Pan: a transcriptional regulator that binds chymotrypsin, insulin, and AP-4 enhancer motifs

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Two cDNAs were cloned that encode proteins (Pan-1 and Pan-2, with predicted molecular masses of 67.4 and 67.7 kD, respectively) that bind to a conserved transcriptional regulatory element present in pancreatic exocrine genes. The cDNAs are virtually identical in the region that encodes the amino-terminal 525 amino acids; they differ only by a 3-nucleotide insertion that results in the addition of a single amino acid. The cDNAs, however, code for related, but divergent, carboxy-terminal regions. Expression of the cloned cDNAs in HeLa cells activates expression of a cotransfected chimeric gene containing multimers of the pancreatic conserved element linked to a minimal promoter. Pan-1 and Pan-2 bind to the consensus sequence CA^C/₆CTG^T/_C, the CACCTGTC motif, which is present in transcriptional control elements of several genes. Both Pan-1 and Pan-2 bind to related motifs present in the rat insulin enhancer as well as a DNA segment containing the SV40 AP-4 element. The Pan-1 and Pan-2 cDNAs display extensive sequence identity with partial cDNA clones encoding E12 and E47, which bind to the immunoglobulin κ E2 cis-active element, and may be derived from the same gene. These factors belong to a set of genetically distinct molecules, including AP-4 and MLTF, that bind to the CACCTGTC motif or related sequences. The Pan/E12,E47 proteins also show structural similarity with the *Drosophila* daughterless protein, MyoD, Myogenin, and Myf-5.

[Key Words: Acinar cells; pancreas; cell-specific expression; DNA-binding protein]

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Exocrine cells of the pancreas synthesize a set of hydrolytic enzymes involved in the digestion of food. The dramatic increase in the levels of these enzymes during pancreatic development is due primarily to an increased rate of transcription (Rutter et al. 1978). Transcriptional enhancers have been identified that appear to determine acinar-specific expression of the various exocrine genes in both cell culture and transgenic animals. A conserved sequence has been identified in the enhancers of the chymotrypsin B, amylase 2A, trypsin I and elastase I exocrine genes, and the 5'-flanking sequences of other exocrine genes. This pancreatic consensus sequence (Swift et al. 1984; Boulet et al. 1986) has been shown to be a critical component of the functional enhancer (Boulet et al. 1986; Kruse et al. 1988).

A pancreas-specific DNase I footprint encompassing the consensus sequence has been identified in the chymotrypsin B, amylase 2A, and trypsin I genes (Meister et al. 1989). Recently, Cockell et al. (1989) identified an apparent pancreas-specific binding activity that interacts with the consensus sequence of the α -amylase 2, elas-

tase 2, trypsin^a, and other pancreatic genes. Competition for binding in gel mobility-shift experiments and methylation interference studies suggest that this chymotrypsin enhancer element contains two distinct binding motifs. One motif contains the sequence, CACCTGTC, and the other, TTTTCCC (TC motif) (Cockell et al. 1989; Meister et al. 1989). These sequences form an apparent bipartite binding motif that is also present in the consensus sequence of other pancreatic genes (Cockell et al. 1989). Multimers of the chymotrypsin B sequence can function as a cell-specific enhancer in gene transfer experiments (Meister et al. 1989). Deletion of sequences containing these elements in the rat chymotrypsin B enhancer virtually abolishes enhancer function (Boulet et al. 1986). A mutation of the related CACCTGTG motif present in the rat amylase 2A enhancer destroys enhancer activity (Boulet et al. 1986). An apparent pancreas-specific binding activity protects the sequence CACCTGTG in the rat elastase I enhancer from DNase I digestion; mutations of this sequence severely decrease elastase gene expression (Kruse et al. 1988). These collective studies suggest a crucial role for this element in the control of pancreas-specific gene expression.

Here, we report the isolation of two cDNAs that encode proteins that selectively bind to the CACCTGTC motif and demonstrate that expression of these cDNAs

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Figure 1. Sequence-specific binding of Pan-1 to the chymotrypsin B gene pancreatic consensus element. (A, top) Schematic representation of five block mutations (A–E) of the chymotrypsin enhancer element (wt). (A, bottom) The specificity of binding of in vitro-produced Pan-1 to the chymotrypsin enhancer element was analyzed in a gel mobility-shift competition assay (Fried and Crothers 1981; Garner and Revzin 1981). Rabbit reticulocyte lysate containing in vitro-translated Pan-1 was incubated with radiolabeled probe (–213 to –186 of the rat chymotrypsin B gene) and 2- or 20-fold molar excess of unlabeled competitor fragment. The complex was subjected to nondenaturing polyacrylamide gel electrophoresis. The five block mutations of the enhancer sequence, A–E, and wild-type (wt) sequence were used as competitors. The lane designated (–) represents reticulocyte lysate alone, without the addition of Pan-1 mRNA used for in vitro translation. The faster migrating complex observed in the (–) lane represents a nonspecific binding activity that is competed by the addition of RNA used for in vitro translation. (B)

Methylation interference analysis of Pan-1 contacts with the chymotrypsin enhancer element. Partially methylated probe (–213 to –186), end-labeled on the noncoding strand, was incubated with in vitro-produced Pan-1 and bound complex was isolated by preparative polyacrylamide gel electrophoresis. After cleavage of methylated guanines, the DNA was analyzed by denaturing polyacrylamide gel electrophoresis. The noncoding sequence is shown with dots adjacent to unmethylated G residues.

in cotransfection experiments activates the expression of a gene containing multimers of the CACCTGTC motif linked to a minimal promoter.

Results

Isolation and characterization of cDNA clones

To study the mechanism of transcriptional regulation, we screened a λ gt11 cDNA expression library prepared from an acinar cell line for cDNAs that encode proteins binding to the pancreatic consensus element. Of 400,000 plaques screened with a multimerized consensus sequence probe, one recombinant phage was isolated that displayed specific binding activity. A second cDNA insert that encodes an apparent full-length protein was obtained by oligonucleotide hybridization screening of the λ gt11 library. The proteins (designated Pan-1 and Pan-2), encoded by the cDNA inserts were examined for their binding specificity. Pan-1, produced in vitro, displayed selective binding to a region containing the CACCTGTC motif in methylation interference and binding competition experiments (Fig. 1A,B). Pan-2 produced by in vitro translation also selectively binds the pancreatic consensus sequence. This binding is abolished by a block mutation of the CACCTGTC motif (see Fig. 6, below). Deletion of the DNA sequences coding for the carboxy-terminal 224 amino acids of Pan-1 abolished the binding ability of in vitro-translated protein, whereas removal of coding sequences for the 23 carboxy-terminal amino acids only decreased the binding affinity (data not shown). Thus, the carboxy-terminal region may be involved in DNA binding.

The 2.7-kb cDNA insert encoding Pan-1 and the 3.0-kb insert encoding Pan-2 were sequenced and found to

be identical except for a region at the 3' terminus and a 3-nucleotide insertion in the Pan-1 cDNA. Therefore, the two cDNAs are probably derived from the same gene. The Pan-1 cDNA contains an open reading frame coding for 638 amino acids (Fig. 2). The Pan-2 cDNA insert contains an open reading frame coding for an apparent full-length protein of 649 amino acids (with a predicted molecular mass of 67.7 kD). The Pan-2 cDNA contains the coding sequences for the initiator methionine and the first nine amino acids, which are absent in the Pan-1 cDNA. With the exception of an additional alanine present at predicted amino acid residue 168 in Pan-1, the two proteins diverge only in a 72-amino-acid section (amino acids 525–597) of the carboxy-terminal region. They regain identity from amino acid 597 to the carboxy terminus at 649 of Pan-2. The divergence at 525 follows a CAG consensus splice donor sequence (Mount 1982). Thus, the two cDNAs may be generated by alternative splicing. The Pan-1 and Pan-2 cDNAs possess extensive nucleotide similarity in the divergent region, suggesting that alternative splicing of an exon duplication is responsible for the unique cDNAs. A DNA sequence present in both Pan-1 and Pan-2 cDNAs hybridizes to single *Bam*HI and *Hind*III genomic restriction fragments (Fig. 3). This result supports the premise that a single gene in the rat codes for the two proteins.

Interestingly, Pan-1 and Pan-2 are very closely related to E12 and E47, factors that bind to the immunoglobulin κ E2 motif and that have been characterized by partial cDNA clones isolated from a human cDNA library (Murre et al. 1989). Pan-1 corresponds to E47 (91% identity over 131 amino acids) and Pan-2 to the E12 clone (83% identity over 440 amino acids). E12 and E47 diverge at the same position as Pan-1 and Pan-2 in the carboxy-terminal region. Therefore, the clones may be de-

rived from the same gene. The differences in amino acid composition between Pan-1/2 and E12/47 may be due to

evolutionary changes in the rat and human forms of the same proteins. In addition, these proteins are closely re-

TGGTGTCACTACACAGGGCTGCCTCTAGGCGCTGGGGAACGCACCCAGTTCGCACACCTGCTGCCCTGGCTGG																			Met	Met	Asn	Gln	Ser	Gln	6																
																			ATG	ATG	AAC	CAG	TCT	CAG	Pan-2																
Arg	Met	Ala	Pro	Val	Gly	Ser	Asp	Lys	Glu	Leu	Ser	Asp	Leu	Leu	Asp	Phe	Ser	Met	Met	Phe	Pro	Leu	Pro	Val	31																
AGA	ATG	GCA	CCT	GTT	GGC	TCG	GAC	AAA	GAA	CTG	AGT	GAC	CTC	CTG	GAC	TTC	AGT	ATG	ATG	TTC	CCG	CTA	CCT	GTG	Pan-2																
GAATTCCA																			CCT	GTT	GGC	TCG	GAC	AAA	GAA	CTG	AGT	GAC	CTC	CTG	GAC	TTC	AGT	ATG	ATG	TTC	CCG	CTA	CCT	GTG	Pan-1
Ala	Asn	Gly	Lys	Gly	Arg	Pro	Ala	Ser	Leu	Ala	Gly	Thr	Gln	Phe	Ala	Gly	Ser	Gly	Leu	Glu	Asp	Arg	Pro	Ser	56																
GCC	AAT	GGC	AAG	GGC	CGG	CCC	GCC	TCC	CTA	GCT	GGA	ACC	CAG	TTC	GCA	GGC	TCA	GGA	CTG	GAG	GAC	CGA	CCC	AGC																	
Ser	Glu	Ser	Trp	Gly	Asn	Ser	Glu	Gln	Asn	Ser	Ser	Ser	Phe	Asp	Pro	Ser	Arg	Ala	Tyr	Ser	Glu	Gly	Ala	His	81																
TCA	GAG	TCC	TGG	GGC	AAC	AGT	GAA	CAG	AAC	AGT	TCT	TCC	TTT	GAC	CCT	AGC	CGG	GCA	TAC	AGC	GAA	GGT	GCC	CAC																	
Phe	Ser	Asp	Ser	His	Ser	Ser	Leu	Pro	Pro	Ser	Thr	Phe	Leu	Gly	Ala	Gly	Leu	Gly	Gly	Lys	Gly	Ser	Glu	Arg	106																
TTC	AGT	GAC	TCC	CAC	AGC	AGC	CTG	CCG	CCT	TCC	ACG	TTC	CTA	GGA	GCT	GGG	CTT	GGA	GGC	AAG	GGC	AGT	GAA	CGG																	
Asn	Ala	Tyr	Ala	Thr	Phe	Gly	Arg	Asp	Thr	Ser	Val	Gly	Thr	Leu	Ser	Gln	Ala	Gly	Phe	Leu	Pro	Gly	Glu	Leu	131																
AAT	GCC	TAT	GCC	ACC	TTC	GGG	AGA	GAC	ACC	AGT	GTT	GGC	ACC	CTG	AGT	CAG	GCT	GGC	TTC	CTA	CCA	GGT	GAG	CTG																	
Gly	Leu	Ser	Ser	Pro	Gly	Pro	Leu	Ser	Pro	Ser	Gly	Val	Lys	Ser	Ser	Ser	Gln	Tyr	Tyr	Thr	Ser	Phe	Pro	Ser	156																
GGC	CTC	AGC	AGT	CCC	GGG	CCA	CTG	TCC	CCA	TCA	GGC	GTC	AAG	AGC	AGC	TCC	CAG	TAT	TAC	ACC	TCA	TTC	CCC	AGC																	
Asn	Pro	Arg	Arg	Arg	Ala	Ala	Asp	Gly	Gly	Leu	---	Asp	Thr	Gln	Pro	Lys	Lys	Val	Arg	Lys	Val	Pro	Pro	Gly	180																
AAC	CCT	CGG	CGG	AGA	GCT	GCA	GAT	GGT	GGC	CTG	---	GAT	ACT	CAG	CCC	AAG	AAG	GTC	CGG	AAG	GTT	CCG	CCT	GGT	Pan-2																
AAC	CCT	CGG	CGG	AGA	GCT	GCA	GAT	GGT	GGC	CTG	GCA	GAT	ACT	CAG	CCC	AAG	AAG	GTC	CGG	AAG	GTT	CCG	CCT	GGT	Pan-1																
Asn	Pro	Arg	Arg	Arg	Ala	Ala	Asp	Gly	Gly	Leu	Ala	Asp	Thr	Gln	Pro	Lys	Lys	Val	Arg	Lys	Val	Pro	Pro	Gly																	
Leu	Pro	Ser	Ser	Val	Tyr	Pro	Ser	Ser	Ser	Gly	Asp	Asn	Tyr	Ser	Arg	Asp	Ala	Thr	Ala	Tyr	Pro	Ser	Ala	Lys	205																
CTT	CCT	TCC	TCG	GTA	TAT	CCA	TCC	AGC	TCA	GGT	GAT	AAC	TAC	AGC	AGG	GAC	GCC	ACA	GCC	TAC	CCC	TCC	GCC	AAG																	
Thr	Pro	Ser	Ser	Ala	Tyr	Pro	Ser	Pro	Phe	Tyr	Val	Ala	Asp	Gly	Ser	Leu	His	Pro	Ser	Ala	Glu	Leu	Trp	Ser	230																
ACC	CCC	AGC	AGC	GCT	TAC	CCC	TCC	CCC	TTC	TAC	GTG	GCA	GAC	GGC	AGC	CTG	CAC	CCC	TCA	GCG	GAG	CTC	TGG	AGT																	
Pro	Pro	Gly	Gln	Val	Gly	Phe	Gly	Pro	Met	Leu	Gly	Asp	Gly	Ser	Ala	Pro	Leu	Pro	Leu	Ala	Pro	Gly	Ser	Ser	255																
CCC	CCT	GGC	CAG	GTG	GGC	TTT	GGG	CCC	ATG	CTA	GGT	GAT	GGC	TCG	GCC	CCC	CTG	CCG	CTT	GCA	CCG	GGC	AGC	AGT																	
Ser	Val	Ser	Ser	Gly	Ala	Phe	Gly	Gly	Leu	Gln	Gln	Gln	Asp	Arg	Met	Gly	Tyr	Gln	Leu	His	Gly	Ser	Glu	Val	280																
TCC	GTG	AGC	AGT	GGT	GCC	TTT	GGG	GGC	CTC	CAG	CAG	CAG	GAT	CGC	ATG	GGC	TAC	CAG	CTG	CAT	GGG	TCT	GAG	GTC																	
Asn	Gly	Thr	Leu	Pro	Ala	Val	Ser	Ser	Phe	Ser	Ala	Ala	Pro	Gly	Thr	Tyr	Ser	Gly	Thr	Ser	Gly	His	Thr	Pro	305																
AAT	GGT	ACG	CTC	CCA	GCT	GTA	TCC	AGC	TTC	TCA	GCT	GCC	CCT	GGC	ACT	TAC	AGT	GGG	ACT	TCT	GGC	CAC	ACG	CCA																	
Pro	Val	Ser	Gly	Ala	Asp	Ser	Leu	Leu	Gly	Thr	Arg	Gly	Thr	Thr	Ala	Ser	Ser	Ser	Gly	Asp	Ala	Leu	Gly	Lys	330																
CCC	GTG	AGC	GGG	GCC	GAC	AGC	CTC	TTA	GGC	ACC	CGA	GGG	ACT	ACA	GCC	AGC	AGC	TCA	GGG	GAT	GCC	CTT	GGG	AAG																	
Ala	Leu	Ala	Ser	Ile	Tyr	Ser	Pro	Asp	His	Ser	Ser	Asn	Asn	Phe	Ser	Pro	Ser	Pro	Ser	Thr	Pro	Val	Gly	Ser	355																
GCA	CTG	GCC	TCC	ATC	TAC	TCC	CCG	GAT	CAC	TCC	AGC	AAT	AAT	TTC	TCG	CCC	AGC	CCC	TCA	ACG	CCT	GTG	GGT	TCA																	
Pro	Gln	Gly	Leu	Pro	Gly	Thr	Ser	Gln	Trp	Pro	Arg	Ala	Gly	Ala	Pro	Ser	Ala	Leu	Ser	Pro	Asn	Tyr	Asp	Ala	380																
CCC	CAG	GGC	CTG	CCA	GGG	ACA	TCA	CAG	TGG	CCC	CGG	GCA	GGA	GCG	CCC	AGT	GCC	TTA	TCC	CCC	AAC	TAC	GAT	GCA																	
Gly	Leu	His	Gly	Leu	Ser	Lys	Met	Glu	Asp	Arg	Leu	Asp	Glu	Ala	Ile	His	Val	Leu	Arg	Ser	His	Ala	Val	Gly	405																
GGT	CTC	CAT	GGC	CTG	AGT	AAA	ATG	GAA	GAT	CGC	TTG	GAC	GAG	GCC	ATC	CAC	GTC	CTC	CGC	AGT	CAC	GCT	GTC	GGC																	
Thr	Ala	Ser	Glu	Leu	His	Gly	Leu	Pro	Gly	His	Ser	Thr	Leu	Thr	Thr	Ser	Phe	Ala	Gly	Pro	Met	Ser	Leu	430																	
ACT	GCA	AGC	GAG	CTC	CAT	GGA	CTG	CTG	CCT	GGC	CAC	AGC	ACG	TTG	ACC	ACA	AGC	TTT	GCG	GGC	CCC	ATG	TCA	CTG																	
Gly	Gly	Arg	His	Ala	Gly	Leu	Val	Ser	Gly	Ser	His	Pro	Glu	Asp	Gly	Leu	Thr	Ser	Gly	Ala	Ser	Leu	Leu	His	455																
GGC	GGG	CGG	CAT	GCA	GGC	CTG	GTC	AGC	GGA	AGC	CAC	CCT	GAG	GAT	GGC	CTC	ACA	AGT	GGT	GCC	AGT	CTT	TTG	CAT																	
Asn	His	Ala	Ser	Leu	Pro	Ser	Gln	Pro	Ser	Ser	Leu	Pro	Asp	Leu	Ser	Gln	Arg	Pro	Pro	Asp	Ser	Phe	Ser	Gly	480																
AAC	CAT	GCC	AGC	CTT	CCC	AGC	CAG	CCC	AGC	TCC	CTC	CCT	GAC	CTC	TCA	CAG	AGG	CCA	CCC	GAC	TCC	TTT	AGT	GGA																	
Leu	Gly	Arg	Ala	Gly	Val	Thr	Ala	Gly	Ala	Ser	Glu	Ile	Lys	Arg	Glu	Glu	Lys	Glu	Asp	Glu	Glu	Val	Thr	Ser	505																
CTC	GGG	AGG	GCA	GGC	GTG	ACT	GCA	GGC	GCC	AGT	GAG	ATC	AAG	CGG	GAG	GAG	AAA	GAG	GAC	GAG	GAG	GAG	ACA	TCA																	
Val	Ala	Asp	Ala	Glu	Glu	Asp	Lys	Lys	Asp	Leu	Lys	Val	Pro	Arg	Thr	Arg	Thr	Ser	Pro	Asp	Glu	Asp	Glu	Asp	530																
GTG	GCA	GAC	GCT	GAG	GAA	GAC	AAG	AAG	GAC	CTG	AAG	GTC	CCA	CGC	ACA	CGT	ACC	AGC	CCA	GAC	GAG	GAC	GAT	Pan-2																	
GTG	GCA	GAC	GCT	GAG	GAA	GAC	AAG	AAG	GAC	CTG	AAG	GTC	CCA	CGC	ACA	CGT	ACC	AGC	---	---	---	AGT	ACA	GAT	Pan-1																
Val	Ala	Asp	Ala	Glu	Glu	Asp	Lys	Lys	Asp	Leu	Lys	Val	Pro	Arg	Thr	Arg	Thr	Ser	---	---	---	Ser	Thr	Asp																	
Asp	Leu	Leu	Pro	Pro	Glu	Gln	Lys	Ala	Glu	Arg	Glu	Lys	Glu	Arg	Arg	Val	Ala	Asn	Asn	Ala	Arg	Glu	Arg	Leu	555																
GAC	CTT	CTC	CCC	CCA	GAG	CAG	AAG	GCC	GAG	CGG	GAG	AAG	GAG	CGC	CGC	GTG	GCC	AAT	AAC	GCC	CGA	GAG	CGC	CTG	Pan-2																
GAG	GTG	CTG	TCC	CTG	GAG	GAG	AAG	GAC	CTG	AGG	GAC	CGG	GAG	AGG	CGC	ATG	GCC	AAT	AAC	GCT	CGG	GAG	CGG	GTG	Pan-1																
Glu	Val	Leu	Ser	Leu	Glu	Glu	Lys	Asp	Leu	Arg	Asp	Arg	Glu	Arg	Arg	Met	Ala	Asn	Asn	Ala	Arg	Glu	Arg	Val																	
Arg	Val	Arg	Asp	Ile	Asn	Glu	Ala	Phe	Lys	Glu	Leu	Gly	Arg	Met	Cys	Gln	Leu	His	Leu	Ser	Thr	Glu	Lys	Pro	580																
CGT	GTG	CGC	GAC	ATC	AAT	GAG	GCC	TTT	AAG	GAG	CTC	GGC	CGC	ATG	TGC	CAG	CTG	CAC	CTC	AGC	ACT	GAG	AAG	CCG	Pan-2																
CGC	GTG	CGG	GAC	ATT	AAC	GAG	GCC	TTC	CGG	GAG	CTG	GGC	CGC	ATG	TGC	CAG	CTG	CAC	CTC	AAG	TCG	GAT	AAG	GCG	Pan-1																
Arg	Val	Arg	Asp	Ile	Asn	Glu	Ala	Phe	Arg	Glu	Leu	Gly	Arg	Met	Cys	Gln	Leu	His	Leu	Lys	Ser	Asp	Lys	Ala																	

Figure 2. (See following page for remainder of figure and for legend.)

Gln Thr Lys Leu Leu Ile Leu His Gln Ala Val Ala Val Ile Leu Ser Leu Glu Gln Gln Val Arg Glu Arg Asn 605
 CAG ACC AAA CTG CTC ATC CTG CAC CAG GCC GTG GCC GTC ATC CTC AGC CTG GAG CAG CAG GTG CGA GAA CGC AAC Pan-2
 CAG ACC AAG CTG CTG ATC CTG CAG CAG GCG GTG CAG GTT ATC CTG GGC CTG GAG CAG CAG GTG CGA GAA CGC AAC Pan-1
 Gln Thr Lys Leu Leu Ile Leu Gln Gln Ala Val Gln Val Ile Leu Gly Leu Glu Gln Gln Val Arg Glu Arg Asn

Leu Asn Pro Lys Ala Ala Cys Leu Lys Arg Arg Glu Glu Glu Lys Val Ser Gly Val Val Gly Asp Pro Gln Leu 630
 CTG AAC CCC AAA GCA GCC TGC TTG AAG CGG CGG GAG GAG GAG AAG GTA TCT GGC GTG GTC GGG GAT CCG CAG CTG

Ala Leu Ser Ala Ala His Pro Gly Leu Gly Glu Ala His Asn Pro Ala Gly His Leu 649
 GCG CTG TCA GCC GCC CAC CCG GGC CTG GGT GAG GCC CAC AAT CCA GCT GGG CAC CTG TGAGCCGTCACAGGGTCTTCGTT

GGACACGGGACCAACATCTCTGCCCGGGTGCACCCAGGATGGTCTGGATGAGACATCTC

Figure 2. Nucleotide sequence of Pan-1 and Pan-2 cDNAs and predicted amino acid sequences. An in-frame TAG termination codon 5' of the presumed initiator methionine of Pan-2 is underlined. A linked *EcoRI* site (GAATTCC) at the 5' end of the Pan-1 cDNA is also underlined. The nucleotide sequences of the Pan-1 and Pan-2 cDNAs are identical except for a GCA insertion that codes for an Ala residue at amino acid 168 of Pan-1 and a carboxy-terminal divergent region extending from amino acid 525 to 597 of Pan-2. The Leu components of a leucine zipper motif are circled. Sequencing of DNA was performed by use of the Sanger dideoxynucleotide method.

lated to the *Drosophila* daughterless protein (Caudy et al. 1988) and share a more limited structural similarity to c-myc (Battey et al. 1983) and proteins implicated in muscle cell differentiation, such as MyoD (Davis et al. 1987), Myogenin (Wright et al. 1989), and Myf-5 (Braun et al. 1989) in the putative DNA-binding domain (Fig. 4) (Murre et al. 1989). This region has been postulated to form an amphipathic helix-loop-helix involved in protein dimerization (Murre et al. 1989).

The protein sequences are distinctive in various regions of the molecule, suggesting the presence of functional domains. Five methionines are clustered at the amino-terminus (Met-Met-5-Met-16-Met-Met). With the exception of the first methionine, all amino-terminal

methionines obey the Kozak rules for efficient initiation of translation (Kozak 1989). Thus, they may serve as alternative sites for the initiation of translation. At the amino terminus, there is a short potential helical structure (which would be eliminated if translation initiated at position 25). The next 467 amino acids are rich in proline (11%) and glycine (14%) residues, thus it is unlikely that there are any extended helical motifs. This region is extraordinarily rich in hydroxy amino acids (serine 18%, tyrosine 3%) that represent potential phosphorylation sites. Tyrosine residues are present only in the region from amino acid 76 to 378 and are especially concentrated (16%) from amino acid 186 to 216. A putative leucine zipper motif (Landschulz et al. 1988) is found in the

Figure 3. Southern blot analysis and restriction map of the Pan gene. (Top: A, B, C, and D) Rat genomic DNA was digested with *EcoRI* (lane 1), *BamHI* (lane 2), *HindIII* (lane 3), *EcoRI* and *BamHI* (lane 4), *EcoRI* and *HindIII* (lane 5), and *HindIII* and *BamHI* (lane 6). (E) Rat genomic DNA was digested with *EcoRI* (lane 1), *BamHI* (lane 2), *HindIII* (lane 3), *HindIII* and *BamHI* (lane 4), *EcoRI* and *HindIII* (lane 5), and *EcoRI* and *BamHI* (lane 6). The transferred DNA was probed with: (A) *HindIII*–*BamHI* fragment from the Pan-1 cDNA coding for predicted amino acids 424–627 and encompassing the 3' divergent region; (B) the *HindIII*–*BamHI* fragment from the Pan-2 cDNA coding for predicted amino acids 423–626 and encompassing the 3' divergent region; (C) the entire Pan-1 cDNA; (D) the entire Pan-2 cDNA; and (E) *EcoRI*–*PstI* fragment from the Pan-1 cDNA coding for predicted amino acids 10–163. (Bottom) Restriction map of the Pan gene.

region from amino acid 385 to 420 [Leu(385)-Leu(392)-Leu(399)-Thr(406)-Leu(413)-Leu(420)]. In this region, similar sequences (Leu/Ile-His-Gly/Val-Leu) are found ending at alternating heptads at leucines 385, 399, and 413. If this region of the protein forms a coiled-coil structure, as is proposed for leucine zipper dimers, histidine residues 383, 397, and 411 would align on one face of the helix. The region surrounding and including the leucine zipper motif (276–457) is rich in histidine residues (6%). The region from amino acid 383 to 457 is especially histidine rich (12%). Two copies of a Ser-Ser-Ser-Gly-Asp repeat are found at positions 188 and 322. This peptide is also found in the human lamin A protein, which shares a limited structural similarity to the DNA-binding region of the E12 and E47 proteins (Fisher et al. 1986; Murre et al. 1989). A second repeat (Pro-Ser-Ser-Val/Ala-Tyr-Pro-Ser) is present at positions 182 and 207. The carboxy-terminal region beginning at amino acid 492 contains several features that distinguish it from the rest of the molecule, including periodic highly charged domains, a strongly hydrophobic domain, and the potential to form extended α helices. The region in the κ E2 binding proteins equivalent to amino acids 515–605 of Pan-2 contains the DNA-binding and dimerization domains (Murre et al. 1989). The carboxy-terminal region contains no tyrosine residues, and serines decrease from 18% to 4%. The abundance of glycine decreases from 12% in the first 490 residues to less than 1%: only 1 glycine is present in the region from amino acid 490 to 623. Highly charged domains in Pan-1 and Pan-2, comprised of both positive and negative charges, begin at positions 493, 508, 536, 551, and 614. Pan-2 contains an additional charged motif (comprised of 6 consecutive negatively charged amino acids) beginning at residue 525. A hydrophobic stretch of amino acids, present from 584 to 597, could form a short α -helix that corresponds to the amphipathic helix II postulated to form the helix-loop-helix structure of the E12- and E47-encoded proteins (Murre et al. 1989).

Expression of Pan cDNAs activates expression of a promoter containing multimers of the CACCTGTC motif

The putative transcriptional regulatory function of Pan-1 and Pan-2 was examined by a test of the ability of these proteins to regulate the expression of a reporter gene comprised of seven repeats of the chymotrypsin

consensus sequence linked to a prolactin minipromoter that drives transcription of the firefly luciferase gene (Chy7–Prl–Luc) (Nelson et al. 1988). This fusion gene is expressed at high levels in the cell lines AR4-2J and 266-6, which synthesize exocrine gene products, but is silent in HeLa cells (Meister et al. 1989). The Pan-2 cDNA driven by a cytomegalovirus enhancer and promoter selectively activates expression of the Ch7–Prl–Luc fusion gene when cotransfected into HeLa cells (Fig. 5). The same effect was observed when the carboxy-terminal region of Pan-2 was replaced with the similar region of Pan-1 in the expression vector. In contrast, cotransfection of a fusion gene encoding a truncated Pan-2 protein lacking 226 amino acids of the carboxyl terminus was unable to activate expression (Fig. 5). Thus, Pan-1 and Pan-2 can apparently function as transcriptional activators.

Pan-1 and Pan-2 bind insulin and SV40 enhancer motifs

Because regulatory elements of the rat insulin I gene and SV40 enhancer contain sequences related to the CACCTGTC motif of the pancreatic consensus sequence (Table 1), we tested whether the Pan-1 and Pan-2 proteins could bind to these elements in gel mobility-shift experiments. In vitro-translated Pan-1 and Pan-2 bind to the rat insulin I “Nir box” (Moss et al. 1988) as well as to a 38-bp region of the SV40 virus that contains the high affinity AP-4 binding element (Mermod et al. 1988) (Fig. 6). Pan-1 binds with a higher affinity than Pan-2 to the chymotrypsin B, Nir, and SV40 elements. Neither protein was able to bind to the chymotrypsin B element containing a mutated CACCTGTC motif. A comparison of the sequences bound by Pan-1 and Pan-2 suggests a binding site comprised of the consensus sequence of $CA^C/CCTG^T/C$ (Table 1). This CACCTGTC motif is present in the amylase 2A, chymotrypsin B, elastase I and trypsin I enhancers, and the 5'-flanking region of other genes expressed in acinar cells of the pancreas (Boulet et al. 1986; Cockell et al. 1989).

Discussion

cDNAs have been isolated independently from an HIT pancreatic endocrine cell line library by expression screening with an insulin Nir element oligomer, which encodes proteins that are highly related to Pan, E12, and

Figure 4. Related amino acids among Pan-1, Pan-2, the *Drosophila* daughterless protein (Caudy et al. 1988), and proteins implicated in muscle differentiation: MyoD (Davis et al. 1987), Myogenin (Wright et al. 1989), and Myf-5 (Braun et al. 1989). The boxed region represents identical amino acids. The regions compared are amino acids 535–607 of Pan-1, 533–605 of Pan-2, 533–605 of daughterless, 75–145 of Myf-5, 100–170 of MyoD, and 72–142 of Myogenin. The protein sequences were aligned using the Doolittle sequence analysis program in the Eu Gene package.

E47. The differences detected are likely to be due to alternative splicing (M. German et al., in prep.). These cDNAs are likely to be derived from the same gene, which encodes Pan-1 and Pan-2. Because a single 3.0-kb transcript is detected in all rat tissues tested (Fig. 7), and cDNAs encoding Pan/E12- and E47-related proteins isolated from lymphoid and pancreatic exocrine and endocrine cell lines, it is likely that Pan/E12,E47 gene products are distributed widely. In addition, apparent binding sites for Pan-1 and Pan-2 are also distributed widely. Other factors also cross-react with the CACCTGTC or related motifs, thereby increasing the binding repertoire and the opportunity for control over the transcriptional regulatory system. Purified AP-4 binds to the CACCTGTC motif present in the amylase 2A and other exocrine gene enhancers (Fodor et al., in prep.) and, conversely, Pan-1 and Pan-2 bind to the AP-4 element in SV40. The adenovirus MLTF likely binds to the CACCTGTC element because the MLTF binding site competes with the chymotrypsin B and insulin Nir elements in binding experiments using pancreatic exocrine and endocrine nuclear extracts (Moss et al. 1988; Meister et al. 1989). However, both the AP-4 gene (R. Tjian, pers. comm.) and the MLTF gene (R. Roeder, pers. comm.) appear to be distinct from the Pan/E12,E47 gene.

Functional specificity therefore may be determined by specific protein-protein interactions. For example, Pan proteins may interact with other factors that bind to the CACCTGTC element or to the contiguous TTTCCC motif. The putative leucine zipper, or carboxy-terminal dimerization domains may provide the structural basis for dimerization as in the case of Fos and Jun (Kouzarides and Ziff 1988). Such dimerization may be the fundamental unit of the activation complex, perhaps by providing a common structural substrate for some other

functional (linking?) protein. The affinities and specificities of the various interacting factors then may determine the functional competence of the complex. The understanding of the structure and function of this fundamental unit may lead to an understanding of the mechanisms underlying specific gene expression.

Materials and methods

Isolation of cDNA clones

An oligo(dT)-primed library was constructed using poly(A)-selected RNA prepared from the rat exocrine cell line AR4-2J. Nick-translated DNA comprised of multimerized consensus sequence was used to probe the library according to Vinson et al. (1988). The probe was composed of two complementary oligonucleotides of the following sequence, which includes rat chymotrypsin gene sequences from -213 to -186:

5'-GATCTCAGGGCACCTGTCCTTTTCCCATGGCCG
AGTCCCGTGGACAGGAAAAGGGTACCGGCCTAG-5'

The Pan-2 cDNA was isolated by hybridization screening of the AR4-2J library with an oligonucleotide complementary to a sequence present in the 5' region of Pan-1 cDNA. The oligonucleotide is comprised of the following sequence:

5'-TAGCGGGAACATCATACTGAAGTCCAGGAGGTC-3'

Plasmid constructions

Plasmids used as templates for in vitro transcription were constructed by cloning Pan-1 and Pan-2 cDNAs into the *EcoRI* site of a pGem-3Z vector (Promega). Expression vector plasmids were constructed by inserting Pan cDNA sequences into the cytomegalovirus enhancer- and promoter-containing expression vector pLPAΔS (gift of M. Walters, unpubl.). A *StuI* site 44 nucleotides 5' of the predicted Pan-2 initiator methionine

Figure 5. Sequence-dependent activation of gene expression by Pan proteins. Reporter plasmid (10 μ g) with (Chy7Prl) or without (Prl) seven copies of the chymotrypsin pancreatic consensus element (-213 to -186) linked to a prolactin minipromoter luciferase fusion gene (Nelson et al. 1988) was cotransfected with 2 μ g of Pan expression plasmids into HeLa cells. Cells were harvested 48 hr later, and luciferase activity was measured. Expression of Pan Δ , Pan-1, and Pan-2 cDNAs was controlled by a cytomegalovirus enhancer and promoter. Pan-1 replaced the carboxy-terminal divergent region of Pan-2 with that of Pan-1. Pan Δ contains amino acids 1-423 of Pan-2, wherein the putative DNA-binding domain is deleted. A Rous sarcoma virus luciferase construct (RSV-LUC; de Wet 1986) was used to monitor the efficiency of transfection. Results shown represent the data from one experiment. Similar results were obtained in three independent experiments.

Table 1. Sequence comparison of Pan binding elements and related enhancer motifs

Species	Enhancer	Sequence	Element
Rat	chymotrypsin B	CACCTGT	Pan
Rat	amylase 2A	CAGCTGT	Pan
Rat	elastase I	CACCTGT	Pan
Rat	trypsin I	CACCTGT	Pan
Rat	carboxy A1	CACCTGC	Pan
Human	immunoglobulin μ	CAGCTGC	μ E2
	heavy chain	CACCTGC	μ E5
Human	immunoglobulin κ	CACCTGC	κ E2
Simian	simian virus 40	CAGCTGT	AP-4
Rat	insulin I	CATCTGT	Nir
		CA $\frac{C}{G}$ CTG $\frac{T}{C}$	consensus

Nucleotide sequences in the pancreatic consensus sequence present in the rat pancreatic exocrine genes: chymotrypsin B, amylase 2A, elastase I, trypsin I, and carboxypeptidase AI (Boulet et al. 1986) are compared to the rat insulin I gene Nir enhancer element (Moss et al. 1988), immunoglobulin enhancer elements (Murre et al. 1989), and the SV40 AP-4 element (Mermod et al. 1988). A proposed Pan consensus binding site is depicted at *bottom*.

codon was converted to a *Bgl*II site by cleavage and ligation to *Bgl*II linkers. Pan-2 cDNA coding for a predicted full-length protein containing *Bgl*II and *Xba*I ends was inserted into the *Bam*HI and *Xba*I sites of pLPAAS. Pan-1 was constructed by the replacement of the *Hind*III–*Xba*I fragment of Pan-2 with the *Hind*III–*Xba*I fragment of Pan-1 (Fig. 2), which resulted in the exchange of the carboxy-terminal divergent region of Pan-2 with that of Pan-1. Pan Δ was constructed by the insertion of a *Bgl*II–*Hind*III fragment of Pan-2 cDNA, which resulted in the deletion of sequences coding for the 226 carboxy-terminal amino acids of Pan-2. The reporter plasmid Chy7Prl contained seven copies of the rat chymotrypsin B consensus sequence (–213 to –186) linked to a prolactin minipromoter luciferase fusion gene PrILuc (Nelson et al. 1988).

Figure 6. Pan-1 and Pan-2 bind chymotrypsin, insulin, and SV40 enhancer elements. In vitro-produced Pan-1 and Pan-2 were incubated with radiolabeled enhancer element probes and binding was analyzed by gel mobility-shift assays. (Lanes *a*) Rabbit reticulocyte lysate containing in vitro-translated BMV control RNA (Promega) and specific enhancer probe; (lanes *b*) lysate containing in vitro-translated Pan-1 and specific enhancer probe; (lanes *c*) lysate containing in vitro-translated Pan-2 and specific enhancer probe. Lanes designated Chymo contain a chymotrypsin B consensus element probe. Lanes designated Insulin contain the rat I insulin Nir element probe. Lanes designated SV40 contain a region that includes the SV40 AP-4 site as probe. Lanes designated B Mut contain chymotrypsin element block mutant B (Fig. 1), which has a mutation of the CACCTGTC sequence of the chymotrypsin B element as a probe.

Gel mobility-shift assays

Linearized template (5 μ g) was transcribed using T7 polymerase according to the manufacturer's directions (Promega). One-third of the RNA produced was used in a 40- μ l in vitro translation reaction using rabbit reticulocyte lysate as recommended by the manufacturer (Promega). The rabbit reticulocyte lysate (3 μ l) was incubated with 200 pg of 32 P 5'-end-labeled probe (10,000 cpm) in a 6- μ l final volume of a binding reaction containing 70 mM potassium acetate, 15 mM Tris (pH 7.8), 1 mM EDTA, 5% glycerol, 5 mM MgCl₂, and 20–100 ng of poly[d(I-C)]. After incubating at room temperature for 15 min, the reaction mix was electrophoresed on a 5% nondenaturing polyacrylamide gel. Identical translation reactions using 35 S-labeled methionine were performed in parallel with the Pan-1, Pan-2, and truncated templates to verify that equivalent amounts of protein were synthesized. The labeled translation reactions were analyzed on 10% acrylamide gels containing SDS. For binding competition experiments, 2- or 20-fold molar excess of competing unlabeled DNA was added to the labeled probe prior to the addition of lysate. The rat chymotrypsin B probe consisted of sequences extending from –213 to –186 (Boulet et al. 1986). The rat insulin Nir element probe consisted of sequences extending from –124 to –93 of the rat insulin I gene (Moss et al. 1988). The SV40 probe containing the high-affinity AP-4 binding site consisted of sequences extending from 279 to 246

Figure 7. Northern blot analysis of Pan transcripts in rat tissues and cell cultures. RNA prepared from rat tissues, the AR4-2J (Jessop and Hay 1980) and 266-6 (Ornitz et al. 1985) pancreatic exocrine cell lines, and the HIT pancreatic endocrine cell line (Edlund et al. 1985) were hybridized with a 32 P-labeled Pan-2 restriction fragment 2.5-kb (*Eco*RI–*Bam*HI fragment). A single 3.0-kb hybridizing species is detected in all samples tested.

of the SV40 viral genome [Mermod et al. 1988]. All probes consisted of complementary annealed oligonucleotides ^{32}P -end-labeled to approximately equal specific activities.

Methylation interference assays

For methylation interference experiments, a preparative binding reaction was conducted using a 10-fold scale-up of the binding conditions described. The radiolabeled probe was partially methylated [Maxam and Gilbert 1980], and the bound complex was visualized and isolated by autoradiography following native gel electrophoresis as described previously. DNA fragments were recovered by electroelution and subjected to piperidine cleavage according to Maxam and Gilbert [1980]. The cleavage products were resolved on 12% polyacrylamide gels containing 8 M urea.

Cell culture and transfections

HeLa cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 4.5 mg of glucose per ml. Transfections were performed using calcium phosphate precipitates containing 10 μg of reporter plasmid and 2 μg of expression plasmid as described [Nelson et al. 1988].

Luciferase assays

Luciferase assays were performed using 50 μg of protein extract as described [Nelson et al. 1988]. Background activity was measured by performing luciferase assays without added extract, and the background activity subtracted from each measured value.

Southern blot analysis

Rat liver genomic DNA (9 μg) was digested with 150 units of *Eco*RI, *Bam*HI, or *Hind*III restriction enzymes, separately or in combination, and electrophoresed on a 0.8 % agarose gel. The DNA was transferred to an Optibond nitrocellulose membrane [Schleicher & Schuell]. The transfer of DNA to the membrane, hybridizations, and washes were performed according to the manufacturer's directions [Schleicher & Schuell]. The final wash was performed using a $0.1 \times$ SSC solution containing 0.1% SDS at 55°C for 30 min.

Northern blot analysis

Total cellular RNA was prepared according to Chirgwin et al. [1979]. Total cellular RNA (15 μg) or poly(A)-selected RNA (pancreas only; 8 μg), were resolved by electrophoresis on a 1.3% agarose gel containing formaldehyde and transferred to a GeneScreen membrane according to the manufacturer's directions [New England Nuclear]. Then, the RNA was hybridized with 5×10^6 cpm per milliliter of ^{32}P -radiolabeled probe. The blotting, hybridization, and wash conditions were carried out according to the manufacturer's directions [New England Nuclear]. RNA size standards (BRL) were used to calculate the size of hybridizing species.

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Note added in proof

Sequence data described in this paper have been submitted to the EMBL/GenBank data libraries.

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