2A). This complex was competed more effectively than the A complex with the Flat-E wild-type competitor (Fig. 2B). In addition, the wild-type IPF-1 element only competed for formation of the B complex (Fig. 2B), which

Fig. 1. Functional Analysis of the A+T-Rich Elements in Rat Insulin-II Gene Expression

Depiction of the wild-type, Flat-E, and IPF-1 mutant rat insulin-II −322 and −238-LUC constructs. ▨, The enhancer region, which spans sequences from −340 to −91, within each construct. ■, The site of the Flat-E (−201 to −196 bp) and IPF-1 (−81 to −77 bp) mutations. Wild-type (WT), mutated Flat-E element (E-MT), and mutated IPF-1 element (I-MT) insulin enhancer/promoter sequences were fused to the LUC reporter gene. Each mutant is named according to the 5′-end point of the rat insulin-II gene sequences; the 3′-end is at 2 bp. The normalized activities of the insulin-LUC constructs in βTC-3, HIT, and αTC-6 cells are the mean ± SD. The relative activity is expressed as the ratio of the luciferase activity with the mutant construct divided by the level of wild-type luciferase activity. (n) indicates the number of times each transfection was repeated.

was also the only specific complex detected with a labeled IPF-1 probe (Fig. 3B, compare lane 8 with lane 1). These results suggest that the B complex formed with both the Flat-E and IPF-1 elements is composed of a similar β-cell factor(s). As the mutant form of the Flat-E element substantially reduced transcription *in vivo* and was not an efficient competitor of the B complex *in vitro*, the factors in this complex appears to mediate Flat-E element-dependent transcription.

An XIHbox 8-Related Protein Is a Component of the B Complex

Many homeodomain-containing transcription factors are key developmental regulators involved in specifying organ formation and cell phenotypes (23). Importantly, the structure and DNA-binding properties of homeodomain-containing factors appear to be evolutionarily conserved (24). As the consensus binding site recognition sequences for homeoproteins is A/T rich (25), we asked whether a *Xenopus* XIHbox 8 homeodomain-related protein is present in the Flat-E complexes formed in mammalian βTC-3 and HIT cell extracts. We thought that an XIHbox 8-like protein might be a transcription factor of the mammalian insulin gene because the expression of this protein is restricted during *Xenopus laevis* development to endodermal cells that will develop into pancreas and closely associated organs (19).

To examine whether XIHbox 8-like proteins are present within the A and/or B complexes formed with the Flat-E element, we tested the effect of adding affinity-purified antibodies against the N- or C-terminal region of the XIHbox 8 protein. The N-terminal polypeptide antiserum was raised to amino acids 1–75 of XIHbox 8 and does not contain any sequences corresponding to the homeodomain, which is located near the center of the protein. However, a portion of this region was used to prepare antibody to the C-terminal region (amino acids 181–271; Fig. 3A). An affinity-purified antibody to an unrelated murine mesoderm-specific homeoprotein, Mox-1 (26), was used as a negative control. Mox-1 did not affect the formation of the Flat-E-protein complexes (Fig. 3B, compare lane 1 with lane 5). However, the B complex was supershifted with the N-terminal XIHbox 8 antibody (Fig. 3B, compare lanes 2 and 3 with lane 1). This is a specific reaction, as the amounts of the A complex and the other nonspecific complexes are not reduced by this antibody. Furthermore, the N-terminal XIHbox 8 polypeptide blocked the ability of the N-terminal XIHbox 8 antibody to supershift the B complex (Fig. 3B, compare lanes 6 and 7 with lane 2). In contrast, the C-terminal XIHbox 8 antibody does not affect B complex formation (Fig. 3B, compare lane 4 with lane 1). The N-terminal XIHbox 8 antibody also supershifted the B complex formed with an IPF-1 element probe (Fig. 3B, lane 9). It thus appears that a *trans*-activator of Flat-E- and IPF-1-mediated activity is a protein(s) antigenically related to the N-terminal region of the *Xenopus* XIHbox 8 protein.

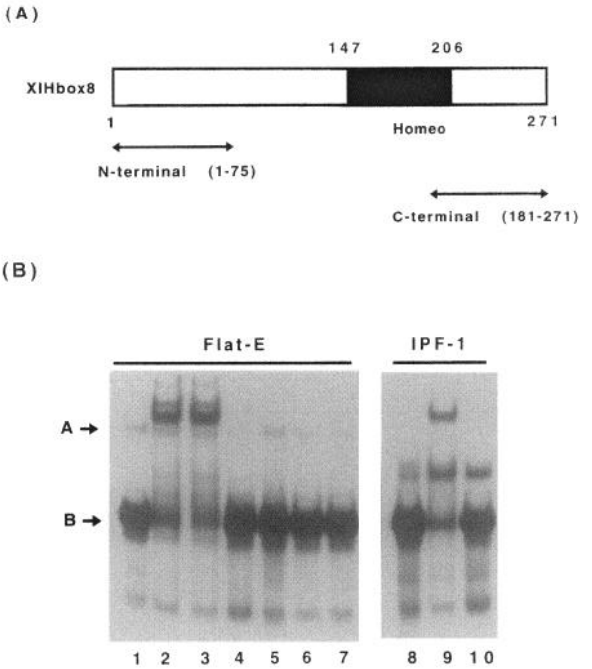

Fig. 3. An Antibody to the N-Terminal Region of XIHbox 8 Protein Inhibits Formation of a β-Cell-Flat E Complex

A, Diagrammatic representation of the XIHbox 8 protein shows the position of the homeodomain region (amino acids 147–206). The N- and C-terminal antibodies were raised to amino acids 1–75 and 181–271, respectively. B, Binding and gel electrophoresis were conducted with Flat-E (lanes 1–7) and IPF-1 (lanes 8–10) element probes with βTC-3 extracts (10 μg). The major bands discussed in the text are labeled. Lane 1, Control; lane 2, plus N-terminal XIHbox 8 antibody (5 μl); lane 3, plus N-terminal XIHbox 8 antibody (6 μl); lane 4, plus C-terminal XIHbox 8 antibody (6 μl); lane 5, plus Mox-1 antibody (5 μl); lane 6, plus N-terminal XIHbox 8 antibody (5 μl) and N-terminal XIHbox peptide (1–75; 1 μl); lane 7, plus N-terminal XIHbox 8 antibody (5 μl) and N-terminal XIHbox 8 peptide (2 μl); lane 8, control; 2, plus N-terminal XIHbox 8 antibody (5 μl); lane 9, plus N-terminal XIHbox 8 antibody (6 μl); lane 10, plus C-terminal XIHbox 8 antibody (6 μl).

The B Complex Contains a 47-kDa XIHbox 8-Related Protein

To identify the XIHbox 8-like polypeptides in cells, the antibodies to the N- and C-terminus of XIHbox 8 were used in Western blot analysis with extracts prepared from HIT, βTC-3, and αTC-6 cells. The presence of a 47-kDa protein was detected with the N-terminal antibody in both insulin-producing β-cell lines, βTC-3 and HIT (Fig. 4). Another XIHbox 8-related protein of approximately 39 kDa was also observed in βTC-3 nuclear extracts, although its levels varied between extract preparations. αTC-6 cells do not appear to express the 47- or 39-kDa proteins, which is consistent with our inability to detect the B complex with αTC-6 cell extracts (Fig. 2A, lane 1). The interaction of the N-terminal antibody with the 47- and 39-kDa proteins in βTC-3 cells is specific, as the antibody did not bind to these

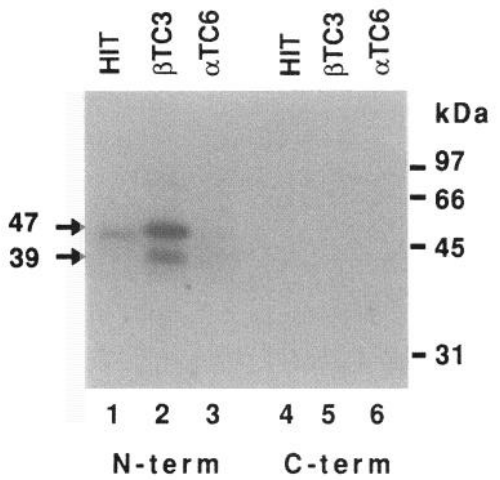

Fig. 4. Western Blot Analysis for XIHbox 8-Related Proteins in βTC-3, HIT, and αTC-6 Cells

Each lane contains 30 μg extract protein. The blot was probed with the antibodies raised to the N-terminal (lanes 1–3) or C-terminal (lanes 4–6) region of the XIHbox 8 protein. The positions of mol wt markers (kilodaltons) and the 47- and 39-kDa XIHbox 8-related proteins are indicated. Lanes 1 and 4, HIT; lanes 2 and 5, βTC-3; lanes 3 and 6, αTC-6.

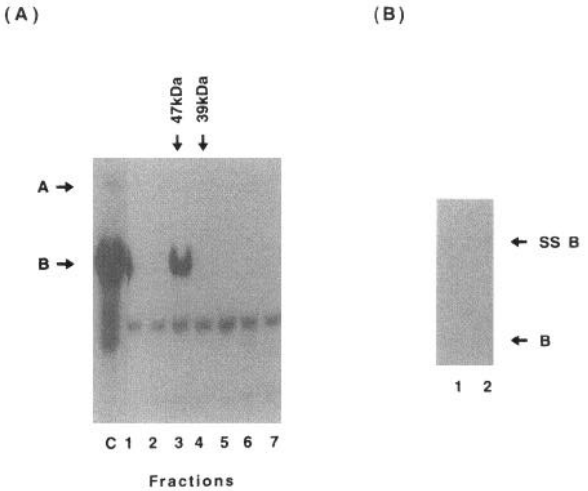

Fig. 5. The B Complex Contains the 47-kDa XIHbox 8 Protein

A, The βTC-3 nuclear extracts (30 μg) were electrotransferred from an SDS-polyacrylamide gel onto an Immobilon PVDF membrane. The proteins were eluted from membrane slices and assayed for Flat-E-binding activity. Each fraction (no. 1–7) represents a different mol wt range. The presence of the 47- and 39-kDa XIHbox 8-related proteins were detected in fractions 3 and 4, respectively, by Western blot analysis with anti-XIHbox 8 N-terminal antiserum. Lane C, βTC-3 nuclear extract alone. The positions of the A and B complexes are indicated. B, Fraction 3 was analyzed for Flat-E binding in the absence (lane 1) or presence (lane 2) of the N-terminal XIHbox 8 antiserum. Note that the B complex is supershifted (SS B) upon addition of the XIHbox 8 antiserum.

proteins after preincubation with the XIHbox 8 N-terminal polypeptide (data not shown). In contrast, no XIHbox 8-related protein products were detected in these extracts with the C-terminal XIHbox 8 antiserum (Fig. 4). As the N-terminal XIHbox 8 antibody also supershifted the B complex in HIT extracts (data not shown), this indicates that this complex also contains the 47-kDa XIHbox 8-related protein. These results corroborate previous studies demonstrating a protein of approximately 50 kDa in the Flat-E element complex from HIT cells (27, 28).

To determine directly whether the B complex contained the 47-kDa XIHbox-8 protein, the proteins from βTC-3 nuclear extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immobilon polyvinylidene fluoride (PVDF) membrane, which was then cut into slices, and the fractionated proteins were eluted. The Flat-E-binding activity of these proteins is shown in Fig. 5A. Binding activity colocalized in fraction 3 with proteins of approximately 50 kDa. XIHbox 8 antibody immunoreactivity to a 47-kDa protein was also detected by Western blot analysis in fraction 3. However, no Flat-E-binding activity was detected in the fraction that contained the 39-kDa protein (Fig. 5A, lane 4). Importantly, we found that the Flat-E binding complex formed with the fraction 3 proteins was supershifted with the N-terminal XIHbox 8 antibody (Fig. 5B). The factor(s) in this fraction also binds to the IPF-1 element (data not shown). These results strongly indicate that the B complex formed with the Flat-E and IPF-1 elements is composed at least in part of the 47-kDa XIHbox 8-like protein.

The 47-kDa XIHbox 8-Related Protein Corresponds to STF-1/IPF-1

Recently, cDNAs encoding two closely related XIHbox 8-like homeoproteins that activate transcription from A/T-rich elements in the somatostatin and insulin genes, respectively, were isolated from rat (STF-1) (20) and mouse (IPF-1) (21) islet cell lines. STF-1 and IPF-1 proteins are almost 95% identical, with the remaining differences represented by conservative amino acid substitutions. The homeodomains of IPF-1, STF-1, and XIHbox 8 are 100% identical (Fig. 6). Importantly, there is over 67% sequence similarity (52% identity) between amino acids 1–75 of XIHbox 8, the region used to make the N-terminal XIHbox 8 antibodies, and the N-terminal regions of IPF-1/STF-1 (Fig. 6). The molecular mass of reticulocyte lysate-translated STF-1 is also approximately the same molecular mass on SDS-polyacrylamide gels as the 47-kDa XIHbox 8-related protein (20).

To test directly whether the 47-kDa XIHbox 8-related protein present within the Flat-E element gel shift complex was IPF-1/STF-1, we used a polyclonal STF-1 antiserum in our gel shift reactions. The STF-1 antiserum was raised against a synthetic peptide corresponding to amino acids 196–214, comprising the last nine amino acids of the homeodomain region (including part of the DNA-binding helix-3), and has been shown to prevent the binding of STF-1 to an A/T-rich element

Fig. 6. Comparison of the XIHbox 8, STF-1, and IPF-1 Proteins

The N-terminal, C-terminal, and homeodomain regions within the XIHbox 8, STF-1, and IPF-1 proteins are aligned (19–21). The sequences within the N- and C-terminal regions of XIHbox 8 span those used in generating the XIHbox 8 antiserum. Several gaps have been introduced in the sequence to maximize the alignment. A *line* represents identity, and conservative changes are marked with *double dots*. The XIHbox 8, STF-1, and IPF1 homeodomains share 100% amino acid identity. The N-terminal regions share 52% sequence identity and 67% sequence similarity, allowing conservative substitutions. Note that the N-terminal similarity falls into several patches of between 10–20 amino acids, which share 80–95% similarity. The C-terminal region, which includes part of the homeodomain, shares 55% sequence identity and 59% sequence similarity with IPF-1/STF-1. The C-terminal sequences from 206–271, which excludes homeodomain amino acids (181–206), has a 37% sequence identity and 43% sequence similarity with the corresponding region of IPF-1/STF-1.

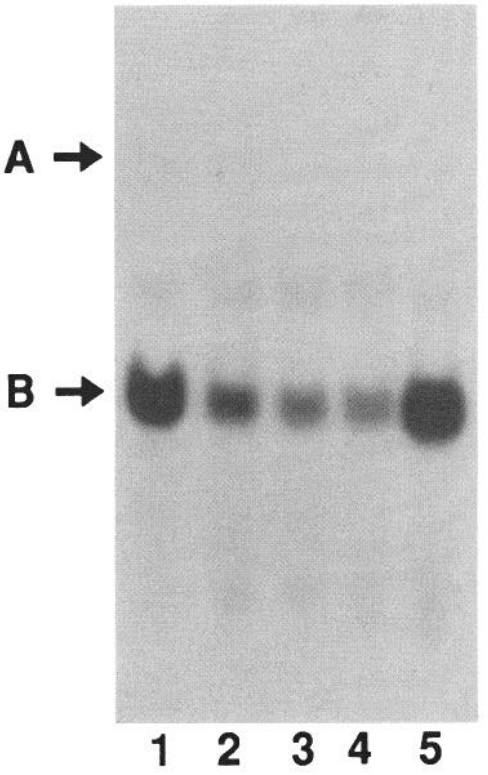

Fig. 7. STF1 Antibody Reduces the Formation of Flat E Complex in βTC-3 Cells

Binding reactions were carried out with Flat E probe and 3 μg of βTC-3 extracts. Extracts were incubated for 10 min at room temperature with STF-1 antibody before the addition of probe. The STF-1 antibody was raised to a synthetic peptide corresponding to amino acids 196–214. The labeled bands are discussed in the text. Lane 1, Control; lane 2, plus 4 μl STF-1 antibody; lane 3, plus 5 μl STF-1 antibody; lane 4, plus 6 μl STF-1 antibody; lane 5, plus 6 μl preimmune serum.

in the somatostatin gene (20). We found that increasing concentrations of STF-1 antiserum reduced the amount of the βTC-3 cell complex containing the 47-kDa XIHbox 8-related protein (Fig. 7), whereas the preimmune serum had no effect (Fig. 7). We conclude that the 47-kDa XIHbox 8-related protein in β-cells corresponds to IPF-1/STF-1.

Mammalian XIHbox 8-Related Proteins Are Localized within the Nucleus of Pancreatic Islet β-Cells

The presence and localization of XIHbox 8-like proteins were examined immunohistochemically in mouse and frog adult pancreas sections, and their distribution compared to that of glucagon (α), insulin (β), and somatostatin (δ) cells of pancreatic islets. In mouse, staining with the affinity-purified antibody to the N-terminal region of XIHbox 8 was found in the nuclei of the majority of cells in the core of the islet (Fig. 8A). XIHbox 8 protein immunoreactivity was also seen in the nuclei of cells at the periphery of the islet, the primary location of the endocrine non-β-cells in adult mouse pancreas (29), indicating that XIHbox 8-related proteins are expressed in other non-β-islet cell types. However, no staining was detected in acinar cells surrounding the islet. Double label immunohistochemistry revealed that XIHbox 8 protein was expressed primarily in β-cells, but was also detected in a subset of α- and δ-cells (Fig. 8, C and D). Thus, over 91% of insulin-producing β-cells contained XIHbox 8 immunoreactivity (Fig. 8B), whereas 3% of α-cells and 15% of δ-cells were positive (Table 1). XIHbox 8 immunoreactivity was not detected in mouse pancreas with antiserum to the C-terminal region of XIHbox 8 (data not shown). In contrast to mice, XIHbox 8 protein expression in adult frog pancreas was detected in the nucleus of both acinar and islet cells (Fig. 9), with an identical pattern for the C-terminal antibody (Fig. 9) and the N-terminal antibody (data not shown).

The immunohistochemical results with mouse islets are consistent with the gel shift results described in Figs. 3 and 5 regarding distribution of the Flat-E and IPF-1 element-binding proteins. These results demonstrate that the mouse 47-kDa XIHbox 8-related protein

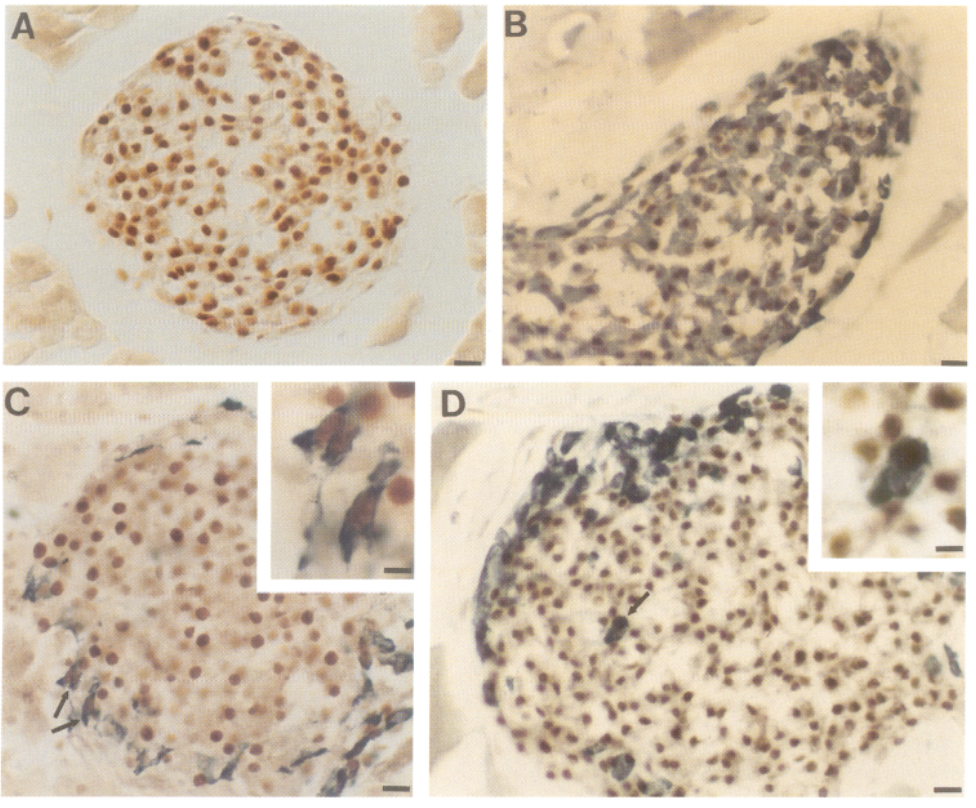

Fig. 8. XIHbox 8-Related Proteins Are Found within the Nucleus of Mouse Islet β-Cells

A, Photomicrograph illustrates immunohistochemical localization of XIHbox 8 in islet cells of adult mouse pancreas. *Bar* = 20 μm. B, Double label immunohistochemical localization of insulin (blue) and XIHbox 8 (brown). Note that most β-cells express XIHbox 8 in their nuclei. *Bar* = 20 μm. C, Immunocytochemical localization of XIHbox 8 (brown) and glucagon (blue). The cell indicated by the *arrow* contains both XIHbox 8 and glucagon. Most α-cells, however, do not express XIHbox 8 protein. *Inset*, High magnification photomicrograph of cell indicated with the *arrow* in C illustrates the presence of XIHbox 8 in the nucleus (labeled with DAB) and glucagon immunoreactivity (blue) in the cytoplasm. *Bar*: *left*, 20 μm; *right*, 4 μm. D, Immunocytochemical localization of XIHbox 8 (visualized with DAB) and somatostatin (blue reaction product). *Inset*, Double labeled cells indicated by *arrows* in the low magnification photomicrograph (*left*) are shown with higher magnification on the *right*. *Bar*: *left*, 20 μm; *right*, 4 μm.

Table 1. Coexpression of XIHbox 8-Related Antigens and Islet Hormones in Mouse Pancreas

XIHbox 8/Insulin	XIHbox 8/Somatostatin	XIHbox 8/Glucagon
91.6 ± 1.2	15.2 ± 1.2	2.68 ± 0.09

Four pancreata were processed for each antigen combination. Four separate counts of at least 1000 β-, γ-, and α-cells were counted at ×1000 magnification with a Nikon microscope under Nomarski optics. The number of cells expressing XIHbox 8 and each hormone is expressed as the mean percentage ±SD of the cells immunoreactive to the hormone.

is an islet nuclear protein present in most, if not all, islet β-cells and a subset of α- and δ-cells.

DISCUSSION

Transcription of genes in eukaryotes is a result of interactions between transcriptional activator proteins bound to DNA and the general transcription factors assembled on the promoter. The interactions between activator proteins bound within the enhancer control region and the transcriptional machinery within the promoter region result from DNA looping, allowing these factors to contact each other (30). A major objective of many studies is to identify and define the molecular mechanisms by which factors cooperate to regulate cell type-specific expression. On the basis of molecular genetic analysis of the insulin gene, it appears that a key control element involved in regulating pancreatic β-cell-type transcription is the ICE, which is found within the enhancer region of all mammalian insulin genes. However, it is also clear that the actions of other transcription factors are necessary for insulin enhancer-mediated activation. Here, we have shown that the evolutionarily conserved A/T-rich Flat-E enhancer element is important in regulating expression of the rat insulin-II gene. A β-cell protein of 47 kDa was shown to specifically interact with the Flat-E element. This protein was found to be antigenically related to XIHbox 8, a homeoprotein that appears to be important in *Xenopus* pancreatic determination (19). As antiserum to STF-1,

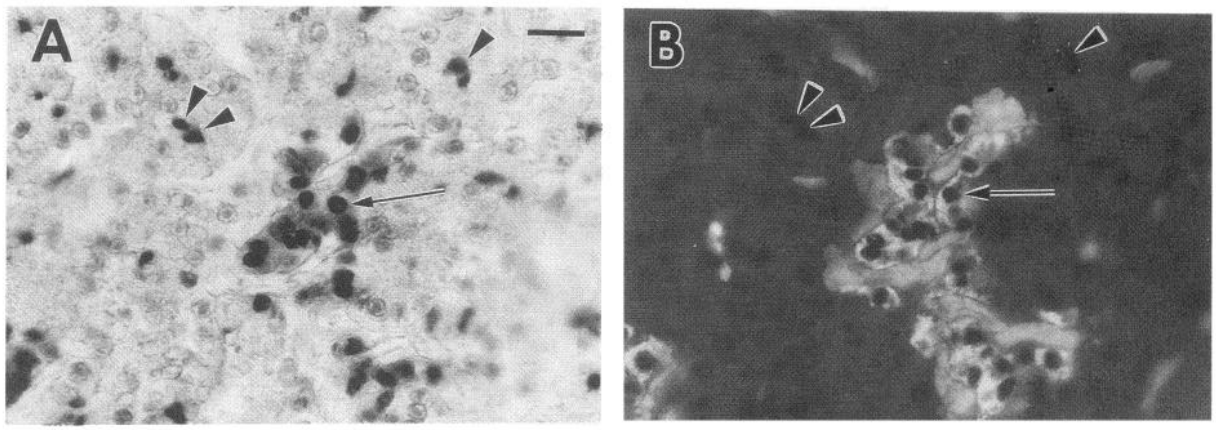

Fig. 9. XIHbox 8 Protein Is Found within the Nucleus of Frog Islet and Acinar Cells

A and B, The same paraffin section of adult frog pancreas was immunostained with the C-terminal XIHbox 8 antibody, detected using alkaline phosphatase-linked secondary antibody (A) and subsequently immunostained with insulin antibody, detected with fluorescein-linked secondary antibody (B). Note that the XIHbox 8 reactivity in A colocalizes to insulin-positive cells (*arrow*) as well as noninsulin-expressing cells (*arrowhead*). N- and C-terminal XIHbox 8 antibodies produced identical distribution of XIHbox 8 protein in adult frog pancreas. *Bar* = 25 μm.

a mammalian XIHbox 8-related protein, blocks the formation of the 47-kDa/Flat-E element complex, we conclude that IPF-1/STF-1 is the mammalian homolog of the *Xenopus* XIHbox 8 protein. These data coupled with the observation that anti-XIHbox 8-immunoreactive staining was present in the nucleus of islet β-cells in mice and frogs suggest that the IPF-1/STF-1 transcription factor is an important regulator of insulin gene expression *in vivo*.

The 47-kDa XIHbox 8-related protein was also found to bind to a conserved A/T-rich region within the insulin promoter region, termed the IPF-1 element (Fig. 3B). Mutations within the IPF-1 element that prevent binding of this 47-kDa protein marginally decrease both rat insulin-I (21) and rat insulin-II expression *in vivo*. Indeed, the IPF-1 transcription factor was cloned as a result of this observation (21). The data presented here show that mutations in the IPF-1 element have less effect on rat insulin-II enhancer/promoter activity than mutations in the Flat-E element (Fig. 1). Interestingly, the Flat-E mutations in βTC-3 cells reduced rat insulin-II enhancer-driven expression to the same extent as an ICE mutation (Fig. 1) (22). We believe that the sensitivity of the insulin enhancer to the ICE and Flat-E mutations reflects the multiple cooperative interactions that mediate the high degree of activity and specificity of this region. Presumably, binding of the 47-kDa IPF-1/STF-1 protein to the IPF-1 element does not play such a central role in facilitating the synergistic interactions between this XIHbox 8-related protein and the general transcription factors.

To determine whether XIHbox 8-related proteins were present in the Flat-E complexes in β-cells, we examined the effects of specific antibodies to the N- and C-terminal regions of the *Xenopus* XIHbox 8 protein on their formation. We found that only the N-terminal region antibodies, raised to a region of the protein that does not contain homeodomain sequences, supershifted the predominant B complex of the Flat-E and IPF-1 elements (Fig. 3B). In contrast, the C-terminal antibody did not influence formation of the Flat-E complexes. Importantly, the N-terminal antibody, but not the C-terminal antibody, also reacted positively in a Western blot analysis of β-cell extracts and immunohistochemically with islet β-cells *in vivo*. The inability of the C-terminal antibody to interact with the 47-kDa protein in gel shift assays (Figs. 2 and 3) or mouse pancreas sections (Fig. 8) presumably reflects the limited sequence conservation in this region of these homeoproteins (Fig. 6; 37% identity with the region of IPF-1/STF-1 C-terminal to the homeodomain). The ability of antiserum to STF-1, the XIHbox 8-related mammalian homeoprotein (20), to inhibit the binding of the 47-kDa IPF-1/STF-1 protein to the Flat-E element also supports this conclusion.

Our immunohistochemical studies indicate that the IPF-1/STF-1 transcription factor is present in the nuclei of the vast majority, and probably all, islet β-cells, and a subset of α- and δ-cells (Fig. 8 and Table 1). In contrast, Ohlsson *et al.* (21) reported a restriction of IPF-1 immunoreactivity to β-cells. The apparent discrepancy between our results and those of Ohlsson *et al.* (21) could be due to differences in treatment of the tissues before immunocytochemical staining. Our tissues were processed by paying particular attention to the recommendations of Brandzaeg (31) and Larsson (32), which are designed to maximize cell and tissue integrity and preserve the subcellular distribution of the appropriate antigens. The lack of cryoprotection and delay between tissue sectioning and fixation (21) might result in decreased sensitivity of the immunohistochemical technique and consequent underestimation of the number of glucagon/IPF-1 or somatostatin/IPF-1 double labeled cells.

The inability to detect STF-1/IPF-1 expression in most islet δ-cells may indicate that this factor is not a *bona fide* regulator of somatostatin transcription *in vivo*. However, our results and those of Ohlsson *et al.* (21) strongly indicate that the IPF-1/STF-1 transcription factor is a critical regulator of insulin gene transcription *in vivo*. A Flat-E-like binding factor of 46 kDa appears to be involved in regulating β-cell expression of the glucokinase gene (27). Therefore, it seems possible that IPF-1/STF-1 may also be necessary for the expression of this gene. The close sequence similarity between the XIHbox 8 and IPF-1/STF-1 proteins suggests that certain components of the transcriptional control mechanism regulating expression of these islet genes may be conserved between frogs and mice.

The XIHbox 8 protein is exclusively expressed in a narrow band of the endoderm in early *Xenopus* embryos (19). As development proceeds, XIHbox 8 proteins are restricted to endodermal cells of the duodenum and the developing pancreas. A similar pattern of expression has been observed for the XIHbox 8-related protein during mouse development (data not shown) (21). These results suggest that IPF-1/STF-1 are essential transcriptional determinants of endocrine cell-specific expression in the developing mammalian islet.

MATERIALS AND METHODS

DNA Constructs

The construction of the rat insulin-II gene enhancer/promoter luciferase expression plasmids (*i.e.* −322 WT and −238 WT) was previously described (22). Each plasmid is named according to the 5′-endpoint of the rat insulin-II gene sequences; the 3′-end point is at 2 bp. Mutations in the A/T-rich regions of the Flat-E (−214-CCTCTTAAGACTCGACTGACCCTAAGGC TAAGT-−182) and IPF-1 elements (−90-GATCCACCCTTCC TGGGACAAACAGC-−65) were created using an *in vitro* oligonucleotide mutagenesis kit (Amersham, Arlington Heights, IL). The underlined nucleotides are the sites of the transversion mutations (A to C and T to G). The sequences of all plasmids were confirmed by DNA sequencing.

Cell Culture and Transfections

The insulin-producing mouse islet βTC-3 (33) and the hamster insulinoma HIT T-15 2.2.2 (9) cell lines were grown in Dulbecco's Modified Eagle's Medium supplemented with 15% (vol/vol) horse serum, 2.5% (vol/vol) fetal bovine serum, and 50 μg each of streptomycin and penicillin per ml. The glucagon-producing mouse islet αTC-6 cell line (34) was grown in Dulbecco's Modified Eagle's Medium containing 10% (vol/vol) fetal bovine serum, streptomycin, and penicillin. The 5′-flanking rat insulin-II gene expression constructs were introduced into βTC-3 cells (10^7 cells/point) by electroporation using a Bethesda Research Laboratories Cell-porator (BRL, Gaithersburg, MD), as described previously (22). Fifty micrograms of the insulin test plasmid and 10 μg pSV2CAT were used per assay. Cells were harvested 40–48 h after transfection. The amount of extract used in the luciferase assay was normalized relative to chloramphenicol acetyltransferase activity. Luciferase and chloramphenicol acetyltransferase enzymatic assays were performed as described by De Wet *et al.* (35) and Nordeen *et al.* (36), respectively. Luciferase activity is defined in arbitrary relative light units. The background luciferase activity from the cloning vector, pSV0ARPL2L (37), was approximately 125 relative light units. Each experiment was repeated several times with at least two different plasmid preparations.

Electrophoretic Mobility Shift Assays

Double stranded ^{32}P-labeled oligodeoxynucleotide probes (50,000 cpm/ng; 0.5 ng) corresponding to rat insulin-II Flat-E (−213-CCTCTTAAGACTCTAATTACCCT-−192) or IPF-1 (−85-ACCCTTAATGGGACAAACAGCA-−64) sequences were incubated with HIT T-15, βTC-3, or αTC-6 protein extracts. The βTC-3 extract was prepared from isolated nuclei (38), and the αTC-6 extract was prepared from whole cells (13). The binding reactions contained 25 mM HEPES (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 μg polynucleotides (deoxyinosine-deoxycytosine), 0.5 μg polynucleotides-deoxy (adenosine/thymidine), 10 μg protein extract, and ^{32}P-labeled probe. The conditions for the competition analyses were the same, except that the specific competitor DNAs were included in the mixture (in the amounts detailed in the figure legends) before the addition of extract. The double stranded Flat-E site mutant used in this analysis corresponded to −213-CCTCTTAAGACTCGACTGACCCT-−192; the mutant nucleotides are underlined. The affinity-purified XIHbox 8 or Mox-1 antibodies were preincubated with β-cell extract for 10 min at room temperature before initiation of the DNA-binding reactions. After incubation of the DNA-binding mixtures at 4 C for 20 min, they were analyzed on a 6% nondenaturing polyacrylamide gel (acrylamide-bisacrylamide ratio, 29:1) at 4 C in TGE buffer (50 mM Tris, 380 mM glycine, and 2 mM EDTA, pH 8.5). After electrophoresis, the gel was dried and subjected to autoradiography.

Western Blot Analysis

Nuclear extract proteins (30 μg) were resolved on 12% SDS-PAGE (39) and electrotransferred to an Immobilon PVDF membrane (Millipore, Bedford, MA). The membranes were first incubated for 1 h in 2% nonfat dry milk in TBST (140 mM NaCl, 0.05% Tween-20, and 10 mM Tris-HCl, pH 8.0), and then with the primary antibody (1:1000 in TBST) for 1 h. After washing three times for 10 min each time in TBST, the membranes were probed for 2 h with [^{125}I]protein-A. The same washing protocol as that described above was followed, and the positions of bound antibodies were detected by autoradiography.

SDS-PAGE Fractionation

Thirty micrograms of βTC-3 protein extract were resolved on 12% SDS-PAGE and electroblotted onto an Immobilon PVDF membrane. The βTC-3 extract lane was cut horizontally into 0.5-cm slices to represent different mol wt classes. The proteins from each fraction were eluted for 4 h at 4 C in 100 μl elution buffer [25 mM HEPES (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 10% (vol/vol) glycerol, and 0.5% (vol/vol) Nonidet P-40], as described previously (40). Each fraction was analyzed for Flat-E element binding in the gel mobility shift assay and by Western blot analysis with the N-terminal XIHbox 8 antibody.

Homeoprotein Antibodies

The N-terminal XIHbox 8 antibody was raised against the first 75 amino acids of XIHbox 8 as a GST/XIHbox 8 fusion protein in the pGEX system described by Smith and Johnson (41). The C-terminal XIHbox 8 antibody was raised to amino acids 181–271 and was described in Wright *et al.* (19). The Mox-1 antibodies were raised to amino acids 2–139 as a GST fusion protein (sequence in Ref. 26). Fusion proteins were induced and purified, rabbits were immunized, and antibodies were affinity purified using techniques described previously (42). The full sequence of the XIHbox 8-predicted protein and the

details of the preparation and specificity of the N-terminal XIHbox 8 antibody will be reported elsewhere.

Immunocytochemistry

Adult CD-1 mice (purchased from Charles River, Lexington, MA) were perfused through the heart with 4% paraformaldehyde buffered to pH 7.4 with 0.1 M PBS. The pancreas was then removed and postfixed for 1 h in the same solution. The fixed tissues were infiltrated overnight in 30% sucrose and mounted in embedding matrix (Lipshaw Co., Pittsburg, PA), and 15- to 20-μm cryostat sections were collected onto gelatin-coated slides. These were then transferred to a Tris-saline solution (TBS; 0.9% NaCl in 0.1 M Tris, pH 7.4) and immunostained using the avidin-biotin-horseradish peroxidase method described previously (1). In brief, the sections were incubated sequentially in 1) 0.3% Triton X-100 in 1% goat serum (Gibco, Grand Island, NY) in TBS for 15 min, 2) a 1:30 dilution of goat serum in TBS for 30 min, 3) an empirically derived optimal dilution of control serum or primary antibody raised in rabbit containing 1% goat serum in TBS for 18 h, 4) a 1:50 dilution of goat antirabbit biotinylated immunoglobulin G (Vector Laboratories, Burlingame, CA) solution in 1% goat serum in TBS for 30 min, and 5) a 1:100 dilution of peroxidase-avidin complex (Vector) for 30 min. After these incubations, the bound peroxidase was visualized by reaction for 6 min in a solution containing 22 mg 3,3′-diaminobenzidine (DAB) and 10 μl 30% H_2O_2 in 100 ml TBS. All incubations were carried out at room temperature. After the DAB step, sections were dehydrated and mounted with Permount (Fisher Scientific, Orlando, FL). For double label immunostaining, sections were first incubated with antiserum to XIHbox 8, and the bound antibody was visualized by DAB (brown precipitate), followed by incubation with antiserum to a hormone that was visualized with the blue reaction product of the Vector SG substrate. Antibodies were used at the following dilutions: guinea pig antibovine insulin (Linco Research, Eureka, MO), 1:400; rabbit antihuman glucagon (Calbiochem, La Jolla, CA), 1:12,000; rabbit antihuman somatostatin (Peninsula Laboratories, Belmont, CA), 1:8,000; rabbit affinity-purified N-terminal XIHbox antibody (amino acids 1–75), 1:500; and rabbit affinity-purified C-terminal XIHbox 8 antibody (amino acids 181–271), 1:100.

The adult frog pancreas was dissected, fixed in 4% paraformaldehyde-PBS, embedded, sectioned, and stained, as described previously (42). Primary antibodies were used at the following dilutions: guinea pig antibovine insulin (Linco Research), 1:400; rabbit affinity-purified N-terminal XIHbox 8 antibody (amino acids 1–75), 1:500; and rabbit affinity-purified C-terminal XIHbox 8 antibody (amino acids 181–271), 1:100.

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