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Developmental Biology

Selective expression of rat pancreatic genes during embryonic development

(pancreatic differentiation/cDNA·mRNA hybridization/developmental gene expression/digestive enzymes)

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ABSTRACT **We present the developmental profiles of the mRNAs of 10 selectively expressed pancreatic exocrine genes and of insulin. The mRNA profiles fall into three related classes, but each profile is in some respect unique. The data on gene expression suggest there are four developmental states of the exocrine pancreas: early morphogenesis and low-level gene expression (the protodifferentiated state), the embryonic differentiated state, a modulated state in neonatal animals, and the adult differentiated state. Each state is characterized by distinct ratios of the exocrine mRNAs and presumably involves a distinct regulatory transition. This complex differentiative program must involve multiple regulatory molecules.**

The differentiated pancreas synthesizes and secretes a defined set of digestive enzymes (from exocrine cells) and hormones (from endocrine cells). Earlier studies in the rat have shown that the synthesis and accumulation of these secretory proteins occur in at least three distinguishable phases, presumably reflecting distinct regulatory transitions (1–3).

The early period of pancreas exocrine development is characterized by histological differentiation without obvious cytodifferentiation. During this period (the protodifferentiated state) low but significant levels of exocrine zymogens are detected (1, 2). Subsequently, there is extensive cytodifferentiation, including the accumulation of specific zymogens and the appearance of zymogen granules (embryonic differentiated state). The zymogen pattern is altered during the postnatal period and again in the mature adult pancreas (mature differentiated state).

Amylase mRNA levels increase more than 1000-fold, roughly in parallel with increases in enzyme levels (4). Thus, the accumulation of amylase seems to be due to increased levels of transcription of the amylase gene (4). A more definitive analysis of gene expression in the pancreas requires specific gene probes. cDNAs for most of the specific pancreatic exocrine and endocrine gene products have now been cloned and sequenced (5–11). We have used these cDNAs as probes to determine the specific mRNA accumulation profiles during pancreatic development. The pancreas-specific mRNAs accumulate in a discontinuous fashion. Significant levels of pancreas-specific mRNAs are found during the protodifferentiated state (12–14 days). Subsequently, large changes in the relative level of gene expression occur during the late embryonic phase, after parturition, and in the mature animal. The relative levels of expression of the various genes are different in each of the four differentiative periods.

MATERIALS AND METHODS

Isolation of RNA. Pancreatic RNA was isolated from embryonic and adult Sprague–Dawley rats by the guanidinium thiocyanate method (12) and was further purified by sedimentation through a layer of 5.7 M CsCl as described (12).

DNA Determination. The DNA content in the pancreas isolated from different ages was determined by a slight modification of the fluorometric method of Kissane and Robbins (13).

Cloned cDNA Probes. Eleven cloned rat cDNAs were used to determine the level of specific mRNA by cDNA·RNA hybridization. These were amylase [1620 base pairs (bp)] (5), carboxypeptidase A1 (1312 bp) (7), carboxypeptidase A2 (580 bp) and carboxypeptidase B (650 bp) (unpublished), chymotrypsin B (350 bp) (9), elastase I (920 bp) (10), elastase II (680 bp) (10), insulin I (350 bp) (11), phospholipase (620 bp) (unpublished), RNase (580 bp) (6), and trypsin I (850 bp) (8). The cross-hybridization of the trypsin I cDNA probe with trypsin II mRNA can be neglected because the trypsin II gene is expressed at <1% the level of trypsin I (8). The insulin I cDNA cross-hybridizes with insulin II mRNA; therefore, the data reflect contributions from both species.

^{32}P-labeled probes (specific activity, 2–4 × 10^9 cpm/μg) were prepared by nick-translating cDNAs (as a plasmid or isolated insert) as described (14), using the four [α-^{32}P]dNTPs at 25 μM each (Amersham, 3000 Ci/mmol; 1 Ci = 37 GBq). For quantitation of 28S rRNA, a cDNA probe was synthesized from electrophoretically purified 28S rRNA by reverse transcription using the random-priming method (15).

Quantitation of mRNA by Dot Blot Analysis. *Nitrocellulose binding of RNA.* RNA was denatured by heating at 60°C for 10 min in 10 μl of 2.2 M formaldehyde/50% (vol/vol) formamide/10 mM phosphate buffer, pH 7.0, and then was diluted in 100 μl of 20× standard saline citrate (SSC; 1× is 0.15 M NaCl/15 mM sodium citrate) and passed through a nitrocellulose filter that has been moistened with water and equilibrated with 20× SSC. A manifold apparatus (Schleicher & Schuell) was used to aid suction (flow rate about 100 μl/min). The filters were baked at 80°C for 2 hr.

Filter hybridization. The baked filters were prehybridized at 43°C for several hours in a sealed plastic bag containing 5 ml of 5× SSC/deionized 50% formamide/0.2% Ficoll/0.2% polyvinylpyrrolidone/0.2% bovine serum albumin/50 mM phosphate buffer, pH 7.0/0.1% $NaDodSO_4$/0.1% sodium pyrophosphate/sonicated salmon sperm DNA (100 μg/ml)/poly(rA) (50 μg/ml). Hybridization was at 43°C overnight with gentle mixing in 5 ml of the above solution containing nick-translated probe (1–5 × 10^7 cpm/ml). After the hybridization, filters were washed sequentially at 50°C in 4× SSC, 2× SSC, and 1× SSC, and 0.1× SSC in 0.1% $NaDodSO_4$ (30 min each). The filter was blotted dry and exposed to x-ray film, and radioactivity was determined by scintillation spectrometry or, for very weak signals, by scanning the autoradiograph with a laser densitometer equipped with an area integrator (LKB).

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Abbreviation: bp, base pair(s).

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Calculations. The concentration of mRNAs was calculated by normalizing hybridization levels relative to the signal of 20-day amylase mRNA, previously determined by solution hybridization to be 2.6×10^{-3} (4). Calculation was made according to the formula

$$C_n = \text{cpm}_n \times (L_R/L_D) \times (2.6 \times 10^{-3}/\text{cpm}_{20A}) \times R/D \times (7.12 \times 10^{-12}),$$

where C_n is the concentration (g per cell) of a particular mRNA; cpm_n is the counts/min (or densitometric value) of the hybridized RNA dot, corrected for the specific activity of the probe used; L_R is the length of the mRNA in bases (see *Results*); L_D is the length of the cloned cDNA fragment (see above); cpm_{20A} is the counts/min for amylase mRNA dot hybridization at 20 days of gestation; R/D is the ratio of total RNA to DNA (0.38 at 12 and 13 days, 0.45 at 14 days, 0.66 at 16 days, 1.10 at 18 days, and 2.40 at 20 days of gestation; 2.30 at birth; 9.30 in adulthood); and the DNA content per cell was taken as 7.4×10^{-12} g (16). The number of mRNA molecules per cell (N_n) is calculated from the formula $N_n = C_n \times 1/(\text{the molecular weight of the particular mRNA}) \times$ Avogadro's number.

RESULTS

Quantitation of mRNA Levels. In order to carry out the projected studies, the hybridization methods had to be specific, sensitive, and effective over a large concentration range (≈4 orders of magnitude). We have adapted dot blot hybridization to meet these specifications by optimizing the conditions of denaturation on the filters and maximizing the specific activities of the probes (see Fig. 1 legend). Hybridization with each cDNA probe was carried out with multiple increasing concentrations of RNA (Fig. 1*A*). The estimations of mRNA content were made from the linear portion of the curve fitted by the method of least squares (Fig. 1*B*). The limit of sensitivity under our conditions is about 0.5 pg as determined with a standard.

The specificity of hybridization is more difficult to control. A blot analysis of electrophoretically separated total RNA was carried out to test the hybridization specificity and to confirm the size of each mRNA. As shown in Fig. 2, each mRNA species was identified as a single band of the expected size. The sizes of mRNA in nucleotides were 1690 for amylase, 1450 for carboxypeptidases A1 and A2, 1550 for carboxypeptidase B, 960 for chymotrypsin B, 1250 for elastase I, 1100 for elastase II, 920 for RNase, 1700 for phospholipase, 970 for trypsin I, and 600 for insulin. Similar blot hybridization data were obtained for several RNA preparations obtained at 16, 18, and 20 days of gestation (data not shown). The hybridization specificity was tested further by comparing the dot blot results (Fig. 3 *A* and *B*) with control hybridizations with nonhomologous sequences. No hybridization was detectable above background when the pancreatic RNAs were hybridized with nick-translated pBR322 DNA (Fig. 1*A*) or when RNAs from nonpancreatic tissue (brain, liver, spleen) were hybridized with a cDNA probe containing sequence for carboxypeptidase A1 (Fig. 3*D*). However, there was detectable hybridization with other probes in some of the other tissues (Table 1). With the amylase and RNase cDNA probes, there was little if any signal with brain, intestine, and kidney but significant signals in the other tissues tested (heart, lung, liver, and spleen). It is not known whether this represents bona fide hybridization with RNA derived from the same or closely related genes or adventitious crossreaction with nonrelated genomic sequences. In these cases, the level of uncertainty corresponds to 50–200 molecules per cell.

There are several known nonpancreatic amylase genes (19). One of these is expressed both in the salivary gland and, at lower levels, in the liver. The mRNA of the salivary gland/liver gene crossreacts with the pancreatic amylase cDNA probe. Thus it is likely that the signal detected in the 16-day liver is due to amylase mRNA. Several of the other pancreatic genes are also members of gene families. There are, for example, at least 20 crossreacting trypsin genes (20) and several chymotrypsin genes (9). At least some of these may be expressed in other cell types and contribute to the signals observed in adult tissues. There are several others of the genes [carboxypeptidase A (7), A2 (unpublished data), B (unpublished data), chymotrypsin B (9), and elastase I and II (14)] that are probably single-copy genes, so that cross-hybridization with transcripts of related genes is not a problem. The specificity of the hybridization of the carboxypeptidases A1 and A2 and elastase I cDNA probes to pancreatic acinar mRNA is evident. Our observations on

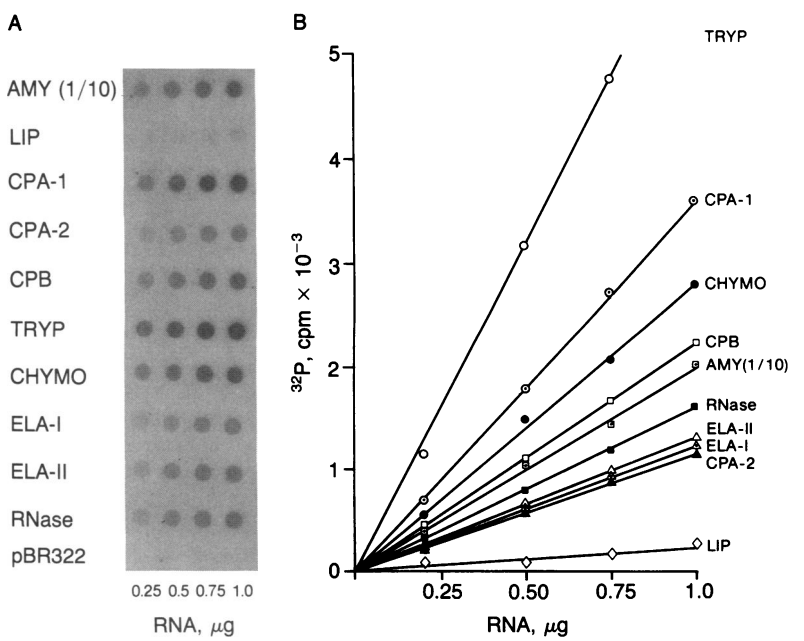

FIG. 1. Quantitation of mRNA by dot blot hybridization. (*A*) Total RNA isolated from adult rat pancreas was denatured, and 0.25-, 0.5-, 0.75-, and 1.0-μg (1/10th as much for amylase probe) samples were spotted onto a nitrocellulose filter and hybridized with various nick-translated cDNA probes as indicated: AMY, amylase; LIP, phospholipase; CPA-1, carboxypeptidase A1; CPA-2, carboxypeptidase A2; CPB, carboxypeptidase B; TRYP, trypsin I; CHYMO, chymotrypsin B; ELA-I, elastase I; ELA-II, elastase II; and RNase. (*B*) Each spot represented in the autoradiograph in *A* was cut out and radioactivity was determined. Each hybridization was corrected for background. Lines were obtained by linear regression.

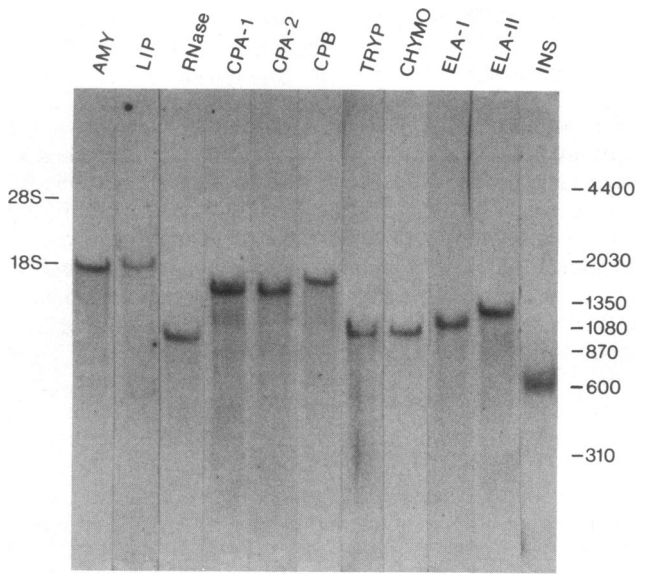

FIG. 2. Blot-hybridization analysis of pancreatic total RNA. RNA (1 μg per lane) isolated from adult rat pancreas was electrophoresed (17) in a 1.5% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose paper, and hybridized with nick-translated cDNA probes (INS, insulin I; see Fig. 1 legend for other abbreviations). Positions of 28S and 18S rRNA are shown at left. The standards (length in bases at right) were denatured restriction fragments of λ DNA cut with *Hin*dIII and φX174 replicative form DNA cut with *Hae* III.

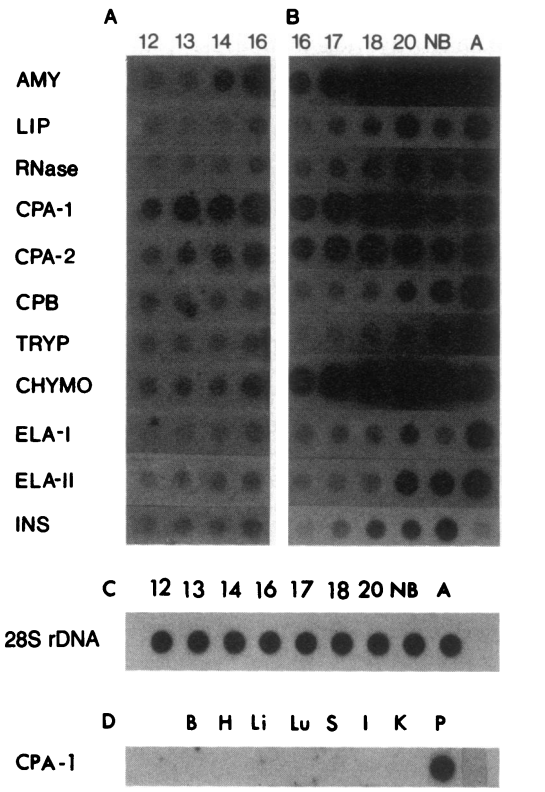

FIG. 3. (*A* and *B*) Changes in the levels of pancreatic mRNAs during differentiation. One-microgram samples of total RNA were spotted and hybridized with cDNA probes (see legends to Figs. 1 and 2 for abbreviations used). Numbers shown above each autoradiograph indicate the days of gestation of the embryo from which pancreatic RNA was isolated; NB and A, newborn and adult, respectively. Exposures for each probe in *B* were individually adjusted to compensate for the differences in the specific activity of each probe and the size of each cDNA relative to the size of the corresponding mRNA. The intensity of each dot, therefore, represents the relative level of mRNA in each RNA sample. (*C*) Changes in the level of 28S rRNA during pancreatic differentiation. Total RNA isolated from the indicated stage was hybridized with ^{32}P-labeled cDNA probe made from 28S rRNA. (*D*) Levels of carboxypeptidase A1 mRNA in nonpancreatic tissue. Total RNA isolated from various tissues (adult unless otherwise indicated) were hybridized with carboxypeptidase A1 probe. B, brain; H, heart; Li, embryonic liver (16 days); Lu, lung; S, spleen; I, intestine; K, kidney; P, embryonic pancreas (14 days).

elastase I are supplemented by the extensive analyses by Swift *et al.* (18).

There is an additional complication in the analysis, especially in the early embryonic stages. Mesenchymal cells are contiguous with the pancreatic epithelium and are difficult to remove. Dissection of the pancreas is performed by a standardized procedure that yields rather similar samples that are contaminated with mesenchymal cells. From DNA and protein analyses (21) of resolved epithelial and mesenchymal cells from pancreas, we estimated that the ratio of pancreatic epithelial cells to mesenchyme is approximately 0.2 at 12 days, 0.3 at 13 days, 0.7 at 15 days, and more than 0.9 thereafter. The values between 12 days and 14 days reported in Fig. 4 were not corrected for contamination with mesenchymal cells. Corrections were made for carboxypeptidases A1 and A2 and elastase I (dotted lines) since there is no apparent cross-hybridization signal in control tissues.

Exocrine Gene mRNA Levels During Pancreatic Development. At 12 days of gestation, the earliest practical stage for measurement of mRNAs by the methods we employed, the pancreas has developed from a diverticulum (detectable at 10 days) to a bulb-like epithelial structure resembling a single pancreatic acinus embedded within a mesenchymal-cell matrix. Both endocrine and exocrine epithelial cells are present contiguously in the epithelial monolayer forming the walls of the primitive acinus (22). Pancreas-specific mRNAs at 12 days are present at about 6–40 molecules per cell (Fig. 4). If

Table 1. Pancreatic mRNA in rat tissues

Tissue	Relative level of mRNA									
	AMY	LIP	RNase	CPA-1	CPA-2	CPB	TRYP	CHYMO	ELA-I	ELA-II
Pancreas (14 days)	1	1	1	1	1	1	1	1	1	1
Brain (adult)	<0.1	0.5	0.1	<0.01	<0.01	0.5	1	0.8	<0.1	0.5
Heart (adult)	0.5	0.5	0.2	<0.01	<0.01	1.0	2	1.5	<0.1	1.5
Lung (adult)	0.5	1	0.5	<0.01	<0.01	2.0	2	1	<0.1	2
Liver (16 days)	0.5	1.5	0.2	<0.01	<0.01	1.5	1.5	0.5	<0.1	0.8
Spleen (adult)	0.5	1.5	1.0	<0.01	<0.01	1.5	2	1.5	<0.1	2
Intestine (adult)	<0.1	0.8	0.1	<0.01	<0.01	0.5	1	0.5	<0.1	1
Kidney (adult)	<0.1	0.8	0.1	<0.01	<0.01	0.5	1	0.5	<0.1	1

The total RNA from various tissues was isolated and mRNA content was determined by hybridization with cDNA probes. On the basis of radioactivity measurement, the level of mRNA in each tissue was normalized to that in 14-day pancreas. Since the RNA/DNA ratios are not known in most tissues, we could not calculate absolute values (molecules per cell). See Fig. 1 legend for abbreviations.

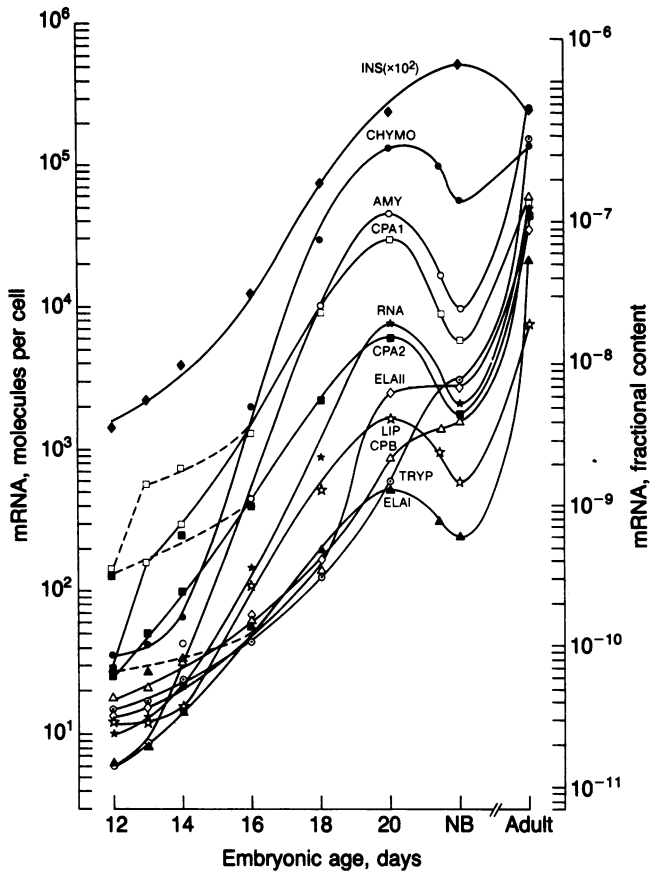

FIG. 4. Changes in the level of digestive-enzyme mRNAs and insulin mRNA during pancreatic differentiation. Levels of mRNAs at each stage are either expressed as molecules per cell (left coordinate, see *Materials and Methods* for calculation) or as fractional content (right coordinate, fraction of total RNA divided by molecular weight of each mRNA). Levels of mRNAs at 12–14 days were not corrected for mesenchymal contamination (see text), except for carboxypeptidases A1 and A2 and elastase I (broken lines). For convenience in plotting, the level of insulin mRNA was multiplied by 10^2. Symbols and abbreviations: open circles (AMY), amylase; filled circles (CHYMO), chymotrypsin B; open squares (CPA1), carboxypeptidase A1; filled squares (CPA2), carboxypeptidase A2; open triangles (CPB), carboxypeptidase B; filled triangles (ELAI), elastase I; open stars (LIP), phospholipase; filled stars (RNA), RNase; open diamonds (ELAII), elastase II; filled diamonds (INS), insulin; circles with a dot (TRYP), trypsin I. NB, newborn.

the mesenchymal cells contain none of these mRNAs, then the level present in the acinar cells (20% of total cells) would be in the range 30–200 molecules per cell. The relative concentrations of the mRNAs bear no relationship to the concentrations at the end of the gestational period (20 days) in the mature pancreas. At 12 days the highest mRNA levels correspond to carboxypeptidases A1 and A2, which are present at intermediate levels in the differentiated cell; the lowest levels are represented by amylase and trypsin, which are present at one of the highest and one of the lowest levels, respectively, in the differentiated pancreas cells (20 days).

The mRNA accumulation profiles (Fig. 4) generally increase during embryonic development to a maximum just prior to birth, decline significantly during the postnatal period, and again increase, to a slightly higher maximum, in the adult. Within these general trends, however, the profiles for individual mRNAs are highly variable and obviously noncoordinate. For example, at 13 days the level of carboxypeptidase A1 mRNA selectively increases; subsequently, the profile follows rather closely that of carboxypeptidase A2 and elastase I mRNAs. The level of amylase mRNA is relatively low at 12 days but then begins to increase rapidly at 13–14 days; subsequently the profile closely follows that of chymotrypsin until birth. In the adult the amylase/chymotrypsin mRNA ratio is different from that in the differentiated exocrine cells of the 20-day embryo. The concentration profiles of mRNAs encoding RNase and phospholipase are rather similar during days 12–18 but differ somewhat at 20 days. The levels of carboxypeptidase B and trypsin mRNAs increase modestly and similarly during embryonic development, only about 50-fold from 12–20 days, and then increase sharply in the postnatal period. Trypsin mRNA increases about 100-fold from birth to adulthood. Elastase II mRNA more or less parallels the levels for carboxypeptidase B and trypsin, during the early phases of development (12–18 days), but increases more rapidly between 18 and 20 days; it remains at a constant level at birth and increases about 10-fold in the adult.

Thus, each gene presents a singular mRNA profile, and significant changes in relative levels of mRNAs occur at each significant developmental stage (12–13 days, 20 days, birth, and adulthood), as shown in Table 2 and Fig. 4.

DISCUSSION

The present work defines the steady-state levels of 10 mRNAs of pancreatic enzymes (zymogens) and insulin during the course of development. It complements and extends the earlier measurements of the accumulation of pancreas-specific proteins (1–3).

The data confirm that the pancreas-specific genes are expressed during the early period of morphogenesis (the protodifferentiated state). This is most evident in the studies with carboxypeptidases A1 and A2 and elastase I, in which there is no detectable background hybridization with the probes, but it is also evident in the developmental profiles of the other mRNAs. The high signals for the 12- to 13-day pancreatic rudiments, obtained with carboxypeptidases A1 and A2 probes, are persuasive evidence for selective expression in the protodifferentiated state. The magnitude of the signal virtually precludes the possibility that the mRNA is derived from a minor cell type (e.g., glucagon- or insulin-producing cells); the carboxypeptidase signal is higher than the insulin hybridization signal (see Fig. 4).

The developmental profiles appear to be unique for virtually every gene, suggesting each gene may to some extent be regulated independently. However, the patterns of expres-

Table 2. Relative steady-state mRNA levels of pancreatic exocrine genes during development

Age	Relative level of mRNA									
	AMY	TRYP	CHYMO	CPB	CPA-1	RNase	CPA-2	ELA-II	ELA-I	LIP
Adult	100	59	56	22	20	18	17	13	8	3
Newborn	4	1	22	0.6	2	0.9	0.7	1	0.1	0.2
20-day	18	0.2	48	0.4	11	3	2	1	0.2	0.6
13-day	0.003	0.006	0.01	0.008	0.06	0.005	0.02	0.006	0.003	0.004

The level of each mRNA (molecules per cell) at each indicated stage was normalized to the level of amylase in adult pancreas. See Fig. 1 legend for abbreviations.

sion of certain sets of genes are similar and the profiles can be grouped into three classes: chymotrypsin B, amylase, RNase, and lipase (class I); carboxypeptidases A1 and A2 and elastase I (class II); and trypsin, carboxypeptidase B, and elastase II (class III). Thus, there may be common coordinating features in the regulatory mechanisms. The relative levels of the mRNAs in the late embryonic pancreas (embryonic differentiated state) are significantly different from those of the early embryonic pancreas (see Table 2) and confirm the existence of a significant regulatory transition during embryogenesis. After parturition, during the neonatal period there are distinct changes in relative levels of expression of the genes during a period of dramatic hormonal and nutritional changes. Specifically, there is a significant decrease in levels of class I and II mRNAs, while the levels of class III mRNAs (trypsin, carboxypeptidase B, elastase II) remain constant or increase during this phase (Fig. 4). The changes are not associated with birth *per se*, since they start to occur before parturition (see chymotrypsin B, amylase, and carboxypeptidase A1 in Fig. 4).

The relative levels of the mRNA species in the mature adult are distinct from earlier periods. The levels of class III mRNAs are selectively increased; the level of trypsin mRNA is ≈100-fold higher than in the embryonic differentiated state. The low level of trypsinogen in the embryonic differentiated state may reflect the selective control of the level of this powerful hydrolytic enzyme, which is the key activator of the other zymogen species.

The steady-state cDNA levels presented here represent the balance between synthesis (transcription) and breakdown (turnover). Earlier studies suggest that the accumulation profile for amylase mRNA during embryogenesis primarily reflects transcription. However, it seems unlikely that all regulations will operate solely at the transcriptional level. The varying ratios and patterns of the mRNAs in each of the four developmental stages (Table 2) indicate that regulation of this set of genes cannot occur via a unitary regulatory mechanism. The levels of expression of the exocrine genes vary by a factor >10,000. Thus there are both qualitative and quantitative differences in expression patterns for each of the genes. We hypothesize that the control of specific gene expression during embryogenesis must involve multiple regulatory elements, which we term "differentiators," that interact with the gene to control transcription. Recent experiments in our laboratory have shown that sequences in the 5′ regions of the genes for amylase, chymotrypsin, and insulin control the expression of a linked reporter function (chloramphenicol acetyltransferase) in differentiated pancreatic cell lines (23–25). Dissection of this control region in the amylase and chymotrypsin genes has revealed a cell-specific enhancer sequence (21, 23). Related sequences have been identified in the 5′ flanking regions of other pancreas-specific genes, suggesting the existence of a pancreas-specific enhancer element. More extensive studies on the insulin gene (24) suggest that in addition to the enhancer, a distinct regulatory element within the promoter region may contribute to cell-specific expression. Both positive and negative regulators may be involved (U. Nir and W.J.R., unpublished work). We postulate that a small set of *trans*-acting differentiators may be operative in pancreatic acinar cells. Inherent differences in affinity of these differentiators for *cis*-acting elements on the various genes may help to explain differences in levels of gene expression. However, the varying levels may also reflect regulation by hormones and/or differing nutritional states. Amylase, for example, is regulated by insulin and by glucocorticoid levels (26). Trypsin, on the other hand, is regulated by intracellular Ca^{2+} ions, by nutritional states, and by compounds that affect protein kinase C (C. Stratowa and W.J.R., unpublished work). Thus, a set of interacting regulatory systems may in combination produce the complex mRNA profiles observed in pancreatic acinar cells.

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