Binding Sites for Hepatocyte Nuclear Factor 3β or 3γ and Pancreas Transcription Factor 1 Are Required for Efficient Expression of the Gene Encoding Pancreatic α-Amylase

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Efficient expression of genes under the control of α-amylase 2 5′-flanking sequences in exocrine pancreatic cells requires, in addition to the pancreas transcription factor 1 binding site (M. Cockell, B. J. Stevenson, M. Strubin, O. Hagenbüchle, and P. K. Wellauer, Mol. Cell. Biol. 9:2464–2476, 1989), another *cis*-acting element at positions −60 to −86. This DNA element, which contains an AT-rich core, constitutes a high-affinity binding site for nuclear proteins present not only in the pancreas but also in other tissues and cell lines derived from the endoderm. Purification of binding activities from pancreatic cells by DNA affinity chromatography reveals several distinct proteins ranging in size from 45 to 54 kDa (p45, p47/48, and p54). All of these proteins interact with the specific DNA sequence upon renaturation in vitro. Protein sequencing, electrophoretic mobility shift assay, and immunoblot analyses identify p54 and p47/48 as members of the hepatocyte nuclear factor 3 (HNF3 [forkhead]) family of transcription factors. p54 belongs to the subfamily of HNF3β proteins, while p47/48 binding activity includes HNF3γ. The cDNAs for two HNF3β proteins differing only in N-terminal amino acid sequences were isolated from a pancreatic cDNA library. The mRNAs encoding the two protein species accumulate to different steady-state levels in poly(A)+ RNA of pancreatic cells. Our results support a model by which the pancreas-specific expression of the α-amylase gene is mediated by a combination of cell-specific and cell lineage-specific transcription factors.

Transcriptional activation of eukaryotic genes throughout organogenesis is mediated by the regulated assembly of protein complexes at promoters and enhancers. The activation of genes encoding tissue-specific proteins in terminally differentiated cells is the final result of this process. The transcription factor complexes which activate the genes in terminally differentiated cells may therefore be expected to contain components that are specific for a particular cell lineage.

Transcription of the genes encoding specific products of the exocrine pancreas requires the binding of a pancreas-specific protein complex, pancreas transcription factor 1 (PTF1), to an enhancer element present in the 5′-flanking regions of these genes (5, 27, 34). It appears unlikely, however, that PTF1 alone will be sufficient to modulate the expression of individual genes of the pancreas in response to different physiological stimuli or different nutritional needs. It may be expected, therefore, that the activator complexes formed in vivo also contain gene-specific components. To understand how such regulatory complexes function, it will be necessary to identify these components and study their individual roles.

The regulatory sequences of exocrine pancreas-specific genes have not yet been thoroughly dissected, although in vitro DNase I footprinting suggests they will all turn out to be relatively complex (5, 9). None of the potential sites of protein-DNA interaction identified by footprinting, other than the PTF1 binding sites, shows any obvious conservation of sequence motifs. Thus, it is difficult to ascertain which sites are biologically relevant from footprint analysis alone.

Functional evidence exists which suggests that *cis*-acting elements other than the PTF1 binding sites do play an essential role in the transcriptional control of pancreas-specific genes. Even though multimers of the PTF1 binding sites from the chymotrypsinogen gene (20) and the elastase 1 gene (26) have been shown to stimulate transcription of reporter genes in cultured cells and transgenic mice, respectively, a single copy of either element is not sufficient to activate transcription. In the minimal elastase 1 enhancer that is capable of conferring high-level, position-independent expression to a homologous promoter in cultured pancreatic cells, two other functional elements (termed B and C) have been identified in addition to the PTF1 binding site (termed the A element [9, 10]). Combinations of any two of these three elements mediate exocrine pancreas-specific expression in transgenic mice (35), while transcription of reporter genes under the control of a multimerized B element is redirected to the endocrine pancreas (11). Multimers of the PTF1 binding site, although able to convey exocrine cell-specific transcription, give a greatly reduced level of transcription in transgenic animals compared with a single copy of the minimal enhancer, and only a small subpopulation of exocrine cells express the transgene (26). The multimerized C element alone is unable to elicit cell-specific transcription (11).

These observations suggest that the natural pancreas-specific enhancers may be of the type described by Tjian and Maniatis (36), in which the relative position and orientation of individual elements direct the assembly of a stereospecific complex whose architecture is required for the correct modulation of strength, specificity, and timing of activation. In order to understand how such multicomponent enhancer complexes

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integrate different regulatory signals, it is essential to identify all of the individual elements and their transcription factors and not only elements that bind transcription factors restricted to the exocrine pancreas.

Although some of the factors involved in regulation of exocrine pancreas-specific transcription have been characterized at the protein level, their sequences have not been reported to date. In this paper, we identify a second regulatory element in the 5′ control region of the α-amylase 2 gene and characterize the binding activities that recognize it. We show that these are members of the hepatocyte nuclear factor 3 (HNF3) family of transcription factors that have recently been implicated as playing a role in determining the fate of embryonic cell populations undergoing commitment to specific lineages (3, 29).

MATERIALS AND METHODS

Tissue culture conditions and preparation of nuclear extracts from cultured cells and tissues. Exocrine pancreatic rat AR42J cells were cultured as previously described (5). All other cell lines described in this paper were cultured in a medium mixture containing 45% F12K medium plus 22.5% each F12 medium and Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Nuclei from rat pancreas, liver, spleen, kidney, salivary gland, and brain cells were prepared essentially as described by Petrucco et al. (23). Amy2-II-binding activities were purified by DNA affinity chromatography from isolated nuclei of tissue and cells in culture as described by Hagenbüchle and Wellauer (8). Proteins were applied to the DNA affinity column in 60 mM KCl–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.9])–0.5 mM EDTA–5 mM MgCl₂–12% glycerol–5 mM dithiothreitol–0.1% Triton X-100 and washed with 4 column volumes of the same buffer (with the exception that it contained 300 mM KCl). The sequence-specific binding activities were eluted with 400 mM KCl in the buffer described above.

Construction of hybrid genes and transient expression. 5′ deletions of the α-amylase 2 5′-flanking region (32) containing a common 3′ end (nucleotide position +15) were produced by restriction enzyme or BAL 31 digestion. *Hin*dIII linkers were added to the blunt-ended α-amylase gene fragments, which were then inserted into the unique *Hin*dIII site of a pSVO-Tk vector containing the 1,477-bp *Bgl*II-*Nco*I fragment of the herpes simplex virus thymidine kinase (TK) gene (nucleotides +253 to +1730 [17]). The *Bgl*II site of this fragment was blunt ended and converted into a unique *Hin*dIII site by linker addition. The blunt-ended oligonucleotide Amy2-IV was inserted into a unique, blunt-ended *Nde*I site of hybrid plasmids containing α-amylase 2 sequences +15 to −100 and +15 to −44 to generate constructs B and C, respectively. Construct D was generated by *Rsa*I excision of nucleotides −44 to −86 from the *Hin*dIII fragment containing α-amylase 2 gene sequences +15 to −224. The resulting *Rsa*I fragments were religated and inserted into the *Hin*dIII site of the pSVO-Tk vector. Construct E was made by site-directed mutagenesis of construct B with the Amy2-II PM1 oligonucleotide as a mutagenic primer in conjunction with the Altered Sites in vitro mutagenesis system (Promega). Construct F was made by inserting a 180-bp restriction fragment containing α-amylase 2 5′-flanking sequences −44 to −224 by blunt-end ligation into a unique *Bgl*II site at position −300 of the α-amylase 1 gene 5′-flanking region that was part of an *Eco*RI fragment containing α-amylase 1 sequences +99 to −500. The modified region was then excised by *Eco*RI, blunt ended, and, after addition of *Hin*dIII linkers, subcloned into the *Hin*dIII site of pSVO-Tk. Construct F Co was made accordingly but without insertion of α-amylase 2 gene sequences prior to subcloning into the expression vector. The pRSV-Tk vector was generated by addition of *Hin*dIII linkers to the 545-bp *Nde*I-*Hph*I restriction fragment of pRSV-CAT containing the Rous sarcoma virus (RSV) promoter (nucleotides +15 to −540 [6]). This fragment was then inserted into the *Hin*dIII site of pSVO-Tk. All hybrid genes used in this study were verified by DNA sequence analysis. Transient expression of hybrid genes in AR42J cells and primer-extended cDNA synthesis were carried out as described previously (5).

EMSA and supershift assays. Binding reactions (20-μl mixtures) were carried out in binding buffer (12% glycerol, 12 mM HEPES [pH 7.9], 60 mM KCl, 0.12 mM EDTA, 5 mM MgCl₂, 5 mM dithiothreitol, 0.1% Triton X-100). Binding reaction mixtures containing isolated nuclei were incubated for 60 min, and those containing purified protein were incubated for 10 min at 20°C in the presence of 0.2 ng of ³²P-end-labelled oligonucleotide (specific activity, 6 × 10⁸ to 8 × 10⁸ cpm/μg). Binding reaction mixtures with nuclear extracts also contained 1 μg of each sonicated single-stranded *Escherichia coli* DNA and poly(dI-dC). For supershift assays, binding reaction mixtures containing proteins and DNA were incubated as described above. Preimmune, HNF3β, and HNF3α antisera (a gift of R. Costa) were added to the binding reaction mixtures at final dilutions of 1:5,000 each. Samples were then incubated for an additional 45 min at 20°C. For antibody-mediated inhibition of binding, purified protein was incubated for 30 min at 20°C with 40-fold dilutions of either preimmune serum or anti-HNF3γ serum (a gift of E. Lai). DNA was added, and the reaction mixture was incubated

for an additional 15 min as indicated above. Samples were analyzed by electrophoresis on 2% agarose gels in 0.5× Tris-borate-EDTA at 4°C.

In vitro renaturation of Amy2-II proteins. For the renaturation of individual proteins, Amy2-II binding activity purified by one round of DNA affinity chromatography was separated on a 7.5% acrylamide gel in the presence of the cationic detergent 16-BAC (benzyldimethyl-*n*-hexadecylammonium chloride) as described by Macfarlane (15). Slicing of the gel, elution (48 h at 4°C), and in vitro renaturation of the protein were performed according to the method of Sommer et al. (34). DNA-binding reactions with gel-purified, renatured proteins were done in binding buffer containing 250 μg of bovine serum albumin per ml and a mixture of protease inhibitors. The renaturation efficiency was estimated to be about 1% of input binding activity.

Western and Southwestern blots. For Western blotting (immunoblotting), proteins purified by one round of DNA affinity chromatography or crude extracts were separated by Tris-glycine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a polyvinylidene difluoride membrane (Immobilon P) as described for the peptide sequencing. The membrane was blocked with 5% (wt/vol) milk in 50 mM Tris-HCl (pH 7.4)–200 mM NaCl. The primary antibody was diluted 1,000-fold and reacted overnight in the same buffer. The secondary antibody, goat anti-rabbit antibody conjugated to horseradish peroxidase, was diluted 1,000-fold and incubated for 30 min. After extensive washing in 50 mM Tris-HCl (pH 7.4)–200 mM NaCl, proteins reacting with the antibodies were visualized with the Amersham enhanced chemiluminescence detection system. For Southwestern (DNA-protein) blotting, SDS-PAGE-separated proteins (10 to 50 ng each) were transferred to nitrocellulose in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 1 h on ice at 200 mA and 60 V. The filter was first incubated for 15 min in preincubation buffer (25 mM Tris-HCl [pH 7.4], 1 mM EDTA, 140 mM NaCl, 2.5% milk powder) and then was incubated for 30 min in the same buffer containing 0.25% milk powder. The filter was then incubated overnight in the latter buffer containing 50 ng of ³²P-labelled DNA probe per ml and sonicated single-stranded competitor *E. coli* DNA (100 μg/ml). The filter was washed four times for 10 min each in fresh buffer without probe or competitor DNA and then was exposed to film.

Amino acid sequencing of cyanogen bromide peptides. Amy2-II peptides (5 μg) purified by one round of DNA affinity chromatography were separated on a Tris–glycine–SDS–12% polyacrylamide gel (13) with a Bio-Rad Protean II minigel apparatus. The proteins were stained for 2 min with 0.05% Coomassie blue R250 in 50% (vol/vol) methanol–10% (vol/vol) acetic acid and then destained in 5% (vol/vol) methanol–7% (vol/vol) acetic acid. The individual bands were cut out and sliced into small pieces. In vitro cleavage of the proteins with cyanogen bromide (2.5 mg/ml in 70% [vol/vol] formic acid) was performed by vigorous shaking for 12 h at room temperature under argon and in the dark. The liquid was removed, and the remaining peptides were eluted with 200 μl of 70% formic acid for 2 h under the same conditions. The pooled eluates were lyophilized twice from H₂O, and the peptides were separated by Tris-Tricine-SDS-PAGE (16% T, 6% C) according to the method of Schägger and von Jagow (30). After electrophoresis, the separated peptides were electroblotted at 200 mA in a Bio-Rad miniblotter for 2 h at 0°C onto a Transblot polyvinylidene difluoride membrane (Bio-Rad) in 10 mM CAPS (pH 11) containing 10% (vol/vol) methanol (16). The peptides were visualized by staining for 1 min with 0.02% (vol/vol) Coomassie blue R250 in methanol-acetic acid. The excised membrane pieces were washed three times for 5 min each in 50% (vol/vol) methanol–5% (vol/vol) acetic acid and rinsed twice with H₂O. The vacuum-dried pieces of filter were stored at −20°C under argon. N-terminal sequencing was carried out on Applied Biosystems 473A and 477A pulsed liquid-phase microsequencers.

Northern analysis and RNase H assay. Total RNA was extracted from tissues and cell lines by methods described by Schibler et al. (33). Total and cytoplasmic poly(A)⁺ RNA was obtained by two rounds of chromatography on oligo(dT) columns. Glyoxylation and electrophoresis of RNA on agarose gels were done according to the method of McMaster and Carmichael (18). RNA was transferred from the gel to a nylon membrane and hybridized overnight at 42°C in solutions containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and ³²P-labelled DNA probe. The final wash of filters was in 0.1× SSC at 65°C. RNase H mapping was carried out essentially as described by Hagenbüchle et al. (7). Five-microliter aliquots of poly(A)⁺ RNA were first digested for 15 min at 37°C with 10 U of DNase I (RNase free, from Boehringer) to eliminate residual DNA. RNA was then hybridized to 100 ng of a 750-bp *Nar*I fragment of HNF3β cDNA for 3 h at the midpoint temperature of the DNA in a solution containing 70% formamide (recrystallized twice) and 0.3 M salt. RNA-DNA hybrids were then precipitated by ethanol, resuspended in 5 μl of 10 mM Tris (pH 7.5)–0.1 mM EDTA, and digested for 15 min at 37°C with 2 U of RNase H (Pharmacia) in a total volume of 50 μl under conditions specified by the supplier. RNase H-resistant material was then extracted with chloropane, precipitated with ethanol, resuspended in H₂O, and digested once again with DNase I to eliminate the cDNA fragment. All enzymatic digestions were carried out in the presence of 60 U of RNase inhibitor RNasin (Promega). RNA was extracted with phenol-chloroform, precipitated, and resuspended in H₂O for denaturation by glyoxal and gel electrophoresis.

Isolation of cDNA clones. A commercial pancreas cDNA library (Stratagene) containing about 2 × 10⁶ independent cDNA clones was screened for HNF3β cDNAs by using the ³²P-cDNA insert of clone K2 (14) as a probe and yielded four positive clones. These were plaque purified, and cDNA inserts were isolated

by the in vivo excision protocol provided by the supplier. The cDNAs in their double-stranded form were then partially sequenced according to the method of Sanger et al. (28) with Sequenase 2.0 (U.S. Biochemical Corp.) and oligonucleotide primers under conditions indicated by the manufacturer. All four clones were found to be independent and to contain cDNA inserts that specify HNF3β. The two longest clones were then fully sequenced on both strands.

RESULTS

DNase I footprint studies of α-amylase 2 gene promoter sequences have shown that sequences −60 to −86 (Amy2-II), lying between the binding site for the exocrine pancreas-specific transcription factor PTF1 (−122 to −157) and the cap site, are a site of protein-DNA interaction (5). To test whether this binding site is functionally significant, we have constructed a series of hybrid genes containing a TK reporter gene under the control of α-amylase 5′-flanking sequences. The various hybrid genes were then used for transient expression assays in differentiated pancreatic AR42J cells in culture (Fig. 1). Flanking sequences up to −224 (construct A) are sufficient to permit efficient transcription of the transfected TK reporter gene in AR42J cells (lane A). Construct B, which contains the PTF1 binding site flanked by pBR322 vector sequences and linked to α-amylase 2 promoter sequences +15 to −100, retains sufficient regulatory information to mediate efficient cell-specific transcription of the reporter gene (lane B). We have previously shown that sequences +15 to −100 (lacking the PTF1 binding site) are not sufficient for transcription of the reporter gene (5). When the PTF1 binding site is linked to α-amylase 2 promoter sequences +15 to −44 (construct C) or when DNA sequences −44 to −86 encompassing the Amy2-II footprint are deleted (construct D), transcription is also abolished (lanes C and D). These results indicate, therefore, that sequences encompassing the Amy2-II footprint harbor a second *cis*-acting element that is essential for efficient transcription of the α-amylase gene in exocrine pancreatic cells. We conclude that both the PTF1 binding site and the Amy2-II footprint domain are essential and that neither alone is sufficient to activate transcription from a residual α-amylase gene promoter.

This notion is supported by the observation that α-amylase 2 flanking sequences −44 to −224 induce efficient transcription from the heterologous α-amylase 1 gene promoter (construct F [Fig. 1, lane F]). The α-amylase 1 gene is under the control of a weak promoter that is active in the liver and the salivary gland (31). No transcription of the TK reporter gene under control of this promoter alone is detected in the transient expression assay (Fig. 1, lane F Co).

In order to determine whether the Amy2-II element defined by footprints and the transient expression assay is sufficient to bind one or more *trans*-acting factors with high affinity, we tested the ability of crude nuclear extracts to retard an oligonucleotide containing the Amy2-II site by EMSA (Fig. 2a). Three differently migrating protein-DNA complexes (C1, C2, and C3), and occasionally a fourth (C4), are observed when crude nuclear extract from an exocrine pancreatic cell line (AR42J) is incubated with the ^{32}P-labelled Amy2-II probe (sequence shown in Fig. 2c). All three are apparently sequence specific, as shown by the fact that binding is inhibited by a 50-fold molar excess of the unlabelled Amy2-II oligonucleotide but not by the same excess of a variety of other oligonucleotides of similar length, including that corresponding to the PTF1 binding site (Amy2-IV). All three binding activities are slightly diminished by an excess of oligonucleotide Amy2-I, which contains the TATA box of the α-amylase 2 promoter. Formation of C1 is inhibited by increasing the molar excess of the probe (see Fig. 2b and 5b), but it is also abolished by competition with oligonucleotide Amy2-V. The DNA se-
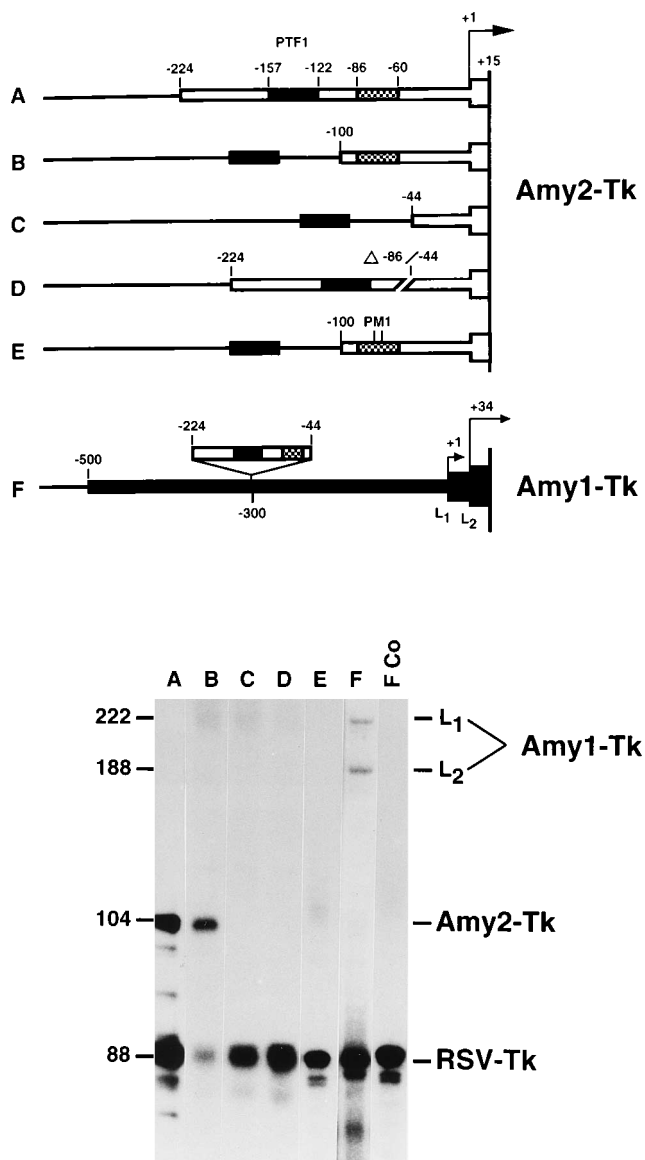

FIG. 1. Primer extension analysis of RNA transcripts from transient expression of hybrid genes in AR42J cells. The maps of hybrid genes A to F are shown at the top. Different portions of the 5′-flanking sequences from α-amylase 2 (Amy2; open boxes) and α-amylase 1 (Amy1; solid boxes) genes were fused to plasmid vectors containing viral TK gene sequences (nucleotides +253 to +1730). The positions of α-amylase 2 gene flanking sequences that correspond to a protein binding site in vitro are indicated by shaded boxes. PM1 in hybrid gene E designates point mutations that were introduced into hybrid gene B by site-directed mutagenesis in vitro (see the text and Fig. 2). Transcription initiation sites are indicated by arrows. AR42J cells were cotransfected with hybrid genes A to F plus the RSV-Tk plasmid as an internal control; and the 5′ ends of RNAs produced from the plasmids were mapped by electrophoresis of primer-extended cDNAs on a 6% sequencing gel. Lanes: A to E, Amy2-Tk plasmids A to E, respectively; F, Amy1-Tk plasmid F into which Amy2 5′-flanking sequences −44 to −224 were inserted; F Co, Amy1-Tk plasmid F lacking Amy2 sequences. The size of the expected products generated by extension of a 40-nucleotide primer within the coding sequence of the TK gene are 88 and 104 nucleotides for RSV-Tk and Amy2-Tk plasmids, respectively. The Amy1 flanking region contains two different transcription start sites (L$_1$ and L$_2$) that yield primer extension products with sizes of 222 and 188 nucleotides, respectively. The numbers shown are in nucleotides.
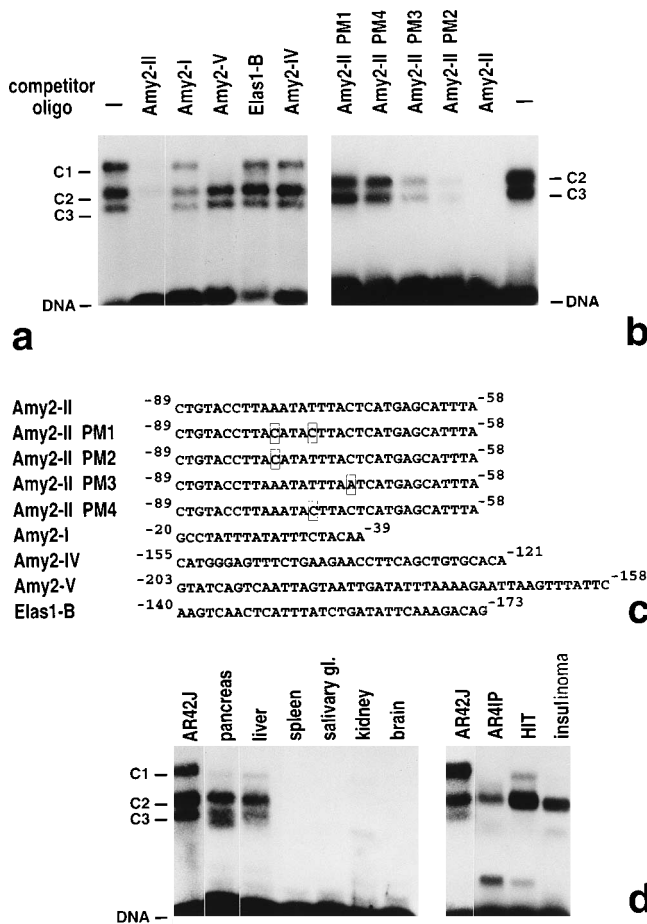
FIG. 2. Sequence specificity and cellular distribution of activities binding to an oligonucleotide (oligo) containing α-amylase 2 5'-flanking sequences −58 to −89 (Amy2-II). (a) Binding of nuclear proteins from AR42J cells to ^{32}P-labelled Amy2-II DNA was analyzed by EMSA. Protein-DNA complexes are designated C1 to C3. The presence of C1 is dependent upon the ratio of protein to DNA probe (see the text). The sequence specificity of protein binding was analyzed by the addition of a 50-fold molar excess of unlabelled homologous and heterologous oligonucleotides. (b) Amy2-II oligonucleotides carrying either double (PM1) or single (PM2 to PM4) point mutations were used as competitors for the analysis of Amy2-II binding by EMSA. Note that complex C1 is absent because binding assays were carried out with five times more radiolabelled DNA than the amount used in panel a (see the text). (c) Sequences of one strand of the oligonucleotides used in panels a and b are shown. Amy2-II PM1 to PM4 are identical to Amy2-II, except at the positions indicated by the boxes. The oligonucleotides Amy2-I, Amy2-IV, and Amy2-V are derived from different regions of the α-amylase 2 gene 5'-flanking sequence (5). Oligonucleotide Elas1-B contains a putative regulatory element from the 5'-flanking region of the elastase 1 gene (8). (d) Cellular distribution of Amy2-II binding activities was examined by EMSA with nuclear extracts prepared from a variety of tissues and cell lines as indicated above each lane.

quences present in Amy2-V define a footprint domain in the α-amylase 2 5'-flanking region (positions −158 to −203 [5]). However, deletion of this region does not affect the transcription efficiency of hybrid genes in transient expression assays (Fig. 1). The oligonucleotide Elas1-B containing 5'-flanking sequences of the elastase 1 gene does not compete for Amy2-II binding activities. Elas1-B contains element B of the elastase 1 enhancer, which has been defined as required but not sufficient for elastase 1 gene transcription in the exocrine pancreas (9, 10) and which has been shown to bind an unidentified cell-specific factor (11).

The fact that oligonucleotide Amy2-I weakly competes for

all of the Amy2-II binding activities allowed us to deduce a potential recognition motif common to both oligonucleotides. Comparison of the sequences shows that the longest stretches of homology between them are two overlapping hexamers, AAATAT and TATTTA (Fig. 2c). A series of oligonucleotides (Amy2-II PM1 to PM4) that differ in sequence from the Amy2-II probe at only one or two positions were synthesized (sequences shown in Fig. 2c). The ability of each to compete for Amy2-II binding activities when present at 50-fold molar excess over the labelled wild-type probe was measured (Fig. 2b). Mutants PM1 (A to C at −79 and T to C at −75) and PM4 (T to C at −75) weakly inhibit the formation of either C2 or C3 while both PM2 (A to C at −79) and PM3 (C to A at −71) compete more strongly. Thus, nucleotide position −75 of the Amy2-II binding site is critical for the binding of activities giving rise to C2 and C3, while positions −71 and −79 are less important. The fact that C2 and C3 behave in a similar fashion in these competition assays strongly suggests that both complexes are formed by proteins which recognize the same sequence motif. An Amy2-Tk hybrid gene carrying the PM1 mutation (construct E) no longer supports transcription in the transient expression assay (see the absence of the 104-bp band in Fig. 1, lane E). This result demonstrates that binding activities generating C2 and/or C3 are essential for efficient transcription from the α-amylase 2 gene promoter.

EMSA was used to determine the cellular distribution of the binding activities detected in AR42J cells (Fig. 2d). Complexes with migration properties identical to those from AR42J cells are detected in nuclear extracts from both pancreas and liver cells. Nuclear extracts prepared from spleen, salivary gland, kidney, and brain cells have no significant Amy2-II binding activity. Nuclear extracts of cell lines derived from exocrine (AR4IP) and endocrine (HIT and insulinoma) pancreatic tumors all contain a binding activity which, when bound to DNA, comigrates with C2 of AR42J cells. No counterparts to AR42J protein-DNA complexes (C1 to C3) were found in HeLa and L cells (data not shown). These results suggest that Amy2-II binding activities have a restricted cellular distribution but, in contrast to PTF1 binding activity, are not strictly limited to the exocrine pancreas.

To elucidate the nature of Amy2-II-binding activities and the relationship between the different protein-DNA complexes, proteins that recognize the Amy2-II oligonucleotide were purified by DNA affinity chromatography. The binding activities were recovered from the column with an overall yield of 75 to 80% of the activities present in the starting material (Fig. 3). However, the relative amounts of the complexes varied somewhat in different preparations. The protein composition of column fractions containing Amy-II binding activity were examined by SDS-PAGE and silver staining. In several independent preparations, predominant bands migrating at 54, 47, 48, and 45 kDa were reproducibly detected in the fractions in which the binding activity eluted (Fig. 3b). In order to assign the various proteins to the Amy2-II complexes observed by EMSA, two independent experimental approaches, both of which rely on renaturation of purified material, were used. Figure 4a shows the result of a Southwestern blot in which samples of purified binding activity were separated by SDS-PAGE and either visualized by silver staining (lane 1) or transferred to a nitrocellulose filter and probed with radiolabelled Amy2-II DNA. A single radioactive band which comigrates with the 54-kDa species is detected when the filter is probed with ^{32}P-labelled Amy2-II oligonucleotide in the presence of a 1,000-fold weight excess of nonspecific single-stranded competitor DNA. This positively identifies p54 as a sequence-specific DNA-binding protein but does not rule out that native
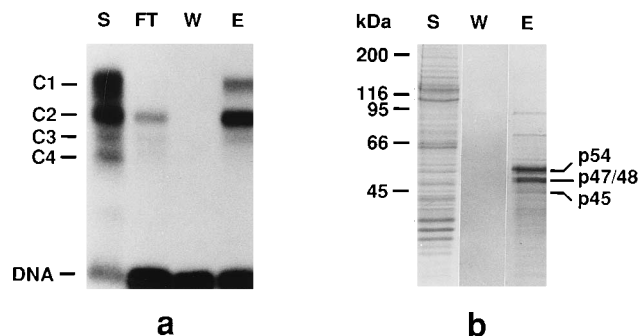
FIG. 3. Purification of Amy2-II binding activities from crude nuclei of AR42J cells by DNA affinity chromatography. (a) The presence of Amy2-II binding activity in different column fractions was monitored by EMSA. S, starting material; FT, flowthrough fraction; W, last wash at 300 mM KCl; E, 400 mM KCl eluate. Lanes FT, W, and E all contain the same volume equivalent of material loaded in lane S. The positions of protein-DNA complexes C1 to C4 are indicated. (b) Samples of S, W, and E were subjected to electrophoresis on an SDS–12% polyacrylamide gel alongside commercial molecular mass markers and were visualized by silver staining. The polypeptides which reproducibly coelute with Amy2-II binding activity in different preparations are indicated on the right.

p47/48 and p45 have DNA-binding activities that are unable to renature after transfer to nitrocellulose. To explore whether any of these proteins have inherent DNA-binding activity that cannot be renatured on filters, affinity-purified proteins were also separated on a denaturing polyacrylamide gel, eluted from individual gel slices, subjected to a renaturation procedure in solution, and assayed for binding to Amy2-II DNA by EMSA. Figure 4b shows that Amy2-II complexes are indeed generated by proteins eluted from slices containing p54, p47/48, and p45, whereas no binding activity is renatured from other parts of the gel.

Renatured p54 gives rise to a complex that comigrates with native C2. Renatured protein from the slice containing p47/48 gives rise to a complex that comigrates with native C3. p45 generates a renatured complex comigrating with native C4. Competition for binding of the renatured proteins by Amy2-II or unrelated oligonucleotides confirmed that the complexes formed have the same specificity as the native complexes with which they comigrate (shown for C2 in Fig. 4c). We conclude from these data that p54 alone yields C2 and p47/48 yields C3. We were unable to separate the bands migrating as the doublet p47/48 and to assay their individual binding activities. Therefore, we cannot distinguish whether only one protein or both proteins are able to generate C3.

The individual proteins from purified Amy2-II binding activity were cleaved with cyanogen bromide, and the resulting peptides were gel purified and subjected to sequence analysis. Two peptides derived from p54 were homologous to regions within the DNA-binding domain of HNF3β, a member of a family of transcription factors first identified in the liver (14). These factors have been divided into three subgroups (α, β, and γ) on the basis of amino acid sequence differences within the DNA-binding domain (see Fig. 6). p47/48 yielded insufficient quantities of peptide from which to obtain sequence information. Peptides derived from p45 were homologous to a region of poly(A)-binding protein which has been previously shown to bind AT-rich sequences in single-stranded DNA (4). We have not ascertained whether, in this case, p45 binding activity is due to the presence of the single-stranded oligonucleotide, but it is unlikely that this protein plays any role in transcriptional control.

In order to establish conclusively which Amy2-II complexes contain the p54 protein, we carried out both supershift and
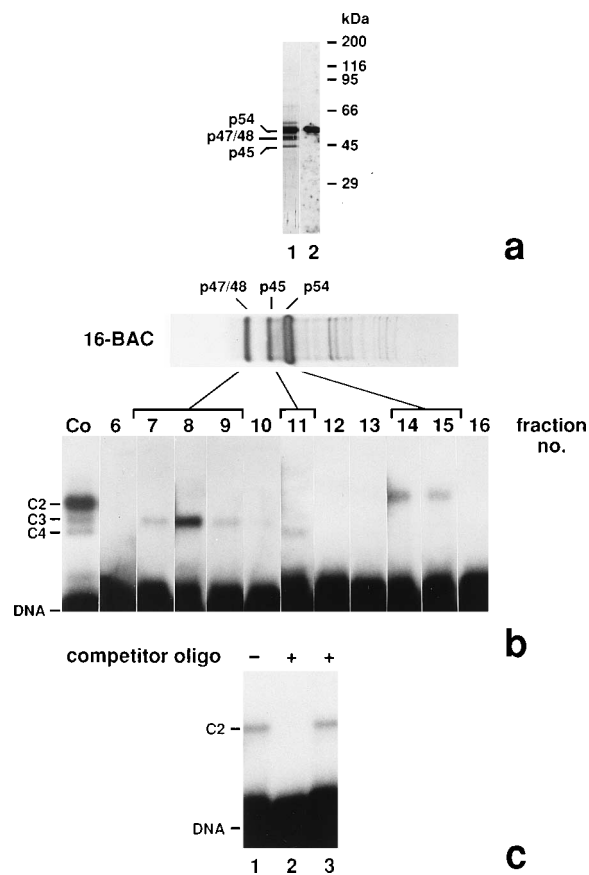

FIG. 4. DNA-binding properties of polypeptides purified by DNA affinity chromatography. (a) Southwestern analysis of purified Amy2-II binding activity. Proteins were fractionated by SDS-PAGE, the gel was cut, and one lane was stained with silver (lane 1). The material in the other lane was transferred to nitrocellulose and incubated with ^{32}P-labelled Amy2-II DNA in the presence of nonspecific unlabelled competitor DNA (lane 2). (b) Renaturation of purified proteins in solution. Amy2-II binding activity was fractionated by 16-BAC–PAGE (see Materials and Methods). Note that p47/48 and p45 migrate in reversed order in this gel system. Proteins were eluted from individual gel slices and renatured separately. The renatured material was then incubated with ^{32}P-Amy2-II DNA, and its potential to form complexes with DNA was analyzed by EMSA. A binding reaction mixture containing purified, native Amy2-II binding activity comigrated alongside as a marker (Co). (c) Sequence specificity of protein-DNA complex formed by renatured p54 protein (from gel slice 14) was tested by carrying out EMSA in the absence (lane 1) or presence of a 50-fold molar excess of unlabelled Amy2-II DNA (lane 2) and Amy2-II PM1 DNA (lane 3). Oligo, oligonucleotide.

Western blot assays with polyclonal antisera raised against HNF3α, HNF3β, and HNF3γ (Fig. 5). C2, but not C3, is lost in favor of a new, more slowly migrating form when a binding reaction mixture containing nuclear extract of AR42J cells is incubated in the presence of HNF3β antibody (Fig. 5a). The migration of none of the complexes is affected by incubation with HNF3α antibody. Binding reaction mixtures containing nuclear extracts derived from tissues and cell lines displaying Amy2-II binding activities gave similar results—namely, that all C2 complexes identified in Fig. 2d contain HNF3β-like proteins. When the supershift experiments were carried out under conditions which favor formation of C1 (i.e., with limiting concentrations of the DNA-binding site), the migration of this complex was also altered by incubation with the anti-HNF3β antibody (Fig. 5b). These observations suggest that p54 may bind as a component of a multimeric protein complex to Amy2-II DNA. Immunoblots of denatured proteins from
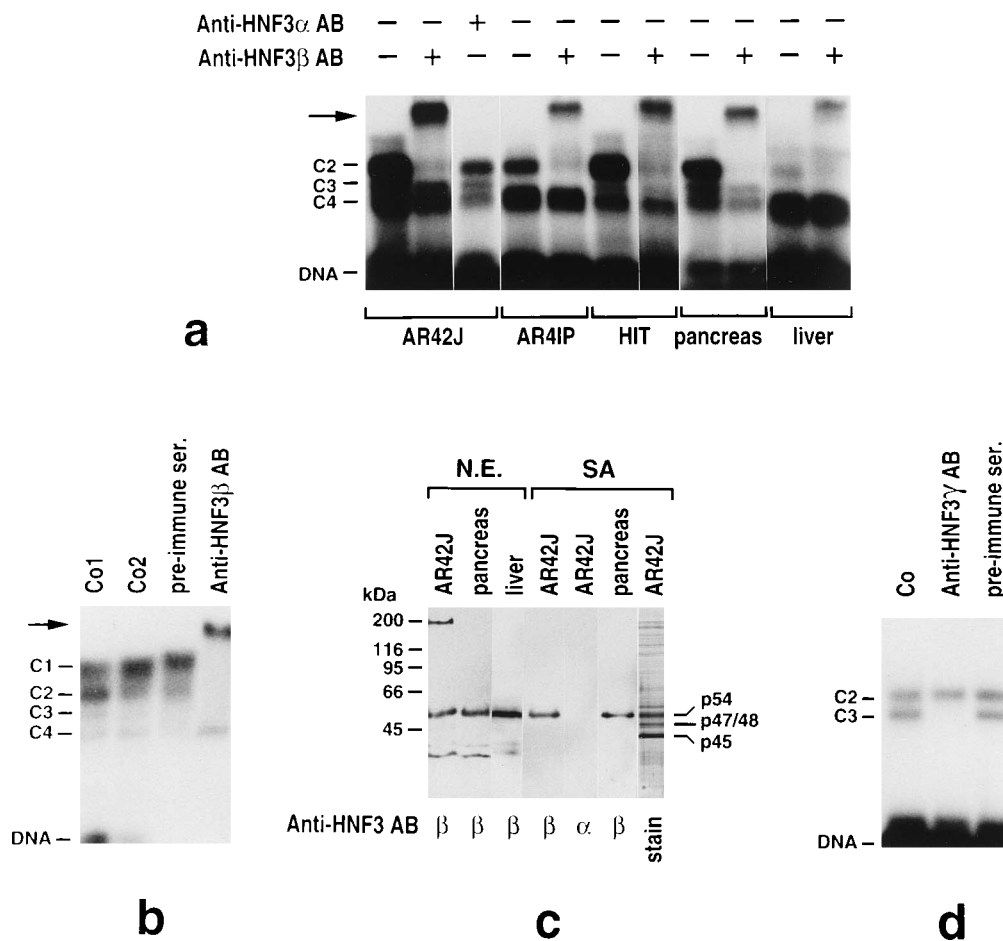
FIG. 5. Identification of HNF3 proteins in Amy2-II binding activity. (a) Binding reaction mixtures containing nuclear extracts from the cell lines and tissues indicated and ^{32}P-Amy2-II DNA were incubated for EMSA in the presence (+) or absence (−) of antisera (AB) directed against HNF3α or HNF3β protein. The positions of C2 complexes that are supershifted by anti-HNF3β antibody are marked by an arrow. (b) EMSA with affinity-purified Amy2-II binding activity was carried out under conditions which favor formation of C1 (see the legend to Fig. 2 and the text). Control binding reaction mixtures each contain about 1 ng of protein and 0.2 ng (Co1) or 0.05 ng (Co2) of DNA probe. Reactions with binding reaction mixtures containing either preimmune serum (ser.) or anti-HNF3β serum were carried out under conditions used for Co2. The position of supershifted C1 is indicated by an arrow. (c) Western blot analysis of crude (N.E.) and purified (SA) Amy2-II binding activities. Proteins from the tissues and cell lines indicated were fractionated by SDS-PAGE and transferred to an Immobilon polyvinylidene difluoride filter. Membrane-bound proteins were incubated with either anti-HNF3β or anti-HNF3α antibodies. One lane of the gel containing purified Amy2-II binding activity was silver stained as a marker. Molecular mass markers were used to align the silver-stained and immunoblotted proteins. (d) EMSA with affinity-purified Amy2-II binding activity that was pretreated with either preimmune serum or anti-HNF3γ antibody. A standard binding reaction is shown as a control (Co).

both purified material and crude nuclear extracts show that the anti-HNF3β serum recognizes a polypeptide with a size of 54 kDa in pancreas, liver, and AR42J cells and that p54 is a major species recognized by the antibody in total nuclear protein (Fig. 5c). These results confirm that p54 is a member of the HNF3β subfamily of transcription factors. The finding that C2 and C3 binding activities share a binding site suggested that C3 might contain yet another member of the HNF3 family. Indeed, formation of C3 complex is abolished by an antibody directed against HNF3γ (Fig. 5d). This antibody does not produce a supershift but blocks the binding of HNF3γ to its cognate sequence (14). Therefore, the binding activity giving rise to C3 includes HNF3γ.

The identification of p54 as a member of the HNF3β subfamily prompted us to search for HNF3β cDNA clones in a pancreatic cDNA library. Screening this library with a radiolabelled cDNA of HNF3β (14) yielded four independent positive cDNA clones. Partial sequence analysis demonstrated that all four contained the DNA-binding (forkhead) domain of HNF3β. The two longest cDNA clones were then completely

sequenced. One of these clones was almost full length and specifies essentially the protein sequence published for HNF3β from liver (termed variant A in Fig. 6 [3, 14]). The second cDNA clone specifies an HNF3β protein (variant B) that differs from the liver species in N-terminal amino acid sequences.

Northern analysis demonstrates that HNF3β mRNAs are as abundant in exocrine pancreatic cells as in other cell types of the endoderm lineage (Fig. 7a). To examine whether the cDNA clone that specifies HNF3β variant B is derived from a genuine mRNA, we carried out the experiment shown in Fig. 7b. A radiolabelled oligonucleotide (probe I) harboring sequences that specify the N-terminal amino acids of variant B detects a species of RNA that has the expected size (2.2 kb) of HNF3β mRNAs upon hybridization to pancreatic poly(A)$^+$ RNA (panel I, lane 1). To prove that this signal does not arise from fortuitous hybridization to an unrelated RNA species having the same size as HNF3β mRNA, we carried out RNase H mapping as outlined in the Fig. 7b. This approach relies on a site-specific cleavage of HNF3β mRNA prior to hybridization of the oligonucleotide probe. Briefly, a 750-bp NarI re-

HNF3β A　　MLGAVKMEGHEPSDWSSYYAEPEGYSSVSNMNASLGMNGM　　40
HNF3β B　　　　　　　LPS-RLPA-C-DA------------------　　31
　　　　　　　NTYMSMSAAAMGSGSGNMSAGSMNMSSYVGAGMSPSLAGM　　80
　　　　　　　--　　71
　　　　　　　SPGAGAMAGMSGSAGAAGVAGMGPHLSPSLSPLGGQAAGA　　120
　　　　　　　--　　111
　　　　　　　MGGGLAPYANMNSMSPMYGQAGLSRARDPKTYRRSYTHAKP　　160
　　　　　　　----------------------------------pep 1 --　　151
　　　　　　　PYSYISLITMAIQQSPNKMLTLSEIYQWIMDLFPFYRQNQ　　200
　　　　　　　--　　191
　　　　　　　QRWQNSIRHSLSFNDCFLKVPR⊙PDKPGKGSFWTLHPDSG　　240
　　　　　　　--　　231
pep 2　　　　NMFENGCYLRRQKRFKCEKQLALKEAAGAGSGGGKKTAPG　　280
　　　　　　　--　　271
　　　　　　　TQASQVQLGEAAGSASETPAGTESPHSSASPCQEHKRGGL　　320
　　　　　　　--　　311
　　　　　　　SELKGTPASALSPPEPAPSPGQQQQAAAHLLGPPHHPGLP　　360
　　　　　　　--　　351
　　　　　　　PEAHLKPEHHYAFNHPFSINNLMSSEQQHHHSHHHHQPHK　　400
　　　　　　　--　　391
　　　　　　　MDLKTYEQVMHYPGGYGSPMPGSLAMGPVTNKAGLDASPL　　440
　　　　　　　--　　431
　　　　　　　AADTSYYQGVYSRPIMNSS　　459
　　　　　　　------------------　　450　　　**a**

HNF3α　　MLGTVKMEGHESNDWNSYYADTQEAYSSVPVSNMN..
HNF3β A　MLGAVKMEGHEPSDWSSYYAEP-EGYSSV-SNMN..
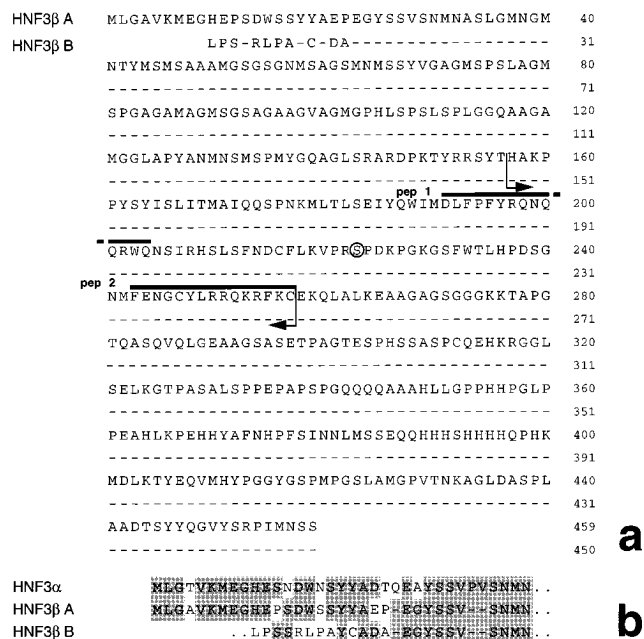HNF3β B　..LPSSRLPAYCADA-EGYSSV-SNMN..　　**b**

FIG. 6. Amino acid sequences deduced from sequencing the two longest cDNA clones obtained from screening a pancreatic cDNA library (a). Amino acids that were determined from direct sequencing of two p54 peptides are overlined (pep 1 and pep 2). Amino acid sequences located between the arrows specify the DNA-binding (forkhead) domain. One protein sequence (HNF3β A) is identical to that published for HNF3β of liver (3, 14), except that it contains a serine rather than an alanine residue at position 223. The second protein sequence (HNF3βB) is colinear with HNF3β A, except for N-terminal amino acids. (b) N-terminal amino acid sequences of HNF3β A and B are aligned for optimal homology to those of HNF3α.

striction fragment of HNF3β cDNA is hybridized under stringent conditions to poly(A)$^+$ RNA, and the resulting mRNA-cDNA hybrids are then digested with RNase H. This procedure generates RNase H-resistant 5′- and 3′-terminal RNA fragments that are derived exclusively from HNF3β mRNA. When these are hybridized to probe I, we observe that the 2.2-kb RNA species is indeed lost in favor of a 460-nucleotide 5′-terminal fragment (panel I, lane 2). The mRNA for variant B is not restricted to the pancreas but is also detected in RNase H-treated poly(A)$^+$ mRNA from liver (panel 1, lane 3). The presence of an additional band at 650 nucleotides suggests that yet another HNF3β-related mRNA species exists in this tissue. The mRNA for variant B apparently represents only a minor species in the pancreas, as demonstrated by the fact that rehybridization of the filter with probe II (specific for variant A) yields considerably stronger signals than that with probe I. Probe II detects the intact 2.2-kb mRNA of variant A (panel II, lane 1) and a 320-nucleotide 5′-terminal fragment in the RNase H-treated sample (panel II, lane 2). The mRNAs for variants A and B thus differ in the lengths of their 5′ ends.

The observation that the two mRNAs for HNF3β variants differ in size at their 5′ ends suggests that they originate from transcription initiation at different sites and/or alternative splicing of a primary transcript. Moreover, our results show that the abundance of the two mRNA species is not the same in the pancreas. We speculate that this differential accumulation may have a profound effect upon the expression of HNF3β target genes, because the variant sequences lie within a region of the protein known to function as a transactivation domain (22).
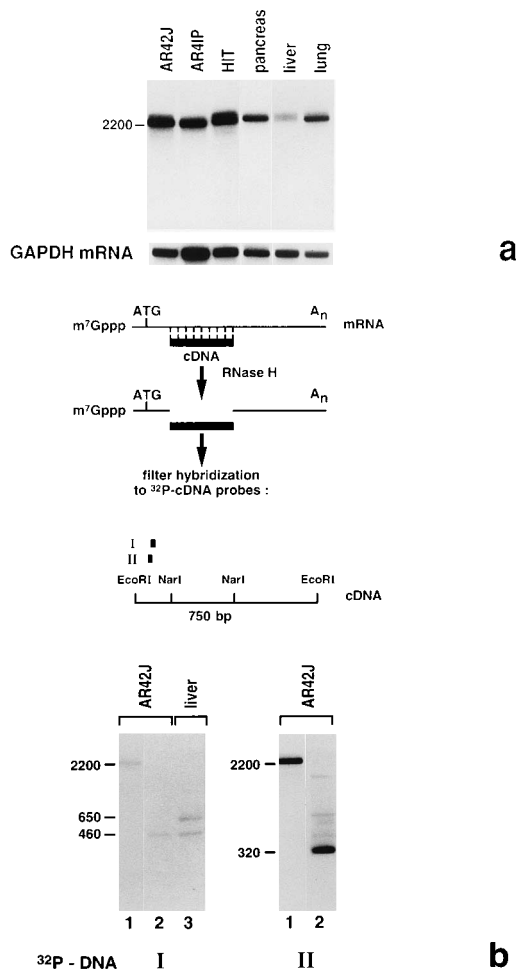
FIG. 7. Identification of HNF3β mRNA-related transcripts in the exocrine pancreas. (a) Distribution of HNF3β mRNA in cell lines and tissues derived from the endoderm. Three micrograms of cytoplasmic or total (pancreas) poly(A)$^+$ RNA was glyoxylated, fractionated on a 1.3% agarose gel, blotted onto a nylon membrane, and hybridized with ^{32}P-labelled HNF3β cDNA. The filter was then rehybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA for an approximate quantitation of HNF3β transcripts. (b) Search for the presence of HNF3β variant B mRNA in pancreatic cells and liver tissue. The schematic representation shows the strategy used for this experiment. Five-microgram aliquots of cytoplasmic poly(A)$^+$ were hybridized in solution to a NarI fragment of HNF3β variant A cDNA. RNA-DNA hybrids were digested with RNase H, and the resulting RNA fragments were separated by electrophoresis on a 2% agarose gel (a). RNA was then transferred to a membrane and hybridized sequentially to radiolabelled DNA probes I and II (lanes 2 and 3). Three micrograms of pancreatic poly(A)$^+$ RNA that had not been hybridized to DNA in solution but digested with RNase H was included as a control for degradation resulting from the enzyme treatment alone (lanes 1). Oligonucleotide probes I and II were designed to discriminate between 5′-terminal fragments of mRNAs encoding HNF3β variants A and B, respectively. The two oligonucleotides have the same size (31 nucleotides), were labelled to the same specific radioactivity, and were used at the same concentration for hybridization. The intensities of the hybridization signals in panels I and II can thus be directly compared. The sizes of the RNA molecules are given in nucleotides and were determined from RNA and single-stranded DNA markers that were included in the gel.

DISCUSSION

We have identified a *cis*-acting regulatory element that is required for the control of α-amylase 2 gene transcription in the exocrine pancreas. The core of this element appears to be an AT-rich motif unrelated to the binding site of PTF1, the cell-specific transcription factor that was previously identified

(5). Two of the activities binding to the AT-rich motif have been identified as belonging to the family of HNF3 transcription factors. Retrospective comparison with a consensus HNF3 binding site established by sequential selection and amplification of binding sites (21) confirms that the Amy2-II oligonucleotide harbors a good HNF3 binding site. The three nucleotides mutated in our series of Amy2-II point mutants PM1 to PM4 correspond to positions 2, 6, and 10 of the 12-bp HNF3 consensus. The HNF3 binding site is apparently essential but not by itself sufficient for efficient expression of the α-amylase 2 gene. This contention is based on results from transient expression of hybrid genes in pancreatic cells, but it is also supported by the observation that cells containing HNF3 but lacking PTF1 do not express the gene (this paper and reference 5).

The HNF3 (forkhead) family of proteins constitutes a class of transcription regulators originally identified as activators that coordinately regulate the expression of a number of genes in the liver by binding to their promoters and enhancers (12, 14). These proteins have more recently been implicated as critical players during embryogenesis at the stage at which body axis formation and commitment to different developmental fates are decided (3, 29). In particular, HNF3α and HNF3β have a role in specifying the formation of definitive endoderm and gut tissue (29). Less is known about the early developmental role of HNF3γ. This protein is expressed in a limited number of organs that originate from the primitive gut (14). Because it is not possible to separate HNF3β and HNF3γ binding activities on the basis of sequence specificity, we have been unable to determine whether only one factor or both factors are involved in transcription of the α-amylase 2 gene. However, one important concept that emerges from our work is that exocrine pancreas-specific expression of the α-amylase 2 gene is likely to be mediated by a combination of cell- and cell-lineage-specific transcription factors rather than by cell-specific transcription factors alone or by cell-specific association of more widely distributed transcription activators.

X-ray crystallographic studies of an HNF3-DNA complex have shown that the HNF3 DNA-binding domain is a structural variant of the helix-turn-helix motif of homeodomain proteins (1, 2). Moreover, all members of the family are likely to adopt a conformation closely resembling the helix-turn-helix DNA-binding domains of the internucleosomal, DNA-packaging histones H1 and H5 (25). The high degree of structural similarity between proteins with such different sequences as HNF3 and H5 is certain to be functionally significant. Support for this hypothesis comes from nucleosome phasing studies of the distal enhancer of the mouse serum albumin gene. It has been demonstrated that HNF3 plays a critical role in the repositioning of nucleosomes over this enhancer and by doing so provokes a cascade of subsequent regulatory events (19).

What is the role HNF3β and/or HNF3γ plays in transcriptional control of the α-amylase 2 gene? These factors are unlikely to precipitate a cascade of further regulatory events in this case. However, the HNF3 binding site, which is located adjacent to the TATA box and thus is in close proximity to the basal transcription machinery, could enhance transcription from the α-amylase 2 gene promoter by recruiting components of the basal transcription complex via HNF3 activation domains. Another possibility is that it may function as an architectural component by stabilizing a nucleosome or another protein particle that favors a particular rotational and translational setting of the surrounding chromatin. This might serve to bring other important players such as PTF1 and basal factors into the correct orientation to allow physical contact between their activation domains. This hypothesis can be ad-dressed only by examining the chromatin structure of the α-amylase 2 gene promoter in vivo and comparing how it changes in different cell types and when the gene in the permissive cell type is in different transcriptional states.

The regulatory strategies of pancreatic genes appear to involve overlapping recognition motifs with specific roles that change throughout development. Kruse et al. (11) have shown that an element, which is itself capable of directing endocrine pancreas-specific transcription when multimerized, constitutes an integral part of the exocrine pancreas-specific elastase 1 enhancer (element B) and that, in this context, its endocrine activation function is suppressed. These authors also identified similar motifs in the 5′-flanking sequences of a number of other genes encoding pancreas-specific products, including one at positions −178 to −188 of the α-amylase 2 gene promoter. In this respect, it is intriguing that the multimeric complex C1, which contains HNF3β (Fig. 5b) and apparently also HNF3γ (unpublished observations), can bind to a sequence in the Amy2-V oligonucleotide (Fig. 2a). Inspection of the sequences of oligonucleotides Amy2-II and Amy2-V for shared motifs suggests a potential core recognition site at positions −175 to −182. The core of this motif would overlap with the sequence which is homologous to the endocrine pancreatic cell-specific element B identified in the elastase 1 gene. At least two transcription factors, one islet cell-specific and one ubiquitous, which recognize this element have been identified (11). However, proteins generating C1 cannot be identical to either of these factors, because their binding is inhibited by the Amy2-V sequence but not by an oligonucleotide containing element B from the elastase 1 enhancer. No negative effect upon transcription of a hybrid gene lacking the Amy2-V region has been observed during transient expression in a pancreatic cell line (see the results with hybrid gene B in Fig. 1). This does not rule out that Amy2-V binding activity has a functional role: for instance, to prevent the binding of other factors that would themselves cause inappropriate activation of transcription.

Does the pancreas use members of the HNF3 family of factors to regulate the expression of genes encoding pancreas-specific products other than α-amylase? Competition studies between candidate binding sites defined by DNase I footprinting of different promoters have so far failed to detect sequences which can compete for Amy2-II binding activities in other genes (unpublished observations). Recent evidence suggests, however, that HNF3 may also be involved in controlling the expression of the glucagon gene in the endocrine pancreas (24). A definitive answer to this question would require that the effect upon transcription of various genes be studied with pancreatic cells in which HNF3 expression has been disabled.

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