Binding of a pancreatic nuclear protein is correlated with amylase enhancer activity

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Received June 23, 1989; Revised and Accepted September 8, 1989

ABSTRACT

The mouse amylase gene *Amy*-2.2 is expressed at high levels specifically in the acinar cells of the pancreas. The region between -172 and -110 of this gene includes sequence elements common to pancreas-specific genes. Nuclear proteins with specific affinity for this region were partially purified from rat pancreas. The consensus element of another pancreas-specific gene, elastase 1, competes for protein binding to the amylase sequences. Binding was localized by DNase I protection to the sequence -156 to -122. Site-directed mutagenesis of this sequence resulted in concomitant loss of protein binding and enhancer activity. Photo-affinity labelling of pancreatic nuclear extracts identified one predominant binding protein with a molecular weight of approximately 75 kDa. The data indicate that binding of this nuclear protein is essential for the enhancer activity of this pancreas-specific element.

INTRODUCTION

Eukaryotic gene transcription is regulated by the interaction of cis-acting DNA control elements with specific DNA-binding proteins (1, 2). Some cis-acting elements are widely distributed, while others are limited to genes with specific patterns of expression. Mammalian differentiation is likely to involve coordinated gene activation by tissue-specific transcriptional factors which recognize cis-acting elements shared by genes which are expressed in the same tissue. Shared cis-acting sequences have already been identified for erythroid-specific (3, 4), pancreas-specific (5-10) and pituitary-specific genes (11). The product of the recently cloned *pit* -1 gene appears to be an example of a transcriptional activator which recognizes a sequence common to pituitary-specific genes (11-13).

The acinar cell of the mammalian pancreas has been a useful model system for analysis of tissue-specific gene expression, because several genes are expressed at high levels specifically in this tissue. A 20 bp pancreatic consensus sequence was identified by sequence comparison of the 5' flanking regions of several of these genes, including amylase, elastase, chymotrypsin, trypsin, ribonuclease, and carboxypeptidase A (5, 7). Expression studies in transgenic mice have been used to localize pancreas-specific determinants of the amylase and elastase genes. We demonstrated that a 227 bp fragment (-208 to +19) of the mouse *Amy*-2.2 pancreatic amylase gene directed pancreas-specific expression of the chloramphenicol acetyltransferase gene (10). A hybrid gene containing the elastase 1 enhancer fragment -205 to -72 ligated to the human growth hormone gene was also expressed specifically in the pancreas of transgenic mice (14). In transfected cells, a pancreas-specific enhancer of the rat amylase gene was localized to the region -235 to -41, by transfer of amylase fragments to the thymidine kinase promoter (7). Sequences between -154 and -115 were essential for enhancer activity (7). In all of these studies, the

functional 5' flanking sequences included a pancreas consensus element. These observations are consistent with a model in which the pancreatic consensus functions as a cis-acting element for pancreas-specific gene expression. The purpose of this study was to investigate the interaction of the consensus element with nuclear proteins from pancreas.

We have investigated protein binding to the mouse *Amy-2.2* enhancer. While this manuscript was in preparation, a similar study of the closely related *Amy-2.1* gene was reported (15). Our results confirm the reported location and specificity of protein binding to the amylase consensus. In addition, we have used site-directed mutagenesis of the binding site to demonstrate a correlation between protein binding and enhancer activity. We have also characterized the enhancer binding protein by photo-affinity labelling.

MATERIALS AND METHODS

Plasmid construction

A *Bgl* II/*Mbo* II fragment (nucleotides -208 to -82) and an *Mnl* I fragment (nucleotides -172 to -110) were isolated from the 5' flanking region of the mouse *Amy-2.2* gene, whose sequence was previously reported (8). These fragments were cloned into the blunted *Pst* I site of the polylinker located 12 bp upstream of position -205 of the rat elastase 1 promoter in the plasmid pE1mCAT (16). This plasmid was kindly provided by Dr. Raymond MacDonald. A synthetic oligonucleotide corresponding to enhancer mutant 1 was cloned into the *Pst* I and *Sal* I sites of the polylinker. Enhancer mutant 2 was constructed by cloning two synthetic oligonucleotides, corresponding to amylase fragments -167 to -138 and -136 to -108, into the *Pst* I and *Sal* I sites of the polylinker respectively. The net increase of 4 bp between the two pancreatic amylase elements I and II in this mutant changes their relative helical orientation. Each construct was sequenced by the dideoxynucleotide method of Sanger to confirm the sequence and orientation of the insert (17).

Cell culture, DNA transfections, and measurement of CAT[†] activity

The mouse pancreatic acinar cell line 266-6 (18) and the rat pancreatic acinar cell line AR42J (19) were transfected in Eagle's minimum essential medium containing 10% fetal calf serum by the calcium phosphate method (20). Four hours after addition of 5 μ g of test plasmid DNA, 266-6 cells were washed and incubated for an additional 44 hours. Subconfluent cultures of AR42J cells were transfected with 10 μ g of test plasmid DNA and grown in medium containing 100 μ M chloroquine. Four hours later, the AR42J cells were shocked with 15% glycerol for 2.5 minutes, rinsed, and incubated for an additional 44 hours. Preparation of cell extracts and CAT enzyme assays were performed according to Gorman (21). Protein concentration was determined by the Bradford method (22). CAT assays of extracts from 266-6 cells contained 180 μ g of protein and were incubated at 37°C for 3 hours; assays of extracts from AR42J cells contained 50 μ g of protein and were carried out for 7 hours.

Preparation and chromatography of nuclear extracts

Nuclei were prepared from rat pancreas by the method of Blobel and Potter (23). In one experiment (Figure 2), dog pancreas was utilized. Pancreatic nuclear protein from rat and dog gave indistinguishable results in gel retardation and DNase I protection assays. Nuclear proteins were extracted by the method of Dignam *et al.* (24). The nuclear extract was dialyzed against buffer containing 10 mM HEPES, pH 7.9, 50 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 1 mM EGTA, 0.5 mM PMSF, 2 mM sodium vanadate, and 20% glycerol. Protein was quantitated by the Bradford method (22). Nuclear extracts were adjusted to

Table 1. Enhancer activity of amylase sequences in pancreatic acinar cell lines AR42J and 266-6. Cell transfections and CAT assays were carried out as described in Materials and Methods. Data represent mean ± S.D. for the indicated number of independently transfected cultures.

Plasmid	CAT activity (% conversion)	
	AR42J cells	266-6 cells
pSVoCAT	0.7 ± 0.2 (4)	0.1 (2)
pE1mCAT	1.5 ± 0.2 (4)	5.3 ± 1.3 (9)
Amy(−208 to −82)E1CAT	9.2 ± 2.0 (4)	32 ± 6.9 (3)
Amy(−172 to −110)E1CAT	12 (1)	31 ± 9.8 (8)
Amy (−110 to −172)E1CAT		28 ± 6.6 (3)

100 mM KCl before passage over a BioRex-70 cation exchange column equilibrated with buffer containing 10 mM HEPES, pH 7.9, 100 mM KCl, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, and 20% glycerol. Proteins were eluted with a linear gradient of KCl (0.1 M to 1.0 M) or, in later experiments, with 0.5 M KCl. The yield after chromatography was 30% of the original activity, with 20-fold purification.

Gel retardation

Isolated DNA fragments or double-stranded oligonucleotides were radio-labelled with [α -³²P]dGTP using the Klenow fragment of DNA polymerase (Boehringer Mannheim). Gel retardation assays were carried out as described by Singh *et al.* (25). Labelled DNA fragments were incubated for 20 minutes at 22°C in a total volume of 20 μ l containing 10 mM HEPES, pH 7.9, 100 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 1 mM EGTA, 10% glycerol, 3 μ g poly (dI:dC), 1.5 μ g *E. coli* DNA, 10 μ g of protein from nuclear extract or 2.5 μ g of partially purified protein, and 0.1–1.0 ng of labelled probe (ca. 30,000 cpm). Electrophoresis through a 6% polyacrylamide gel (acrylamide/bisacrylamide ratio, 25:1) was carried out in 45 mM Tris, pH 8.0, containing 45 mM borate and 1 mM EDTA for 2.5 hours at 30 mAmps. The gel was dried and visualized by autoradiography.

DNase I footprinting and G-methylation interference

Radio-labelled probe was prepared from the plasmid containing the 126 bp *Bgl* II/*Mbo* II fragment in pE1mCAT (described above). After digestion of plasmid DNA with *Hind* III, either the coding strand was labelled with T4 polynucleotide kinase (Boehringer Mannheim), or the noncoding strand was labelled with the Klenow fragment of DNA polymerase. Following digestion with *Sal* I, the labelled fragment was isolated from a 6% acrylamide gel.

For DNase I footprinting, binding reactions contained 20 mM HEPES, pH 7.9, 50 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 20% glycerol, 7 μ g of column-purified nuclear protein, 4.5 μ g poly (dI:dC), 3 μ g *E. coli* DNA and 100,000 cpm of

Figure 1. The amylase pancreatic consensus is divided into two elements. The consensus elements of the mouse Amy-2.2 gene (8) and the rat elastase 1 gene (16) are aligned for comparison. The rectangles enclose amylase elements I and II (7–9), which have also been designated the A box and the B box (15).

Figure 2. Gel retardation assay and ion-exchange chromatography of nuclear extract from pancreatic tissue. **A.** Fifteen mg of pancreatic nuclear protein was fractionated on a 2 ml BioRex-70 column and eluted with a linear gradient of KCl, 0.1 M to 1.0 M. A one μ l aliquot of each 450 μ l fraction was assayed by gel retardation with the labelled 63 bp enhancer fragment, as described in Materials and Methods. Column fractions are numbered below the gel. NE, unfractionated nuclear extract. The arrow marks the start of the KCl gradient. **B.** Nuclear extract was incubated in the presence of a 250-fold molar excess of the indicated unlabelled competitor. Lane 1, without protein; lane 2, without competition; lane 3, self competition; lane 4, competition by the unrelated pBR322 fragment +3728 to +3846 (28).

radiolabelled DNA fragment. After 20 minutes of incubation, MgCl_2 and CaCl_2 were added to 5 mM and the DNA digested for 30 seconds with 0.01 units of DNase I. EDTA was added to a final concentration of 5 mM and the reactions were loaded onto a 6% polyacrylamide gel (acrylamide/bisacrylamide ratio, 25:1). Following electrophoresis, both bound and free bands were excised and eluted in 0.5 M ammonium acetate containing 1 mM EDTA and 0.1% sodium dodecylsulfate. DNase I digestion products were extracted with phenol:chloroform, precipitated, and analyzed by autoradiography after electrophoresis on an 8% polyacrylamide/8 M urea sequencing gel.

For G-methylation interference (26), the labelled probe was modified with dimethyl sulfate in 50 mM sodium cacodylate, pH 8.0 containing 1 mM EDTA at 22°C for 5 minutes. The methylation reaction was stopped with 1.5 M sodium acetate, pH 7.0 containing 1 M β -mercaptoethanol, and the DNA was precipitated. The methylated probe was incubated with column-purified nuclear protein as described above for DNase I footprinting and subjected to gel electrophoresis to separate protein-bound from free probe. DNA recovered from the gel electrophoresis was cleaved at methylated guanine residues by treatment with 1 M piperidine for 30 minutes at 90°C. After precipitation, cleavage products were analyzed on a sequencing gel as described above.

Photo-affinity labelling

The method of Chodosh *et al.* (27) was used to cross-link the pancreatic proteins to the enhancer fragment. Single-stranded oligonucleotides corresponding to sequences -167 to -139 of the coding strand or -109 to -134 of the noncoding strand were annealed to the complementary strand of the 63 bp *Mnl* I fragment extending from -172 to -110.

Figure 3. Protection of enhancer sequences by pancreatic nuclear protein. Coding and noncoding strands of the 126 bp *Bgl* II/*Mbo* II fragment (-208 to -82) were end-labelled and assayed as described in Materials and Methods in the presence (+) or absence (-) of 7 μ g of column-purified pancreatic protein. Free and bound fractions were preparatively isolated from an acrylamide gel. Maxam and Gilbert G + A sequencing reactions were used to identify the protected sequences. A. DNase I protection. (To visualize the upstream boundary of the protected region on the noncoding strand, a longer exposure of this gel was obtained.) B. Methylation interference. G-residues were methylated prior to the binding reaction, as described in the text. C. Summary. Residues protected from DNase I digestion are boxed; methylated guanine residues which prevent protein binding are marked. Consensus elements I and II are underlined.

Figure 4. Competition for binding of pancreatic nuclear proteins. The elastase consensus element is described in Figure 1. Mutated amylase enhancers M1 and M2 are described in the text. Gel retardation assays were carried out with the labelled 63 bp amylase enhancer fragment. 1, 100-fold molar excess; 2.5, 250-fold molar excess; 5, 500-fold molar excess. Lane 7, no competitor.

The oligonucleotide primer was extended by the Klenow fragment of DNA polymerase in the presence of dATP, dGTP, [α - 32 P]dCTP, and 5-bromo-2'-deoxyuridine triphosphate (BrdU). The resulting probes were labelled between -136 and -110 [32 P] BrdU probe 1) and between -172 and -138 [32 P] BrdU probe 2) (sequences in Figure 1). Protein binding reactions were carried out as described for gel retardation using 30 μ g of protein from nuclear extracts and 50,000 cpm of labelled probe. After the samples were irradiated under UV light for 30 minutes, unprotected DNA was digested with 50 units of micrococcal nuclease and 0.01 unit of DNase I at 37°C for 30 min. The covalently-labelled proteins were separated by electrophoresis through 10% polyacrylamide containing sodium dodecyl sulfate and visualized by autoradiography.

RESULTS

Enhancer activity of a 63 bp amylase fragment

The *Amy*-2.2 promoter fragment (-208 to +19) is sufficient to direct pancreas-specific expression of CAT in transgenic mice (10), but is expressed at a very low level in transfected pancreatic cell lines (unpublished observations). The low level of expression has made it difficult to carry out deletion analysis to identify the functional elements of this promoter. To circumvent this difficulty, we have used a heterologous pancreas-specific promoter. Restriction fragments from the *Amy*-2.2 promoter were cloned upstream of the elastase 1 promoter in the plasmid pE1mCAT (16) as described in Methods. This plasmid contains the functional elastase promoter fragment -205 to +22, including enhancer domains sufficient for acinar cell specific expression in transfected cells (16 and Table 1). Expression

of the resulting constructs was tested by transfection of the pancreatic acinar cell lines AR42J and 266-6. Several amylase fragments increased the basal activity of pE1mCAT (Table 1). Amylase fragment -208 to -82 increased CAT activity approximately six-fold. The 63 bp amylase fragment -172 to -110, tested in both orientations, was comparable in activity to the larger fragment. The smaller fragments -167 to -139 and -130 to -108 had little effect on CAT activity (data not shown). The active 63 bp fragment was therefore used in subsequent assays to detect enhancer binding proteins. This *Amy*-2.2 enhancer is very similar in sequence and position to the previously described enhancer of the rat amylase gene (7).

The pancreatic consensus sequences of *Amy*-2.2 and elastase 1 are compared in Figure 1. The 20 bp elastase consensus is typical of other pancreas-specific genes (7, 15). The best alignment of amylase with the other genes is obtained by dividing the consensus into two smaller elements, which are inverted in position and separated by 17 nucleotides. These regions have been designated element I and element II (8, 9) or box A and B (15) of the amylase enhancer. The data in Table I demonstrate that constructs with two pancreatic consensus elements are expressed at a higher level than the original elastase construct containing a single consensus.

The amylase enhancer binds nuclear proteins from pancreatic tissue

In order to detect nuclear proteins with affinity for the amylase enhancer, the 63 bp fragment was labelled and incubated with nuclear extract from pancreatic tissue. After gel electrophoresis, a retarded complex was observed (Figure 2A, lane NE). The DNA binding activity was partially purified by chromatography on Bio-Rex 70 (Figure 2A). Column-purified protein from rat pancreas was used in subsequent assays. The sequence specificity of binding is demonstrated by the competition observed in the presence of a 250-fold molar excess of the unlabelled 63 bp fragment (Figure 2B, lane 3), while an equivalent excess of an unrelated DNA fragment did not compete for binding (lane 4).

The DNase I protection assay was used to localize the bound protein. In the presence of nuclear protein, nucleotides -156 to -122 on the coding strand and -159 to -120 on the noncoding strand were protected from DNase I digestion (Figure 3A). The border at residue -159 of the noncoding strand was visible on a longer exposure of the autoradiograph in Figure 3A. Methylation of guanine residues at positions -152 and -150 of the coding strand prevented protein binding (Figure 3B). The protection and interference data indicate that consensus elements I and II are included in the protein binding site (Figure 3C).

The elastase consensus element competes for protein binding with the amylase enhancer

The different arrangement of the pancreatic consensus element in the elastase and amylase genes is shown in Figure 1. To determine whether the same protein could recognize both versions of the consensus, we tested the ability of the elastase element to compete for binding of the amylase element. The double-stranded oligonucleotide corresponding to the elastase 1 sequence -122 to -92 (Figure 1) was an effective competitor for binding to the labelled amylase enhancer (Figure 4, lanes 4 to 7). We conclude that the protein detected in the band shift assay can bind both of these functionally related sequences, in spite of their differences in organization.

Protein binding is necessary for enhancer activity

Mutations were introduced into the amylase consensus by chemical synthesis. Mutant 1 was generated by substituting the sequence AAGGA for the highly conserved pentanucleotide -133 TCACC -129 in consensus element II (Figure 1). The mutated

Figure 5. Effect of mutagenesis on protein binding. Mutated enhancers M1 and M2 are described in the text. –, without protein; +, with 2.5 μ g of protein.

enhancer did not produce a specific protein complex in gel retardation assays (Figure 5), and did not compete effectively with the wild type enhancer sequence for protein binding (Figure 4, lanes 7 to 10).

In mutant 2, the thymidine residue at position –137 (Figure 1) was replaced with the pentanucleotide GTCGA. Neither consensus element is changed in mutant 2, but the distance between them is increased by 4 basepairs. This mutation was also defective in protein binding (Figure 5) and did not compete for binding of the wild type enhancer (Figure 4 lanes 11–13).

To determine whether the inability to bind protein would influence enhancer activity, the mutated enhancers were cloned upstream of the elastase promoter and tested for

Table 2. Effects of mutation on enhancer activity. The enhancer activity of synthetic oligonucleotides corresponding to mutants 1 and 2 (Figure 1) was compared with the wild type sequence. Transfection of 266-6 cells and CAT assays were carried out as described in Materials and Methods. Data represent mean $\pm$ S.D. for the indicated number of independently transfected cultures.

Plasmid	CAT activity (% conversion)
Amy (–172 to –110) EICAT	42 $\pm$ 15 (6)
Amy (mutant 1) EICAT	3.5 $\pm$ 0.6 (6)
Amy (mutant 2) EICAT	6.0 $\pm$ 2.8 (6)

Figure 6. Photo-affinity labelling of the enhancer-binding proteins. Pancreatic nuclear protein was incubated with the 63 bp [32 P] BrdU-labelled probes 1 and 2, described in the text. Covalently labelled proteins were separated on 10% polyacrylamide gels containing sodium dodecyl sulphate. A. Arrows indicate the positions of the molecular weight markers β -galactosidase, phosphorylase B, albumin, ovalbumin and carbonic anhydrase. The estimated size of the covalently labelled DNA/protein complexes are indicated at the left. Lane 1, probe 2; lanes 2–4, probe 1. The reaction in lane 4 did not include nuclear protein. B. Specificity of covalent labelling. Probe 1 was incubated with nuclear protein in the absence of competitor (–) or in the presence 50-fold molar excess of unlabelled competitors.

expression in transfection assays. The activities of both mutants were significantly reduced when compared with the wild type sequence (Table II).

Photo-affinity labelling of enhancer binding proteins

The double-stranded probes were labelled with [α - 32 P]dCTP and 5-bromo-2'-deoxyuridine triphosphate between nucleotides –136 and –110 (probe 1) and between –172 and –138 (probe 2), as described in Materials and Methods. UV cross-linking of nuclear proteins to each of these probes produced a labelled complex of approximately 75 kDa (Figure 6A). This observation indicates that pancreatic nuclei contain one major protein with affinity for the amylase enhancer. We did not observe any protein with preferential affinity for element I or element II, since both probes generated the same labelled bands. Labelling of the 75 kDa protein was specifically competed in the presence of a 50-fold molar excess of unlabelled enhancer, but was not competed by an unrelated DNA fragment (Figure 6B). The minor bands of 54 and 33 kDa visible on the gels may be the result of degradation of the 75 kDa protein, or the binding of additional proteins with lower affinity or lower abundance in the nuclear extracts.

DISCUSSION

Comparison of 5' flanking sequences provided the first evidence for a consensus element common to all genes expressed in the acinar cells of the pancreas (5). Subsequent functional studies showed that fragments containing this consensus act as pancreas-specific enhancers in cultured cells (7, 15, 16) and in transgenic mice (10, 14). We have now demonstrated that the consensus sequences within the mouse *Amy*-2.2 promoter are included within a binding site for a pancreatic nuclear protein, and that protein binding appears to be required for enhancer activity. Protein binding has been characterized by gel retardation, DNase I footprinting, and G-methylation interference. UV cross-linking to the amylase enhancer labelled a nuclear protein with an estimated molecular weight of 75 kDa.

The amylase gene differs from the other pancreas-specific genes, such as elastase, in that the consensus sequence of the amylase gene is divided into two elements which are separated by 17 bp. The individual elements I and II of the amylase consensus do not act independently as binding sites in gel retardation assays, and do not enhance expression at a level comparable to the intact enhancer (unpublished observations). [³²P] BrdU probes labelled in element I or element II did not cross-link different nuclear proteins. Our results indicate that the amylase enhancer comprises a single functional unit, with a binding site that is recognized by a single protein. The same protein can bind the enhancer of the elastase gene, in spite of the difference in sequence organization represented in Figure 1.

Our DNase protection and elastase competition studies agree with the recent report by Cockell *et al.* (15). By these criteria, the rat pancreatic protein which we characterized appears to be the same as the protein isolated from AR42J cells, which was designated PTF1 (15). The protected region of the *Amy*-2.2 gene differs at 11/39 nucleotides from the *Amy*-2.1 gene studied by Cockell *et al.*, yet the footprints and guanine contact residues of the two are virtually identical. Cockell *et al.* also demonstrated that PTF1 interacts with the trypsin, chymotrypsin and carboxypeptidase genes. These pancreas-specific structural genes are unlinked in the human and mouse genomes, and have therefore evolved separately for more than 80 million years. During this time, the pancreas-consensus elements have diverged substantially in sequence while retaining affinity for PTF1.

Boulet *et al.* observed altered enhancer activity after introduction of mutations within elements I and II of the rat amylase enhancer (7). We have found that mutations within and adjacent to element II destroy protein binding and also reduce enhancer activity. The concomitant loss of enhancer activity and protein binding by these mutants provides functional evidence for a positive effect of protein binding on gene expression. Confirmation of the proposed function will depend upon the future isolation of pure PTF 1 protein and/or cloning of the PTF 1 gene.

ACKNOWLEDGEMENTS

We thank Dr. Raymond MacDonald for the gift of plasmid pE1mCAT, and Drs. Linda Samuelson and Deborah Gumucio for critical reading of the manuscript. This research was supported by USPHS Research Grant DK36089 and Training Grant HD07247. GH was supported by National Research Service Award DK 08137. TMJ acknowledges support from the Rackham School of Graduate Studies, University of Michigan.

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¹**Abbreviations used:** CAT, chloramphenicol acetyltransferase; EGTA, [ethylenbis-(oxyethylenetriolo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; PMSF, phenyl methyl sulfonyl fluoride.

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