

Pancreatic beta cells express a diverse set of homeobox genes

(Lim motif/*Lmx* gene/*Nkx* gene/*Alx* gene/*Vdx* homeobox)

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ABSTRACT Homeobox genes, which are found in all eukaryotic organisms, encode transcriptional regulators involved in cell-type differentiation and development. Several homeobox genes encoding homeodomain proteins that bind and activate the insulin gene promoter have been described. In an attempt to identify additional beta-cell homeodomain proteins, we designed primers based on the sequences of beta-cell homeobox genes *cdx3* and *lmx1* and the *Drosophila* homeodomain protein Antennapedia and used these primers to amplify inserts by PCR from an insulinoma cDNA library. The resulting amplification products include sequences encoding 10 distinct homeodomain proteins; 3 of these proteins have not been described previously. In addition, an insert was obtained encoding a splice variant of engrailed-2, a homeodomain protein previously identified in the central nervous system. Northern analysis revealed a distinct pattern of expression for each homeobox gene. Interestingly, the PCR-derived clones do not represent a complete sampling of the beta-cell library because no inserts encoding *cdx3* or *lmx1* protein were obtained. Beta cells probably express additional homeobox genes. The abundance and diversity of homeodomain proteins found in beta cells illustrate the remarkable complexity and redundancy of the machinery controlling beta-cell development and differentiation.

The beta cells in the pancreatic islets of Langerhans are distinguished by their ability to produce insulin. The cell-specific expression of insulin derives, at least in part, from regulation of the insulin gene promoter by a unique set of nuclear proteins that bind to the promoter and activate it in beta cells. Mapping of transcriptionally active sequences within the insulin promoter has identified several important sequence elements within the rat insulin I (1, 2), rat insulin II (3, 4), and human insulin promoters (5) and has led to the recognition of beta-cell nuclear protein complexes that bind these elements (4–11) (for reviews, see refs. 12 and 13).

Many of the critical sequence elements of the insulin promoter fall into two groups: the E box elements and the (A+T)-rich elements. The proximal human and rat insulin I promoters contain two E boxes called IEB1 (at ≈ -100 bp relative to the transcription start site) and IEB2 (at ≈ -230 bp) (2, 5, 6, 14). The rat insulin II promoter contains only the highly conserved IEB1 (15, 16). The E boxes bind a protein complex that appears unique to the nuclei of beta cells and a few other neuroendocrine cells (6–10, 15). This complex is formed by dimerization of the ubiquitous helix–loop–helix proteins Pan1 and Pan2 with a smaller, more selectively expressed protein (17–20).

There are three important A/T elements in the proximal promoter, all conserved in the known mammalian insulin promoters. The FLAT element (11) [also called E2 (8, 10) or CT2 (9)] is immediately downstream of the IEB2 element; the

RIPE3B element (16) and the P1 element (8) [also called CT1 (9)] lie on either side of the IEB1 element. The A/T elements and the E boxes function synergistically: none of the elements can function in isolation, but combination of an E box and an A/T element results in dramatic activation of transcription (11, 16, 19). A number of complexes from beta-cell nuclei bind to the A/T elements (6, 8–11, 16, 19). Some proteins in these complexes have been cloned, and they all contain homeodomains. The A/T-binding proteins that have been cloned include *cdx3* (21), IPF1 (22) [also called STF1 (23) or IDX1 (24)], the Lim-homeodomain proteins *lmx1* (21) and *isl1* (25), and the Pou-homeodomain protein HNF1 α (26). Interestingly, the *lmx1* and Pan proteins can synergistically activate the insulin promoter when bound to their adjacent binding sites (21).

Based on evidence that the A/T-binding proteins cloned so far do not account for all the beta-cell nuclear complexes that bind the A/T elements (H.O. and M.S.G., unpublished data), we assumed that beta cells contained additional homeodomain proteins. We attempted to identify additional beta-cell homeodomain proteins by PCR. We report here the PCR amplification and cDNA cloning of several additional beta-cell homeodomain proteins. ¶

MATERIALS AND METHODS

PCR and cDNA Cloning. DNA was purified from the λ gt11 HIT (hamster insulinoma) T15 M2.2.2 cDNA library (17) by SDS treatment and phenol/chloroform extraction. The DNA was mixed with oligonucleotide primer pairs and subjected to 35 cycles of PCR using *Taq* DNA polymerase. Primer sequences were as follows: *lmx*, GGGGATCCRTTYTGRAAC-CANACYTG; *cdx*, GGGGATCCRTTYTGRAACCADATYTTNAC; *antp*, GGGGATCCRTTYTGRAACCADATYTTDAT; *lacZ* forward, ATTGGTGGCGACTCCTG; *lacZ* reverse, CACCAGACCAACTGGTAATG. The resulting products were cut with appropriate restriction endonucleases and resolved by agarose gel electrophoresis. Multiple discrete bands were purified individually, ligated into pBlueScript KS+, and sequenced. Full-length cDNA clones were obtained as described (21) using the PCR fragments to screen the HIT cDNA library. Sequence of each cDNA was obtained by the Sanger dideoxynucleotide chain-termination sequencing method.

Northern Blot Analysis. The cell lines include the beta-cell lines HIT T15 M2.2.2 (1) and β TC3 (27), the alpha-cell lines INR1-G9 (28) and α TC1.9 (29), the fibroblast lines BHK21 (2) and NIH 3T3 (30), the pituitary line ATT20 (31), and the pancreatic exocrine line 266-6 (32). The two alpha-cell lines do not express detectable levels of insulin mRNA (29). Cells were grown to $\approx 75\%$ confluence before isolating RNA.

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¶The sequences reported in this paper have been deposited in the GenBank data base [accession nos. X81403 (*alx3*), X81405 (*en-2*), X81407 (*lmx2*), X81408 (*Nkx2.2*), X81409 (*Nkx6.1*), and X81410 (*Vdx*)].

Tissues were obtained from young adult male Syrian hamsters. Poly(A)⁺ RNA was isolated with the OnTrack mRNA isolation kit (Biotex Laboratories, Houston). Ten micrograms of each RNA was heat-denatured, separated on a formaldehyde-agarose gel, and blotted onto GeneScreen nylon membrane (New England Nuclear). The membranes were probed at high stringency with the random primer ³²P-labeled cDNAs shown, as directed by the membrane manufacturer. The membranes were stripped and reprobed with each cDNA. A Syrian hamster β_2 -tubulin cDNA probe (J. Wang and M.S.G., unpublished data) was used as a control for mRNA quality.

Homeobox Gene Names. To avoid biasing our evaluation of these genes, we chose names based on their derived amino acid sequences rather than on assumptions about their expression or function. Where possible, we followed the consensus recommendation on vertebrate homeobox gene nomenclature (33). Although the mouse homologue of *Lmx2* has been recently published with the name *Lim1* (34, 35), we chose to retain the name *Lmx2* because it distinguishes Lim-homeobox genes from genes that only contain Lim domains.

RESULTS

We designed three degenerate DNA oligonucleotide primers based on the amino acid sequence of helix 3 from the homeodomains of *lmx1*, *cdx3*, and the *Drosophila* protein Antennapedia. These oligonucleotide primers were paired with either of two primers complementary to the *lacZ* gene sequences on either side of the *EcoRI* site in λ gt11, and this pair of primers was used to amplify sequences from a λ gt11 HIT (hamster insulinoma) cDNA library by PCR. The PCR products were subcloned, and 119 inserts were sequenced (Table 1).

We identified 10 different homeobox sequences (Table 1). Only two of these genes, *isl1* (25) and *IPF1* (22), have been described previously in beta cells. Of the other eight genes, homologues of five have been described from other species in other tissues. The three remaining genes, *alx3*, *Nkx6.1*, and *Vdx*, have not been described previously. Engrailed-2 (*en-2*) has been described previously in the developing and adult mid/hindbrain (36, 37), but the sequence of the beta-cell *en-2* PCR fragment diverges from published *en-2* cDNA sequences at a known splice junction upstream of the homeobox (data not shown). This sequence probably represents another splice form of *en-2* rather than an unspliced mRNA because the upstream sequence has no homology to the published *en-2* intron sequence (38).

The subcloned PCR fragments of six of these genes were used to screen the HIT cDNA library for longer inserts. Interestingly, *IPF1*, which was the most abundant cDNA by PCR, was less abundant than *Nkx2.2*, *Nkx6.1*, or *Lmx2* by hybridization screening of the same library. Full-length coding sequences of four cDNAs were obtained, and their

Table 1. Classification of PCR products by DNA sequence

Primer	Total number sequenced	Homeodomain	Clones, no.
lmx	49	<i>isl1</i>	1
		<i>lmx2</i>	2
		<i>alx3</i>	2
cdx	42	HOXA4	4
		HOXA13	1
		IPF1	5
		<i>Nkx2.2</i>	1
		<i>Nkx6.1</i>	1
Antp	28	<i>en-2</i>	1
		<i>Vdx</i>	1

A *Alx3*
 MAPMDPERCAPFAVGPAAAGDEPPGPQGTSDASPHLHPAPPRGPR
 LSRFPACGPLEPYLPEPAKPPAKYLQDLGPAPVNLNGGHFYEGPAEAEKA
 SKAASFPQLPVDRCRGGPRDGP SNVQSGSPGCLASLRVPLSPGLPDMELA
 KSKSKRRNRRTTFSTFOLEELEKVKFOKTHYVDVAREOLALRTDLTEARV
 QVWFONRRAKWRKRERYGKMQEGRNPFPTTAYDISVLPRDTSHPQLQNSLW
 PGSGSGSPGGPCLMSPEGIPSPCMSPYSHSHGNVAGFMGVPASPAAHPGI
 YSIHGFPFALGGHSFEPSPDGDYKPSLVSRLVKPKPEPSSLNWT

Lmx2
 MVHCAGCKRPIILDRFLNVLDRAWHVCKVQCECKNLTEKCF SREGKLY
 CKNDFRCFGTKCAGCAQGISPSDLVRRARSKVFLNCFCTMCMNKQLST
 GEELYI IDENKFVCKEDYLSNSSVAKENSLHSATGSDPSLSPDSQDPSQ
 DDAKDESANVSDKEGGSNENDDQNLGAKRRGRPTTIKAKOLETLKAAFA
 ATPKPTRHIREOLAQETGLNMRVIOVWFONRRSKERRMKQLSALGARRHA
 FFRSPRRMRPLVDRLEPGEIIPNGPFSFYGDYQSEYYPGGNYDFPQGP
 PSSQAQTPVDLPFVFPSSGSGTFLGGLDHP LFGHHPSSAQRFDTILAHF
 PGDSPSPEPSLPGPLHMSMAEVFGPSPFFSSLSVNGGASVGNHLSHPPEM
 NEAAVW

Nkx2.2
 MSLTNTKTGFSVKDILDLPTNDEEGSVAEGPEESEGPEPAKRAGPLGQ
 GALDAVHSLPLKSPFYDSSDNPYTRWLASTEGLQYSLHGLAASAPPQDSS
 SKSPEPSADESPDNKETPGGGDAGKRRRVLFSKAQTYELERFRRO
 RYLSAPEREHASLIRLTPTOVKIWFONHRYKMKRARAEGKMEVTPLPSP
 RCVAVPVLVRDGPCHALKAQDLAAATFQAGIPFSAYSQSLQHMQYNAQ
 YSSASTPQYPTAHP LVQAQQWTW

Nkx6.1
 MLAVGAMEGRQSAFLLSSPPLAALHSAEMKTPLYPATYPLPTGPPSS
 SSSSSSSPSPPLGAHNPGLKPPAAGLSSLSLSPQQLSAATPHGINDI
 LSRPMPVASGAALP SASP SGSSSSSSSASATSASAAAAA AAAAAA
 SSPAGLLAGLPRFSSLSLSPPPPGLYFSPSAAA VAVGRYPKPLAELPGR
 APIFWPGVMQSPWRDARLACTPHQGSILLDKDGKRKHTRPTFSGOOIFA
 LEKTFEOTKYLAGEPERARLAYS LGMTESOVKVFONRRTKWRKHAEMA
 TAKKKQDSETERLKGTSENEEDDDYNKLPDPSNDDDKITQLLKKHKSS
 GGLLLHASEAEGSS

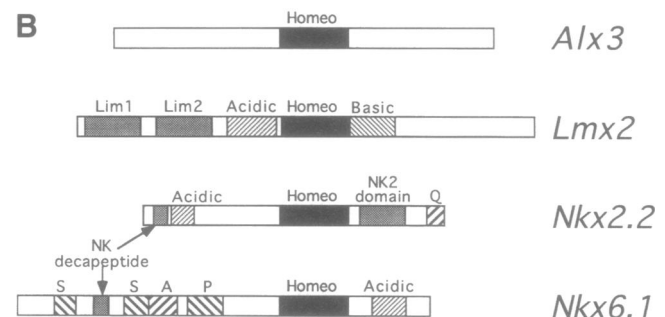


Fig. 1. Derived amino acid sequences of homeobox cDNAs. (A) Amino acid sequences with homeodomains underlined. (B) Schematics depicting homeodomain primary structures. Q, glutamine-rich; S, serine-rich; A, alanine-rich; P, proline-rich.

predicted amino acid sequences are shown in Fig. 1. The homeodomains of the three previously undescribed genes are compared in Fig. 2. Tissue distributions of the corresponding mRNAs were determined by Northern blot with hamster and mouse mRNA (Fig. 3 and Table 2).

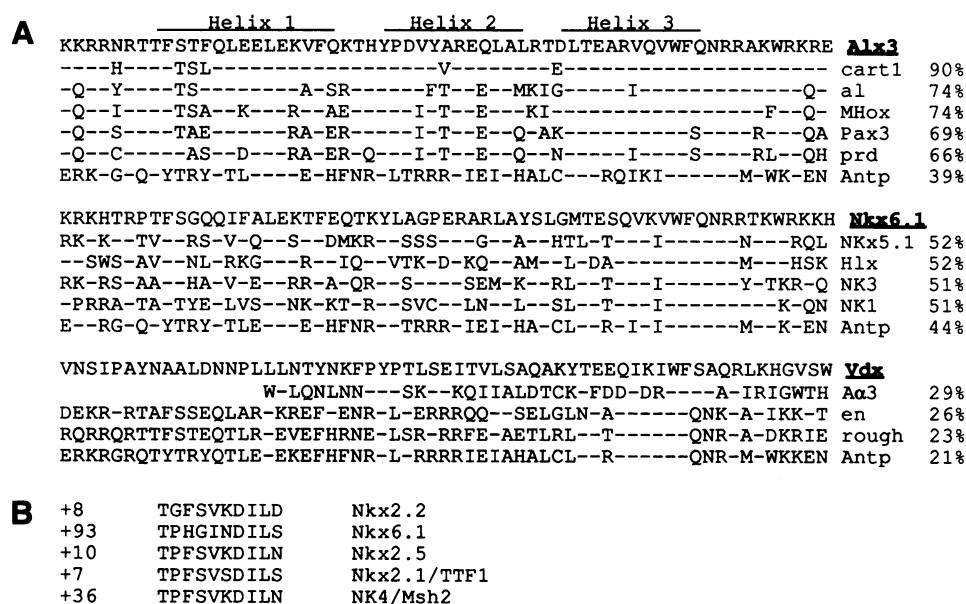


FIG. 2. Homeodomain comparisons. (A) The 61-amino acid homeodomains are compared among related proteins; dashes represent identities. Aa3, Aa3 mating type protein from *S. commune*; prd, paired. (B) Sequences of related decapeptides from the amino-terminal regions of the NK genes. Number is the position of first amino acid relative to initiator methionine in each protein.

The cloning of Lim1, the mouse homologue of Lmx2, has been reported recently (34, 35). The hamster and mouse amino acid sequences are 99.5% identical. Like Lmx1, Lmx2 has two cysteine-rich Lim domains. Similar Lim domains have been shown to complex with divalent zinc cations (39), and the Lim domains in Lmx1 are required for the synergistic interaction with the Pan proteins (21). Despite the presence of the Lim domains, the amino acid sequences of Lmx1 and Lmx2 are not closely related, even within the homeodomains (34, 35). Although Lmx2 expression is not ubiquitous, its mRNA was seen in most of the cell lines and tissues tested by Northern blot (Table 2). In this regard also, Lmx2 is different from Lmx1, which has been detected only in beta cells (21).

The homeobox gene *Alx3* is related to the *Drosophila* gene *aristaless* (*al*) (40) (Fig. 3). *Alx3* encodes a paired class homeodomain; but like *aristaless* and the two other members of this subclass, *cart1* (41) and *mHox* (42), it lacks a paired box. *Cart1*, a cartilage-specific homeobox gene product, shares additional amino acid sequence homology with *Alx3* outside the homeodomain. This homology is strongest (38% identity) over the region between the homeodomain and the

carboxyl terminus; the amino-terminal halves of the two proteins are only 11% identical. In addition to HIT cells, *Alx3* is expressed in the mouse pancreatic exocrine cell line 266-6 and is abundant in pancreatic exocrine cells. *Alx3* mRNA was not observed in the mouse beta-cell line β TC3, but the β -tubulin cDNA control also produced a low signal in β TC3 RNA, suggesting that the β TC3 RNA contained less intact mRNA than the other samples. *Alx3* mRNA was not observed in either alpha-cell line tested.

We obtained two cDNAs that fall into the Nkx class. The NK homeodomain proteins were originally described in *Drosophila* (43), and a number of related *Nkx* genes have been described in mammals. A partial sequence of mouse *Nkx2.2* has been published (44). The full-length *Nkx2.2* coding sequence reveals at the amino terminus a conserved decapeptide that is also found in several other NK-related proteins (Fig. 2B). Expression of *Nkx2.2* has been reported (44) in the mammalian brain, but our data show that it also is expressed in the pancreas and pancreatic islet alpha and beta-cell lines (Table 2).

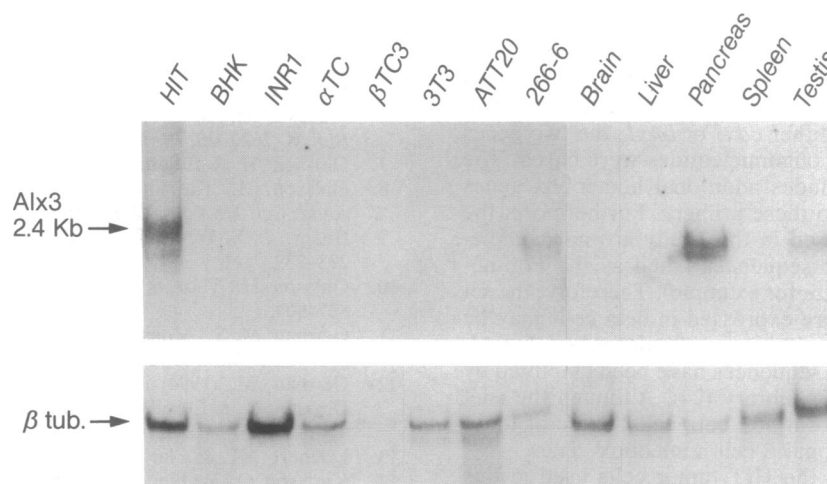


FIG. 3. Expression of *Alx3* mRNA. By Northern blot analysis, 10 μ g of poly(A)⁺ RNA from the cell lines and tissues shown was hybridized to the *Alx3* cDNA probe. β tub., β_2 -tubulin.

Table 2. Tissue distribution of homeobox RNAs by Northern blot analysis

Probe	Hamster RNA					Mouse RNA					Size, kb			
	Tissue					Cell line			Cell line					
	Brain	Liver	Spleen	Testis	Pancreas	HIT	INR1	BHK	β TC3	α TC1.9		ATT20	266	3T3
alx3				+	+	+						+		2.4
lmx2	+	+		+	+	+	+	+	+	+	+	+	+	4.2
Nkx6.1						+			+	+				3.4
Vdx	+					+	+	+	+	+	+	+	+	4.3
Nkx2.2	+				+	+	+		+	+	+			2.4
IPF1						+			+			+		2.4
β_2 tubulin	+	+	+	+	+	+	+	+	+	+	+	+	+	

Nkx6.1 defines another homeobox class that is distantly related to the other NK homeobox genes (Fig. 2A). Like *Nkx2.2*, *Nkx6.1* also encodes the amino-terminal NK decapeptide (Fig. 2B). Except for a short (4 amino acid) homology after the conserved decapeptide in *Nkx2.2* and *Nkx2.5*, *Nkx6.1* has no further homology to the other NK proteins outside the homeodomain. The amino-terminal half of *Nkx6.1* contains several stretches of repeated serines, alanines, and prolines. Similar repeats have been seen in other transcription factors, but their function is not known. In a limited survey (Table 2), we found *Nkx6.1* mRNA only in islet cells—the two beta-cell lines and α TC but not INR1. Because islets comprise only a small fraction of the pancreas, the low abundance of *Nkx6.1* in pancreatic RNA is consistent with selective islet expression. Given the large number of cells and tissues not tested, however, it should not be assumed that *Nkx6.1* expression is absolutely limited to pancreatic islets.

Only incomplete cDNAs were obtained for the very divergent homeobox, *Vdx*. Despite the marked divergence of the *Vdx* homeodomain sequence, helix 3 closely resembles other known homeodomains (Fig. 2A). Ala-52, although a conservative substitution for asparagine, has been seen previously only in fungal homeodomain proteins, such as the *Aa3* mating type homeobox gene from *Schizophyllum commune* (45) (Fig. 2). Interestingly, two other features of the *Vdx* homeodomain—the absence of any basic amino acids at the amino end of the homeodomain and the short helix 1—also are shared by some fungal homeodomain proteins. *Vdx* mRNA was found in all cell lines tested, but in a limited survey of hamster tissues, it was only abundant in brain (Table 2).

DISCUSSION

Our data demonstrate that beta-cells express a remarkable variety of homeobox genes, and the genes identified so far may only represent a fraction of the total set of beta-cell homeobox genes. Despite identifying 10 different beta-cell homeobox genes, including three previously undescribed genes, we did not obtain either *cdx3* or *lmx1*, the two genes on which the PCR primer oligonucleotides were based. The HIT library probably includes additional homeobox genes that are complementary to these primers. Furthermore, the oligonucleotide primers used in this study are not complementary to all homeobox sequences (such as the Pou-homeodomain protein HNF1 α , for example). Therefore, the set of homeobox genes that are expressed in beta cells may be considerably larger than the 13 genes so far described. In fact, nine additional homeobox sequences have been amplified by PCR from a rat islet cDNA library (24). Although the islet library includes cDNAs from non-beta cells, some of these probably represent additional β -cell homeobox genes.

It should be noted that the HIT tumor cells used in this study may synthesize and secrete insulin, but in some other regards they differ from normal beta cells. Therefore, some of the genes described here may not be expressed in normal

beta cells. All previous transcription factor genes that have been cloned from insulinoma tumor cell lines, however, have also been expressed in normal beta cells, and we expect that the majority of these homeobox genes are also expressed in normal beta cells.

The large set of homeobox genes expressed in beta cells suggests remarkable redundancy. The individual functions of the expressed proteins, however, may be quite divergent, so that function may not be as redundant as sequence. The paucity of structural similarities outside their homeodomains and their distinct patterns of expression suggest that the roles of the different proteins are quite divergent. When the patterns of expression of the genes described here are compared with the patterns of previously described beta-cell homeobox genes, no single pattern emerges. Each homeobox gene has a distinct pattern of expression. The distinct, but overlapping, expression patterns of these genes contribute to the combinatorial code that determines the unique development and final phenotype of the beta cell. The beta-cell homeobox genes will be valuable reagents for dissecting this code as well as for studying islet development and differentiation.

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