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Tissue-Specific and Insulin-Dependent Expression of a Pancreatic Amylase Gene in Transgenic Mice

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The regulatory properties of mouse pancreatic amylase genes include exclusive expression in the acinar cells of the pancreas and dependence on insulin and glucocorticoids for maximal expression. We have characterized a murine pancreatic amylase gene, *Amy-2.2*^{y}, whose promoter sequence is 30% divergent from those of previously sequenced amylase genes. To localize sequences required for tissue-specific and hormone-dependent activation, we established two lines of transgenic mice. The first line contained a single copy of the complete *Amy-2.2*^{y} gene as well as 9 kilobases of 5′-flanking sequence and 5 kilobases of 3′-flanking sequence. The second line carried a minigene which included 208 base pairs of 5′-flanking sequence and 300 base pairs of 3′-flanking sequence. In both lines the transgene was expressed at high levels exclusively in the pancreas. Both constructs were dependent on insulin and induced by dexamethasone. Thus, the transferred genes contained the sequences required for tissue-specific and hormonally regulated expression.

Pancreatic amylase (*Amy-2*) genes are members of a multigene cluster which also includes the genes for salivary amylase (*Amy-1*). Although the coding regions of *Amy-1* and *Amy-2* are 90% homologous (21), the promoters associated with each type of gene are dissimilar. *Amy-1* can be transcribed from two distinct promoters: a strong, parotid-specific promoter located 7 kilobases (kb) upstream of the first coding exon, and a weaker promoter active in liver (52). Each *Amy-2* gene, in contrast, is associated with a single promoter adjacent to the structural gene.

Amy-2 expression is restricted to the exocrine pancreas, where amylase accounts for 15 to 25% of protein synthesis and mRNA concentration (8, 44). Pancreatic amylase genes have been shown to be hormonally regulated in vivo by insulin and by glucocorticoids. Production of pancreatic amylase is reduced in human diabetic patients (11), and amylase mRNA is greatly reduced in diabetic rats and mice (14, 29). Administration of insulin restores amylase expression in these diabetic animals. Induction of pancreatic amylase by glucocorticoids has been demonstrated in neonatal mice (48) and in cultured cells (13, 31).

Recent studies have identified gene regions which appear to be required for pancreas-specific gene expression. Walker et al. (50) demonstrated that *cis*-acting sequences upstream of the rat chymotrypsin gene preferentially increase expression in transfected cells derived from a tumor of the exocrine pancreas. Using chimeric genes in transgenic mice, Ornitz et al. (38) demonstrated that a 213-base-pair (bp) region upstream of rat elastase is sufficient to direct expression of human growth hormone in the pancreas. Swift et al. (45a) identified a conserved sequence element upstream of five genes which are expressed specifically in the exocrine pancreas. Deletion of this element resulted in loss of pancreatic cell specificity in transfection assays (9). This sequence is therefore a likely candidate for mediation of pancreas-specific expression in vivo.

With regard to mechanisms of hormonal regulation, substantial evidence indicates that binding of the glucocorticoid receptor to a small sequence element common to several induced genes is involved in direct induction of transcription (28, 51). This sequence element is lacking from some glucocorticoid-inducible genes, which are induced by an indirect cycloheximide-inhibited mechanism (2, 4). Less is known about the mechanisms of gene regulation by insulin. The abundance of several proteins and mRNAs is affected by insulin levels in diabetic animals and in cultured cells (23, 36, 39). Negative and positive effects of insulin on transcription of phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate kinase, respectively, have been demonstrated (18, 37). DNA sequences mediating these effects have not yet been identified.

The mouse genome contains two classes of pancreatic amylase genes, designated *Amy-2.1* and *Amy-2.2*. In strain YBR, the *Amy-2.1*^{y} allele encodes isozyme A_1 and *Amy-2.2*^{y} encodes isozyme B_1 (20). The *Amy-2.1*^{a} allele, originally isolated from inbred strain A/J (44), encodes the pancreatic amylase isozyme A_2 that is common to most inbred strains. The *Amy-2.2*^{a} allele of strain A/J is not expressed (22). To characterize *cis*-acting sequences required for pancreas-specific and insulin-dependent expression, we have sequenced the 5′ regions of the two amylase genes from mouse strain YBR. Both genes are pancreas specific, but *Amy-2.2* demonstrates more extreme dependence on insulin (14). Since transgenic mice provide an expression system for localizing *cis*-acting regulatory sequences (38a), transgenic lines carrying the *Amy-2.2*^{y} gene have been produced. The product of this gene is an electrophoretic isozyme that can easily be distinguished from the endogenous pancreatic amylase of the recipient mouse strain. Transgenic mice which express the gene can be conveniently identified by electrophoretic analysis of urinary amylase. Both the intact *Amy-2.2*^{y} gene and a shortened minigene were expressed heritably in the pancreas of transgenic lines. We have demonstrated that sequences required for pancreas-specific, hormone-dependent expression of *Amy-2.2*^{y} are present in the transferred DNA fragments.

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MATERIALS AND METHODS

DNA sequencing. Restriction fragments containing the 5′ regions of *Amy-2.1*^{y} and *Amy-2.2*^{y} were isolated from previously described mouse genomic clones (20, 35). A 1.15-kb *Eco*RI-*Hin*fI fragment and a 1.9-kb *Eco*RI-*Hin*dIII fragment were isolated from subclones of cosmid clones cPamE1 (*Amy-2.1*^{y}) and cPam23 (*Amy-2.2*^{y}), respectively. The fragments were sonicated, size fractionated, and cloned into M13mp8 by the procedure outlined by Bankier and Barrell (3). Phage DNA was sequenced with dideoxy nucleotides (43), [α-^{35}S]dATP, buffer gradient gels (7), and acrylosilane to bond gels to one plate for drying (17). In addition, some 5′-end-labeled fragments were sequenced by the method of Maxam and Gilbert (33), with a citrate A+G reaction (27). Sequences were determined from both strands for 87% of the *Amy-2.2*^{y} fragments (1,005 of 1,153 bp) and 79% of the *Amy-2.1*^{y} fragments (911 of 1,153 bp). The remaining sequences were determined by repeated sequencing of a single strand.

The 3′ ends of both *Amy-2.1*^{y} and *Amy-2.2*^{y} were sequenced from subclones containing exon j of each gene as described previously (20). Sequences were analyzed with the computer programs of Pustell (International Biotechnologies, Inc.).

Preparation of DNA for microinjection. The intact *Amy-2.2*^{y} gene was isolated on a 43-kb *Nru*I-*Mlu*I fragment from the cosmid clone cPam23 (previously designated cos23) (35). As a result of rearrangement during cloning at a site 9 kb upstream of *Amy-2.2*, this cosmid carries 15 kb of noncontiguous mouse genomic DNA (D. L. Gumucio, Ph.D. thesis, University of Michigan, Ann Arbor, 1986, p. 92). This *Amy-2.2*^{y} gene, also isolated on cosmid cPamE24, has been described previously (20). The fragment was purified by agarose gel electrophoresis, electroeluted into dialysis tubing, and dialyzed against 10 mM Tris hydrochloride, pH 7.5, containing 0.1 mM EDTA. The DNA was diluted with dialysis buffer to 6 μg/ml for microinjection into fertilized mouse eggs obtained from a (C57BL/6J × CBA/CaJ)F_1 female after mating with a CBA/CaJ male (see below).

A minigene was constructed by partial *Eco*RI digestion of the *Amy-2.2*^{y} cDNA clone pADPb1 (20). Full-length linear products were isolated and ligated to a 4.2-kb *Eco*RI fragment of cPam23 containing the 5′ flank, exon a, intron 1, and part of exon b. Clone L1, with the proper site and orientation of insertion, was identified by restriction analysis. After partial digestion of L1 with *Taq*I, full-length linear products were isolated. A 2.7-kb *Taq*I fragment from cPam23 containing part of exon j and 3′-flanking sequences was ligated into the linearized L1 DNA. Clone L2, with proper site and orientation of insertion, was also identified by restriction analysis. This clone was digested with *Bgl*II to produce the 2.4-kb fragment designated AM-0.21/0.3 (see Fig. 2). This amylase minigene contains 208 bp of 5′-flanking sequence and approximately 300 bp of 3′-flanking sequence. The 2.4-kb *Bgl*II fragment was gel purified and dialyzed as described above. The DNA was diluted to 1 μg/ml prior to microinjection into fertilized mouse eggs of strain CBA/CaJ × C57BL/6J.

Microinjection of DNA. Embryos were obtained from superovulated 12- to 14-g females 20 h after treatment with human chorionic gonadotropin. Zygotes were dissected from the oviducts into WM640 medium containing 0.1 mM EDTA (1, 26), washed, and removed to a microdrop of medium under paraffin oil in a microinjection chamber. Microinjection into the male pronucleus was performed with Nomarski optics (10, 25). After microinjection, the zygotes were incubated to the morula stage and then transferred to (BALB/cByJ × 129/SvJ)F_1 host females (24).

Southern blot analysis. Genomic DNA was isolated from 2-cm samples of tails as described previously (19; C. Sabatos, N. Copeland, and N. Jenkins, personal communication). DNA concentrations were determined by measurement of absorbance at 260 nm. To detect the microinjected cosmid DNA, genomic DNA was digested with *Eco*RI and *Pst*I, producing a 750-bp vector fragment. Restriction fragments were separated by electrophoresis on 1.2% agarose gels and transfered to nitrocellulose filters as described previously (47). Filters were hybridized with the 750-bp *Eco*RI-*Pst*I fragment purified from pJB8 (see Fig. 2). Probes were labeled by nick translation or by random oligomer-primed labeling (16).

To detect minigene sequences, genomic DNA was digested with *Taq*I. An 850-bp internal coding fragment was detected by hybridization with a cDNA probe containing the *Eco*RI-*Cla*I fragment from clone pMSa104 (21) (see Fig. 2).

Hormone treatment. To induce diabetes, 6- to 10-week-old mice were treated with six daily injections of streptozotocin (40 mg/kg) as described previously (14). Four weeks after injection, diabetic animals with urinary glucose levels of >2% were identified by using Diastiks (Miles Laboratories, Inc.). Loss of the B_1 isozyme from urinary amylase was used to confirm diabetic status (14). Insulin was administered to diabetic animals for 14 days by subcutaneous implantation of Alzet pumps (model 2001), which released insulin at a rate of 1.2 U/day. The pumps contained a solution of regular insulin (Eli Lilly and Co.), 62.5 U/ml in diluent, plus glutamic acid (7 mg/ml).

Dexamethasone was administered by subcutaneous injection of 0.1 mg of dexamethasone per kg of body weight (48). Six-day-old animals were injected once daily for 3 days.

Electrophoresis of amylase isozymes. Isozymes were separated by electrophoresis on 7% polyacrylamide gels at pH 8.1, as described previously (8, 14). Amylase activity was visualized with a starch-iodine stain, which can detect as little as 5 ng of amylase protein. Urine samples were mixed with glycerol and tracking dye and applied directly to the gels. Urine from diabetic animals was concentrated by lyophilization prior to electrophoresis.

Preparation of tissue samples. Tissue homogenates (10%, wt/vol) were prepared in 0.25 M sucrose–50 mM Tris hydrochloride (pH 7.5) with a Polytron homogenizer. In vivo labeling was carried out by intraperitoneal injection of 250 μCi of [^{35}S]methionine (8). After a 15-min labeling period, amylase was purified from pancreatic homogenates by precipitation with high-molecular-weight oyster glycogen. After electrophoresis of the ^{35}S-labeled amylase as above, the gel was treated with 50% trichloroacetic acid, followed by En^{3}Hance (New England Nuclear Corp.), and dried on Whatman paper for autoradiography. For quantitation, individual lanes were cut from the dried gel, sectioned, rehydrated, solubilized in Protosol (New England Nuclear), and counted in a liquid scintillation counter after addition of 10 ml of ACS scintillation fluid (Amersham).

Northern blot analysis. Total cellular RNA was purified by the CsCl cushion method of Chirgwin et al. (12). The integrity of the RNA was evaluated from the appearance of the 28S and 18S rRNAs on nondenaturing agarose gels. Electrophoresis was through a 1.5% agarose gel containing formaldehyde (32); transfer to nitrocellulose and hybridization were carried out as described by Thomas (49). Northern blots were probed with pADPb1, a cDNA clone complemen-

tary to *Amy-2.2*^{y} mRNA (20). Probes were labeled by nick translation to 1×10^8 to 3×10^8 cpm/μg or by random oligomer-primed labeling to 1×10^9 cpm/μg.

Quantitation of *Amy-2.2* mRNA by solution hybridization. To specifically detect the *Amy-2.2*^{y} transcript in the presence of an excess of *Amy-2.1* mRNA, we took advantage of sequence divergence in the 3′ untranslated regions of the two transcripts. A 20-base synthetic oligomer, 5′-TGTGCTCTCTACATGTCTTT-3′, was synthesized by the University of Michigan Oligonucleotide Facility. The oligomer is complementary to the *Amy-2.2*^{y} mRNA and differs from the *Amy-2.1*^{y} transcript at the eight underlined positions.

The oligomer was labeled to a specific activity of 6×10^6 cpm/pmol in a 5-μl reaction mixture containing 50 to 100 μCi of [γ-^{32}P]ATP at >5,000 Ci/mmol, 20 ng of gel-purified oligomer, and 5 U of T4 polynucleotide kinase. The reaction was incubated at 37°C for 45 min. Specific activity was determined by adsorption to DE81 paper. Unincorporated nucleotide was removed by three successive ethanol precipitations with 10 μg of tRNA added as carrier. Solution hybridization to RNA samples was carried out by the method of Durnam and Palmiter (15) with the following modifications. Each RNA sample was brought to 50 μg of RNA with tRNA, formamide was replaced by SET (15) to a final concentration of 0.2×, each reaction received 1×10^4 to 2×10^4 cpm of labeled oligomer, and hybridizations were carried out without paraffin oil at 50°C in Eppendorf tubes submerged in a water bath. S1 nuclease (50 to 100 U/ml) digestions were carried out at 37°C for 1 h. Trichloracetic acid precipitates were incubated on ice for at least 1 h. Nitrocellulose filters were used to collect the precipitates; GF/C filters did not retain them efficiently.

The abundance of *Amy-2.2*^{y} mRNA from strain YBR was estimated to be 10^5 molecules per acinar cell, assuming that 5% of total pancreatic RNA is mRNA, that amylase mRNA accounts for 15% of the pancreatic mRNA in strain YBR, and that 60% of the amylase RNA in YBR is the product of *Amy-2.2* (8). The following values were used: 1,572 bases of length per *Amy-2* mRNA (21); 6.4 pg of DNA per mouse cell; and an RNA-DNA ratio of 8.4 in mouse pancreas (38, 46).

RESULTS

Comparison of promoter regions of *Amy-2.1*^{y} and *Amy-2.2*^{y}. The nucleotide sequences of the 5′ portions of *Amy-2.1*^{y} and *Amy-2.2*^{y} are presented in Fig. 1. A 233-nucleotide sequence of perfect identity began at nucleotide −8 and extended through exon a into the first intron. Upstream of nucleotide −8, the two genes diverged markedly. The degree of divergence was approximately 30% between −8 and −400 and 50% in the remaining 5′-flanking region. There were several short conserved sequences within this region. Two of these corresponded to elements in the rat pancreatic amylase gene which are required for cell type-specific expression in a transfection assay (9). These regions of the rat and mouse genes are compared in Table 1.

Since pancreatic amylase is known to be inducible by glucocorticoids, we searched our sequences for the core of the glucocorticoid response element, TGT(T/C)CT (41, 51). One homologous sequence, in the inverted orientation, was detected (Fig. 1). This element was located in an unusual position between the TATA box and the mRNA cap site.

Production of transgenic mice carrying an intact *Amy-2.2*^{y} gene. A 43-kb DNA fragment containing the intact *Amy-2.2*^{y} gene (Fig. 2) was microinjected into fertilized mouse eggs. Twenty-nine liveborn animals were tested for expression of the transferred gene by electrophoretic analysis of urine samples collected at the time of weaning. The B_1 isozyme which is encoded by *Amy-2.2*^{y} was present in the urine of individuals 2670 (Fig. 3A) and 2797 (not shown). The presence of the transferred DNA was directly demonstrated by hybridization of genomic DNA with a probe (shown in Fig. 2) homologous to the cosmid cloning vector. The predicted 750-bp *Eco*RI-*Pst*I fragment was detected in individual 2670 (Fig. 3B). Comparison with quantitative standards indicated that a single copy of *Amy-2.2*^{y} was present (Fig. 3C). In addition, a third transgenic individual, 2845, was detected by Southern blot analysis, but neither he nor his offspring expressed the transgene in any of the tissues analyzed.

Mouse 2670, a female, was the founder of the transgenic line A70. Progeny carrying the transgene were identified by electrophoresis of urine. Autosomal Mendelian transmission of the transgene to 50% of offspring has been observed during several generations of outbreeding. In this line there was concordance between the presence of the vector fragment in genomic DNA and detection of the B_1 pancreatic amylase isozyme in urine.

Homozygous A70 animals were obtained by crossing two hemizygous transgenic animals. Homozygotes were identified by the increased intensity of hybridization on Southern blots. Homozygosity was confirmed by test crosses in which two putative homozygotes transmitted the transgene to 100% of their offspring (23 of 23 and 8 of 8). The homozygous animals were healthy and fertile.

Tissue-specific expression of *Amy-2.2*^{y} in A70 mice. Homogenates were prepared from a variety of tissues from the progeny of mouse 2670 and analyzed by electrophoresis followed by staining for amylase activity (Fig. 4). The B_1 isozyme product of the *Amy-2.2*^{y} transgene and the endogenous A_2 isozyme were clearly visible in pancreatic homogenates. The product of the transgene was not detectable in any of the other tissues.

Northern blot analysis was carried out on RNA isolated from various tissues. (The normal transcript of the *Amy-2.2*^{y} gene cannot be distinguished from other amylase transcripts by this method [34].) No unusual transcripts were detected in transgenic mouse pancreas (Fig. 4C). Amylase mRNA was detectable in as little as 0.01 μg of RNA from transgenic pancreas. In contrast, amylase mRNA was not detectable in 10-μg samples of RNA from the other tissues.

Regulation of the transgene in diabetic mice of line A70. To determine whether the transferred *Amy-2.2*^{y} retained its marked insulin dependence, A70 mice were treated with streptozotocin, which induces diabetes by destruction of the insulin-producing β-cells of the pancreas. Electrophoresis of urine from control and diabetic mice demonstrated a preferential loss of the B_1 isozyme (Fig. 5A). The effect on the rate of synthesis of pancreatic amylase was evaluated by in vivo labeling with [^{35}S]methionine. Purified amylase was then analyzed by electrophoresis and autoradiography (Fig. 5B). In untreated controls, labeling of the B_1 isozyme was clearly detected. Quantitation of gel slices by liquid scintillation counting demonstrated that the amount of total amylase synthesis associated with the B_1 isozyme in untreated A70 mice was 6.9 ± 0.6% (mean ± standard deviation, $n = 3$). In diabetic animals, synthesis of the B_1 isozyme could not be detected (Fig. 5B).

An oligonucleotide complementary to the 3′ noncoding sequences of the *Amy-2.2*^{y} mRNA was used to measure the abundance of *Amy-2.2*^{y} transcripts (see Table 2). This 20-mer differs at eight nucleotides from the corresponding

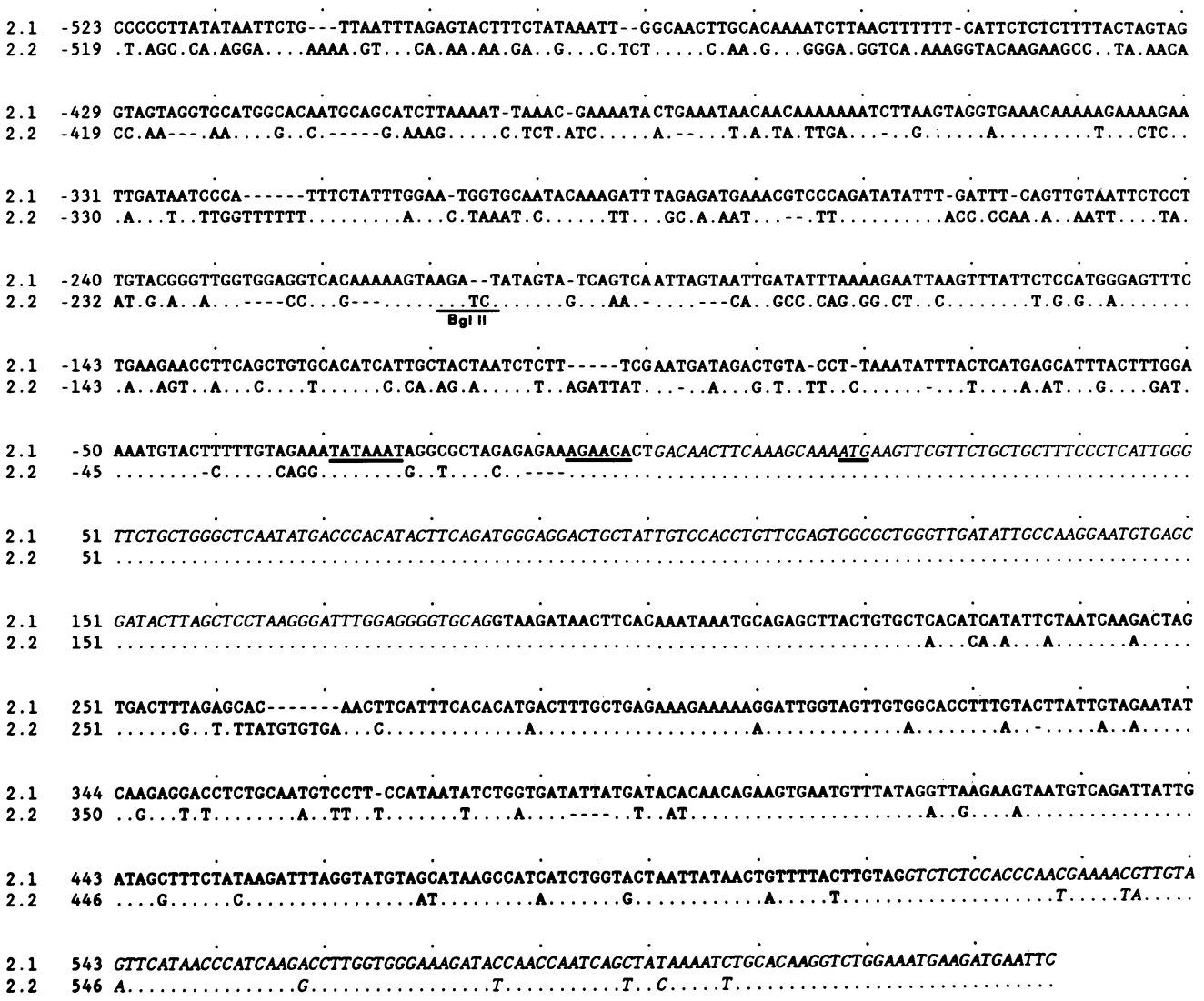

FIG. 1. Sequences of the 5′ regions of two murine pancreatic amylase genes. Dots represent nucleotides which are identical in the two genes. Dashes indicate deletions introduced to maintain alignment. Position +1 is the mRNA cap site of *Amy-2.1*[a] (21). The sequences of exons a and b are italicized. Underlining indicates the positions of the TATA box (−30 to −24), glucocorticoid response element core (−8 to −3), and AUG initiation codon (+18 to +20). The *Bgl*II site at −208 of *Amy-2.2*[y] was used to generate the amylase minigene.

sequence of the *Amy-2.1*[y] transcript (see Materials and Methods). The specificity of the probe for *Amy-2.2*[y] was demonstrated by using two inbred strains. RNA from strain YBR/Ki, which expresses *Amy-2.2*[y], protected the probe from nuclease digestion, while RNA from strain C57BL/6J did not protect the probe. The abundance of *Amy-2.2*[y] transcripts in RNA from line A70, with one gene copy per diploid genome, was one-third of that in strain YBR/Ki, with two gene copies per genome. Reduced abundance of *Amy-2.2*[y] transcripts was observed in diabetic A70 mice after treatment with streptozotocin, and treatment with insulin resulted in the reappearance of transcripts. This result indicates that a *cis*-acting sequence present in the transgene mediated the regulation of amylase by insulin.

Production of transgenic mice carrying an *Amy-2.2* minigene. To localize the sequence required for the observed

TABLE 1. Conserved sequence elements upstream of pancreas-specific genes[a]

Gene	Sequence (positions) I	Sequence (positions) II
Rat *Amy-2*	(−154) T TC CAT GAG AGT TT C (−140)	(−129) C AGC TGT (−122)
Mouse *Amy-2.1*[y]	(−158) C - - - - - - G - - - - - - - (−144)	(−131) - - - - - - - (−125)
Mouse *Amy-2.2*[y]	(−158) - - G - G - - - - - - - - - (−144)	(−131) - - C - - - - (−125)

[a] The two rat sequences were shown to be required for preferential expression of transfected constructs in AR42J cells (9). Sequence II is also conserved in several other rat pancreas-specific genes (45a). Dashes represent identity of the mouse genes with the rat sequence.

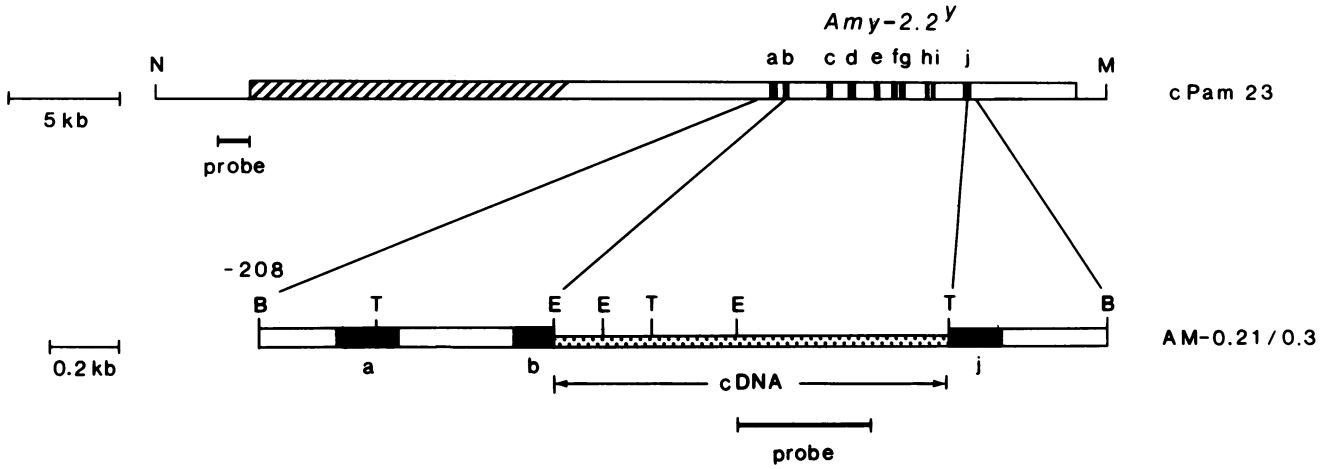

FIG. 2. Structures of DNA fragments used for microinjection. The 43-kb *Nru*I-*Mlu*I fragment of cosmid cPam23 contains the entire *Amy-2.2*^{y} gene with 9 kb of 5′-flanking sequence and 5 kb of 3′-flanking sequence, 15 kb of noncontiguous mouse genomic DNA (hatched area), and 4.2 kb of pJB8 vector sequence (single line). Exons are represented by solid boxes a through j. The 2.4-kb amylase minigene, AM-0.21/0.3, was constructed by insertion of genomic fragments from this cosmid into an *Amy-2.2*^{y} cDNA clone. The stippled area is derived from the cDNA. Abbreviations: N, *Nru*I; M, *Mlu*I; B, *Bgl*II; T, *Taq*I; E, *Eco*RI.

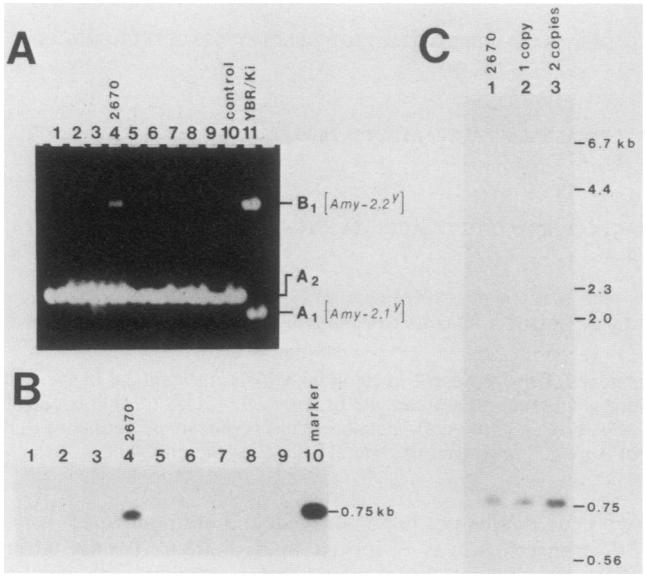

FIG. 3. Expression of *Amy-2.2*^{y} in transgenic line A70. Fertilized eggs were microinjected with 100 to 200 copies of the 43-kb cosmid fragment shown in Fig. 2. (A) Urine was collected from mice 21 days after birth. Three-microliter samples of urine were analyzed by polyacrylamide gel electrophoresis at pH 8.1. Gels were stained to visualize amylase activity with a starch-iodine stain. Lanes: 1 to 9, littermates from one microinjection experiment; 10, recipient mouse strain; 11, YBR mouse urine containing authentic B_1 isozyme. (B) Southern blot analysis of genomic DNA. DNA from the littermates described for panel A was digested with *Eco*RI and *Pst*I. The blot was probed with a vector fragment. The marker lane contained genomic DNA from an untreated animal mixed with cPam23 DNA. (C) Comparison of genomic DNA from transgenic mouse 2670 with quantitative standards. Southern blot analysis was carried out as above. The copy standards contained 10 μg of genomic DNA from an untreated control animal mixed with 9.5 or 19 pg of pBR322 DNA, corresponding to one and two copies per diploid genome, respectively. The blot was probed as described for part B.

tissue specificity and insulin dependence of the *Amy-2.2*^{y} gene, we constructed a minigene by adding two fragments of genomic DNA to an *Amy-2.2*^{y} cDNA fragment (Fig. 2). In addition to the complete coding sequence, the minigene contained 208 bp of 5′-flanking sequence, intron 1, and approximately 300 bp of 3′-flanking sequence. The protein product encoded by the minigene was identical to that of the intact *Amy-2.2*^{y} gene. A 2.4-kb minigene DNA fragment was purified from agarose gels and microinjected into fertilized eggs. Genomic DNA from one of 12 liveborn animals, 2852, contained a diagnostic 0.85-kb *Taq*I restriction fragment (Fig. 6, lane 5). Mouse 2852, the founder of line M52, transmitted the 0.85-kb fragment to approximately 25% of his progeny. The low frequency of transmission is suggestive of genetic mosaicism in 2852. Most of the offspring carried approximately 75 copies per diploid genome (Fig. 6, lanes 1 to 3), while a few offspring gave a less intensely hybridizing band corresponding to approximately 4 copies per diploid genome (Fig. 6, lane 4). Both high-copy and low-copy phenotypes were stably transmitted to 50% of subsequent generations, suggesting a single site of insertion in each case. Whether the two phenotypes were the result of two independent insertion events is under investigation.

The B_1 isozyme was not detectable in urine from mouse 2852, but all of his progeny carrying the transgenic DNA did produce detectable B_1 amylase. The level of expression was similar in the high-copy and low-copy progeny.

Tissue-specific and insulin-dependent expression of the amylase minigene. To determine whether the minigene includes the sequences required for pancreas-specific expression, tissue homogenates were prepared and tested for amylase activity (Fig. 4B). The B_1 isozyme was present in pancreatic homogenates, but was not detectable in the other tissues tested. In both high-copy and low-copy individuals, the B_1 isozyme constituted approximately 0.5% of the total pancreatic amylase.

The abundance of the *Amy-2.2*^{y} transcript in various tissues of M52 mice was compared by the oligonucleotide protection assay (Table 3). The abundance of the transcript in pancreas exceeded that in other tissues by more than

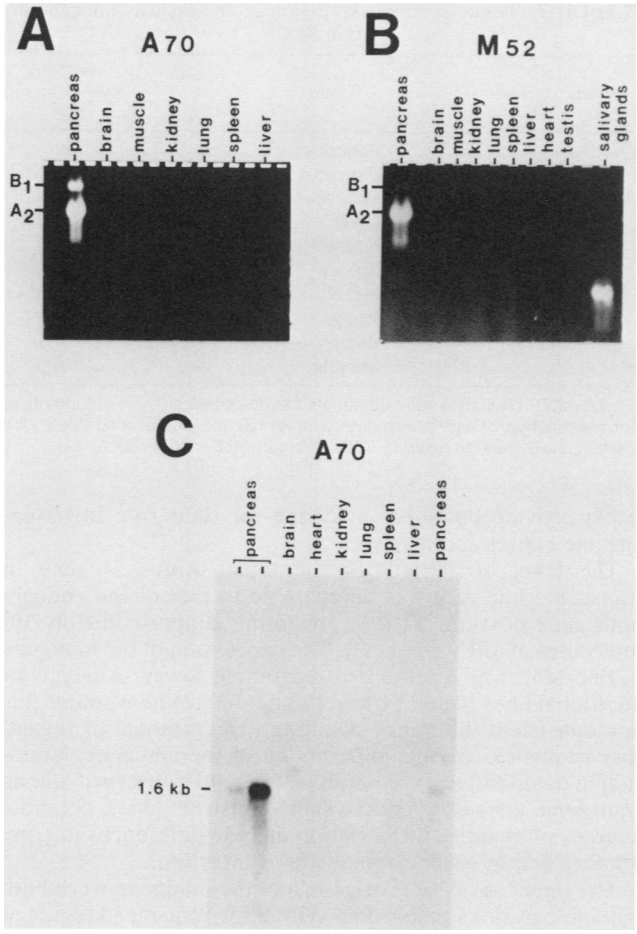

FIG. 4. Pancreas-specific expression in transgenic mice. (A and B) Ten-microliter samples of 0.05% tissue homogenates from strain A70 and M52 mice were analyzed by polyacrylamide gel electrophoresis. Gels were stained for amylase activity. Isozyme positions are indicated. (C) Northern blot analysis of total RNA from tissues of line A70. After electrophoresis in the presence of formaldehyde and transfer to nitrocellulose, blots were hybridized with nick-translated pancreatic amylase cDNA. Pancreas lanes, 10 and 100 ng; other tissues, 10 μg.

100-fold. After treatment with streptozotocin, the *Amy-2.2*^{y} transcript in pancreatic RNA was reduced to undetectable levels (Table 2, experiment 2). These results demonstrate that the sequences mediating tissue specificity and insulin dependence were present in the minigene.

Correct initiation of transcription. Pancreatic RNA was tested for its ability to protect an end-labeled genomic fragment which included the cap site of the mRNA from nuclease digestion. The pattern of bands protected by RNA from lines A70 and M52 was identical to that seen in nontransgenic mice, even after very long exposure of the autoradiographs, indicating that transcription was initiated correctly (data not shown).

Induction of amylase by glucocorticoid treatment. After treatment of 6-day-old animals with dexamethasone, the total amylase activity in pancreas was increased three- to fourfold. To determine whether the B_1 isozyme had been induced, samples of pancreatic homogenates were analyzed electrophoretically (Fig. 7). It is evident that the B_1 isozyme was induced by dexamethasone in both A70 and M52 mice.

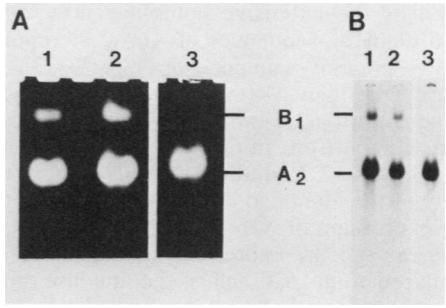

FIG. 5. Reduced expression of *Amy-2.2*^{y} in diabetic mice from the A70 transgenic line. Diabetes was induced by treatment with streptozotocin. (A) Amylase activity in urine samples. Lane 1, 2 μl of control urine; lane 2, 4 μl of control urine; lane 3, 100 μl of urine from diabetic A70 mouse. (B) Pancreatic amylase was labeled in vivo with [^{35}S]methionine, purified, and analyzed by electrophoresis. The gel was dried and X-ray film was exposed. Lane 1, control (55,000 cpm); lane 2, control (35,000 cpm); lane 3, diabetic (38,000 cpm).

DISCUSSION

The two nonallelic pancreatic amylase genes *Amy-2.1*^{y} and *Amy-2.2*^{y} are closely linked in strain YBR. The production of B_1 isozyme in transgenic mice carrying the *Amy-2.2*^{y} gene confirmed the previous assignment of amylase genes and isozymes (20). We sequenced approximately 1.1 kb of both genes, including 500 nucleotides upstream of the cap sites. The two genes diverged markedly upstream of nucleotide −8. Based on 30% divergence of the flanking sequence, assuming a mutation rate of 7×10^{-9} substitutions per site per year for noncoding sequences (40), the time of divergence of these two genes can be estimated to be 43 million years ago. This value exceeds our earlier estimate of 11 million years ago, which was based on the rate of silent substitutions in coding sequences (20). The difference between these two estimates may be explained by gene conversion events affecting the coding sequences. Evidence for one such conversion event was provided by the perfect identity of a 233-bp region of the two genes, which included exon a and part of intron 1 (Fig. 1).

Previous comparison of restriction maps and intronic sequences indicated that *Amy-2.2*^{y} is allelic with the silent *Amy-X* gene from strain A/J (20). This indication was

TABLE 2. Expression of transgenes in diabetic mice[a]

Expt no.	Strain	Treatment	*Amy-2.2* mRNA (cpm/μg)
1	YBR/Ki	None	12,000
	C57BL/6J	None	17
	A70	None	3,900
	A70	Streptozotocin	7
	A70	Streptozotocin plus insulin	2,100
2	YBR/Ki	None	8,000
	C57BL/6J	None	20
	M52	None	160
	M52	Streptozotocin	2

[a] A 20-base oligonucleotide specific for *Amy-2.2*^{y} was ^{32}P labeled and hybridized with total pancreatic RNA. After S1 nuclease digestion, the protected fragment was counted. The amount of RNA in each assay varied between 10 ng and 50 μg. Values were derived from multiple points in the linear range of the assay (200 to 1,200 cpm). The abundance of *Amy-2.2*^{y} transcripts in YBR/Ki pancreas is approximately 10^5 molecules per cell. Values are derived from representative individuals.

strengthened by the extensive homology now evident between the 5'-flanking sequences of *Amy-2.2*^{y} reported here and the corresponding sequence of *Amy-X* (22). The two genes differ by less than 2% (three single-base insertions and three base substitutions) within the 434-bp flanking sequence available for comparison. In the same region, the nonallelic *Amy-2.1*^{y} and *Amy-2.2*^{y} differed by 30%. It is not clear whether any of the identified differences are responsible for the lack of expression of *Amy-X* in strain A/J.

The pancreas-specific expression of the intact *Amy-2.2*^{y} gene in transgenic line A70 and of the amylase minigene in line M52 demonstrates that sequences regulating expression during differentiation are located near this structural gene. In the amylase minigene, noncoding sequences available for a regulatory role have been limited to 208 bp upstream of the cap site, one intron, and approximately 300 bp downstream of the polyadenylation signal. The upstream sequence of the minigene included two small regions with homology to rat amylase sequences that have been shown to function in pancreatic cell-specific enhancement in transfected cells (9). One of these sequence elements is also conserved in rat pancreatic protease genes (45a). The presence of these elements within the amylase minigene is consistent with their function in pancreas-specific gene expression. The base substitutions in *Amy-2.1*^{y}, *Amy-2.2*^{y}, and the protease genes presumably represent changes which are compatible with function. Site-directed mutagenesis within these elements could provide definitive evidence for their role in tissue-specific expression in vivo.

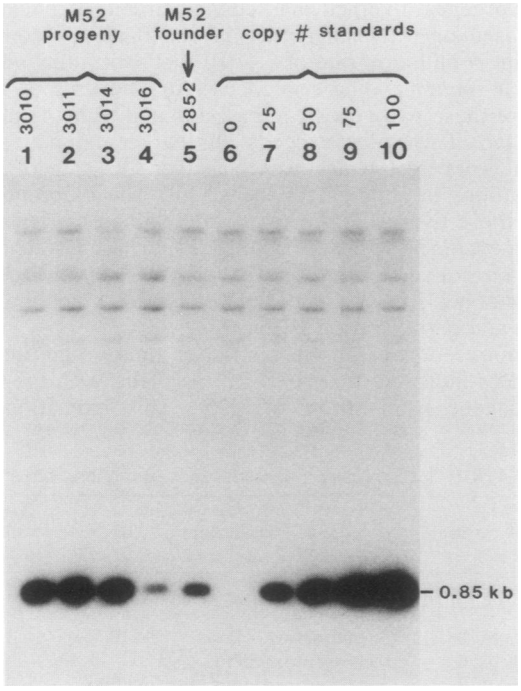

FIG. 6. Southern blot analysis of genomic DNA from transgenic line M52. The founder of this transgenic line, animal 2852, carried the 0.85-kb *Taq*I fragment derived from the microinjected minigene AM-0.21/0.3 (lane 5). Between 10 and 20 copies of the minigene were present in the sample of DNA from 2852. Some progeny contained 75 copies of the minigene per diploid genome (lanes 1 to 3), while others contained 4 copies per genome (lane 4). Copy standards contained 10 μg of genomic DNA from a control animal mixed with 5.2 pg of minigene DNA per copy. Fragments from endogenous amylase genes are visible near the top of the gel. The probe is a cDNA fragment (Fig. 2).

TABLE 3. Tissue-specific expression of an amylase minigene in strain M52[a]

Strain	Tissue	*Amy-2.2* mRNA (cpm/μg)
YBR/Ki	Pancreas	5,500
C57BL/6J	Pancreas	12
M52	Pancreas	83
	Liver	<0.2
	Kidney	<0.3
	Spleen	<0.6
	Heart	<0.3
	Lung	<0.1
	Brain	<0.1
	Muscle	<0.3

[a] *Amy-2.2*^{y} transcripts were quantitated as described in Table 2, footnote *a*. For nonpancreatic tissues, duplicate samples (20 to 50 μg) of total RNA were assayed. Less than 0.1 cpm/μg was detected in the carrier tRNA.

The level of activity of the intact *Amy-2.2*^{y} gene in transgenic line A70 was comparable to that of the endogenous gene in strain YBR/Ki, resulting in approximately 10^5 molecules of mRNA per cell. The expression of the minigene in line M52 was an order of magnitude lower. Analysis of additional lines would be required to determine whether this is a consistent difference resulting from removal of regulatory sequences during construction of the minigene. Variation in quantitative expression is frequently observed among transgenic lines carrying the same construct (38a); potential sources of quantitative variation include differences in copy number and in chromosomal site of insertion.

The intact amylase transgene and the minigene were both inducible by dexamethasone. Within the sequenced region of *Amy-2.2*^{y} there is a single copy of the postulated glucocorticoid response element core sequence, TGTTCT. It will be of great interest to determine whether this sequence, located between the TATA box and the cap site, is functional in mediating the glucocorticoid induction.

We have demonstrated that the *Amy-2.2*^{y} gene and minigene retain sequences which mediate the loss of amylase mRNA in diabetic animals. The concentration of *Amy-2.2*^{y} transcript was restored by treatment of the diabetic mice with insulin. These results provide evidence that *cis*-acting sequences mediate the response to diabetes and insulin. Either the transcription or the stability of the amylase mRNA may be affected. Although the effects of diabetes and insulin on pancreatic amylase were first described in 1963

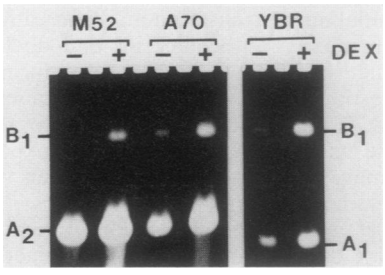

FIG. 7. Induction of *Amy-2.2*^{y} by dexamethasone. Six-day-old mice were treated with dexamethasone (DEX) on 3 successive days, and pancreatic amylase was analyzed on day 4. For each strain, equal amounts of pancreatic homogenate from treated and control animals were applied to the gel.

(11), it is still unclear whether the effect of insulin is direct or is mediated by other hormones or second messengers. The effects of insulin on transcription of PEPCK in cultured liver cells (18) and transcription of pyruvate kinase in the liver of diabetic rats (37) have been demonstrated, but the nature of the nuclear effector is not known. Sequence comparison of *Amy-2.2*^{y} with the recently published 5′-flanking region of PEPCK (5) did not reveal any striking homologies which might be related to regulation by insulin. However, comparison with a larger number of insulin-regulated genes might be more fruitful. Localization of insulin-responsive sequences by transfer to other promoters will be the goal of future work.

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LITERATURE CITED

1. **Abramczuk, J., D. Solter, and H. Koprowski.** 1977. The beneficial effect of EDTA on development of mouse one-cell embryos in chemically defined medium. Dev. Biol. **61**:378–383.
2. **Addison, W. R., and D. T. Kurtz.** 1986. Nucleotide sequences required for the regulation of a rat α_{2u}-globulin gene by glucocorticoids. Mol. Cell. Biol. **6**:2334–2346.
3. **Bankier, A. T., and B. G. Barrell.** 1983. Techniques in nucleic acid biochemistry. Elsevier, Ireland.
4. **Baumann, H., and L. E. Maquat.** 1986. Localization of DNA sequences involved in dexamethasone-dependent expression of the rat α_1-acid glycoprotein gene. Mol. Cell. Biol. **6**:2551–2561.
5. **Beale, E. G., N. B. Chrapkiewicz, H. A. Scoble, R. J. Metz, D. P. Quick, R. L. Noble, J. E. Donelson, K. Biemann, and D. K. Granner.** 1985. Rat hepatic cytosolic phosphoenolpyruvate carboxykinase (PEPCK): structures of the protein, messenger RNA, and gene. J. Biol. Chem. **260**:10748–10760.
6. **Ben Abdeljlil, A., J. C. Palla, and P. Desnuelle.** 1965. Effect of insulin on pancreatic amylase and chymotrypsinogen. Biochem. Biophsy. Res. Commun. **18**:71–75.
7. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
8. **Bloor, J. H., M. H. Meisler, and J. T. Nielsen.** 1981. Genetic determination of amylase synthesis in the mouse. J. Biol. Chem. **256**:373–377.
9. **Boulet, A. M., C. R. Erwin, and W. J. Rutter.** 1986. Cell-specific enhancers in the rat exocrine pancreas. Proc. Natl. Acad. Sci. USA **83**:3599–3603.
10. **Brinster, R. L., H. Y. Chen, M. E. Trumbauer, M. K. Yagle, and R. D. Palmiter.** 1985. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc. Natl. Acad. Sci. USA **82**:4438–4442.
11. **Chey, W. Y., H. Shay, and C. R. Shuman.** 1963. External pancreatic secretion in diabetes mellitus. Ann. Intern. Med. **59**:812–821.
12. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**: 5294–5299.
13. **Darlington, G. J., C. C. Tsai, L. C. Samuelson, D. L. Gumucio, and M. H. Meisler.** 1986. Simultaneous expression of salivary and pancreatic amylase genes in cultured mouse hepatoma cells. Mol. Cell. Biol. **6**:969–975.
14. **Dranginis, A., M. Morley, M. Nesbitt, B. B. Rosenblum, and M. H. Meisler.** 1984. Independent regulation of nonallelic pancreatic amylase genes in diabetic mice. J. Biol. Chem. **259**: 12216–12219.
15. **Durnam, D. M., and R. D. Palmiter.** 1983. A practical approach for quantitating specific mRNAs by solution hybridization. Anal. Biochem. **131**:385–393.
16. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
17. **Garoff, H., and W. Ansorge.** 1981. Improvements of DNA sequencing gels. Anal. Biochem. **115**:450–457.
18. **Granner, D., T. Andreone, K. Sasaki, and E. Beale.** 1983. Inhibition of transcription of the phosphoenolpyruvate carboxykinase gene by insulin. Nature (London) **305**:549–551.
19. **Grosschedl, R., D. Weaver, D. Baltimore, and F. Costantini.** 1984. Introduction of a μ immunoglobulin gene into the mouse germ line: specific expression in lymphoid cells and synthesis of functional antibody. Cell **38**:647–658.
20. **Gumucio, D. L., K. Wiebauer, A. Dranginis, L. C. Samuelson, L. O. Treisman, R. M. Caldwell, T. K. Antonucci, and M. H. Meisler.** 1985. Evolution of the amylase multigene family. YBR/Ki mice express a pancreatic amylase gene which is silent in other strains. J. Biol. Chem. **260**:13483–13489.
21. **Hagenbüchle, O., R. Bovey, and R. A. Young.** 1980. Tissue-specific expression of mouse α-amylase genes: nucleotide sequence of isoenzyme mRNAs from pancreas and salivary gland. Cell **21**:179–187.
22. **Hagenbüchle, O., U. Schibler, S. Petrucco, G. C. Van Tuyle, and P. K. Wellauer.** 1985. Expression of mouse *Amy*-2^{a} alpha-amylase genes is regulated by strong pancreas-specific promoters. J. Mol. Biol. **185**:285–293.
23. **Hill, R. E., K.-L. Lee, and F. T. Kenney.** 1981. Effects of insulin on messenger RNA activities in rat liver. J. Biol. Chem. **256**:1510–1513.
24. **Hoppe, P. C., and D. R. Coman.** 1983. Reduced survival *in utero* from transferred mouse blastocysts compared with morulae. Gamete Res. **7**:161–167.
25. **Hoppe, P. C., and K. Illmensee.** 1977. Microsurgically produced homozygous-diploid uniparental mice. Proc. Natl. Acad. Sci. USA **74**:5657–5661.
26. **Hoppe, P. C., and S. Pitts.** 1973. Fertilization *in vitro* and development of mouse ova. Biol. Reprod. **8**:420–426.
27. **Hudspeth, M. E. S., W. M. Ainley, D. S. Shumard, R. A. Butow, and L. I. Grossman.** 1982. Location and structure of the *var 1* gene on yeast mitochondrial DNA: nucleotide sequence of the 40.0 allele. Cell **30**:617–626.
28. **Karin, M., H. Holtgrere, A. Haslinger, R. I. Richards, P. Kranter, H. M. Westphal, and M. Beato.** 1984. Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein II_A gene. Nature (London) **308**:513–519.
29. **Korc, M., D. Owerbach, C. Quinto, and W. J. Rutter.** 1981. Pancreatic islet-acinar cell interaction: amylase messenger RNA levels are determined by insulin. Science **213**:351–353.
30. **Krumlauf, R., R. E. Hammer, S. M. Tilghman, and R. L. Brinster.** 1985. Developmental regulation of α-fetoprotein genes in transgenic mice. Mol. Cell. Biol. **5**:1639–1648.
31. **Logsdon, C. D., J. Moessner, J. A. Williams, and I. D. Goldfine.** 1985. Glucocorticoids increase amylase mRNA levels, secretory organelles, and secretion in pancreatic acinar AR42J cells. J. Cell Biol. **100**:1200–1208.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
34. **Meisler, M. H., T. K. Antonucci, L. O. Treisman, D. L. Gumucio, and L. C. Samuelson.** 1986. Interstrain variation in amylase gene copy number and mRNA abundance in three

mouse tissues. Genetics **113**:713–722.
35. **Meisler, M. H., and D. L. Gumucio.** 1986. Pancreatic amylase: molecular genetics and evolution, p. 249–263. *In* P. Desnuelle, H. Sjostrom, and O. Noren (ed.), Molecular and cellular biology of digestion. Elsevier Biomedical Press, Amsterdam.
36. **Noguchi, T., H. Inoue, and T. Tanaka.** 1982. Regulation of rat liver L-type pyruvate kinase mRNA by insulin and by fructose. Eur. J. Biochem. **128**:583–588.
37. **Noguchi, T., H. Inoue, and T. Tanaka.** 1985. Transcriptional and post-transcriptional regulation of L-type pyruvate kinase in diabetic rat liver by insulin and dietary fructose. J. Biol. Chem. **260**:14393–14397.
38. **Ornitz, D. M., R. D. Palmiter, R. E. Hammer, R. L. Brinster, G. H. Swift, and R. J. MacDonald.** 1985. Specific expression of an elastase-human growth hormone fusion gene in pancreatic acinar cells of transgenic mice. Nature (London) **313**:600–602.
38a. **Palmiter, R. D., and R. L. Brinster.** 1986. Germline transformation of mice. Annu. Rev. Genet. **20**:465–499.
39. **Peavy, D. E., J. M. Taylor, and L. S. Jefferson.** 1978. Correlation of albumin production rates and albumin mRNA levels in livers of normal, diabetic, and insulin-treated diabetic rats. Proc. Natl. Acad. Sci. USA **75**:5879–5883.
40. **Perler, F., A. Efstratiadis, P. Lomedico, W. Gilbert, R. Kolodner, and J. Dodgson.** 1980. The evolution of genes: the chicken preproinsulin gene. Cell **20**:555–566.
41. **Renkawitz, R., G. Schütz, D. von der Ahe, and M. Beato.** 1984. Sequences in the promoter region of the chicken lysozyme gene required for steroid regulation and receptor binding. Cell **37**:503–510.
42. **Ross, S. R., and D. Solter.** 1985. Glucocorticoid regulation of mouse mammary tumor virus sequences in transgenic mice. Proc. Natl. Acad. Sci. USA **82**:5880–5884.
43. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5468.
44. **Schibler, U., A.-C. Pittet, R. A. Young, O. Hagenbüchle, M. Tosi, S. Gellman, and P. K. Wellauer.** 1982. The mouse α-amylase multigene family: sequence organization of members expressed in the pancreas, salivary gland and liver. J. Mol. Biol. **155**:247–266.
45. **Stewart, T. A., P. K. Pattengale, and P. Leder.** 1984. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/*myc* fusion genes. Cell **38**:627–637.
45a. **Swift, G. H., C. S. Craik, S. J. Stary, C. Quinto, R. G. Lahaie, W. J. Rutter, and R. J. MacDonald.** 1984. Structure of the two related elastase genes expressed in the rat pancreas. J. Biol. Chem. **259**:14271–14278.
46. **Swift, G. H., R. E. Hammer, R. J. MacDonald, and R. L. Brinster.** 1984. Tissue-specific expression of the rat pancreatic elastase I gene in transgenic mice. Cell **38**:639–646.
47. **Takeuchi,T., D. L. Gumucio, T. Yamada, M. H. Meisler, C. D. Minth, J. E. Dixon, R. E. Eddy, and T. B. Shows.** 1986. Genes encoding pancreatic polypeptide and neuropeptide Y are on human chromosomes 17 and 7. J. Clin. Invest. **77**:1038–1041.
48. **Takuma, T., M. Nakanishi, Y. Takagi, T. Tanemura, and M. Kumegawa.** 1978. Precocious differentiation of mouse parotid glands and pancreas induced by hormones. Biochim. Biophys. Acta **538**:376–383.
49. **Thomas, P. S.** 1980. Hydribization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
50. **Walker, M. D., T. Edlund, A. M. Boulet, and W. J. Rutter.** 1983. Cell-specific expression controlled by the 5′-flanking region of insulin and chymotrypsin genes. Nature (London) **306**:557–561.
51. **Yamamoto, K. R.** 1985. Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. **19**:209–252.
52. **Young, R. A., O. Hagenbüchle, and U. Schibler.** 1981. A single mouse α-amylase gene specifies two different tissue-specific mRNAs. Cell **23**:451–458.