Cloning and Expression of the Rat p8 cDNA, a New Gene Activated in Pancreas during the Acute Phase of Pancreatitis, Pancreatic Development, and Regeneration, and Which Promotes Cellular Growth*

Gustavo Vidal Mallo‡, Fritz Fiedler‡‡, Ezequiel Luis Calvo†, Emilia Mariana Ortiz‡‡‡, Sophie Vasseur‡, Volker Keim‡‡ §§, Jean Morisset‡¶, and Juan Lucio Iovanna‡

To characterize at the molecular level the pancreatic emergency program set up by the pancreatic cells in response to pancreatitis, we have developed a strategy in which the phenotype of the pancreatitis affected pancreas is established by characterization of a large number of its transcripts. Herein, we describe the cloning, sequence, and expression of a new gene, named p8, which is strongly activated in pancreatic acinar cells during the acute phase of pancreatitis, in developing pancreas and during pancreatic regeneration. In acinar cells, p8 mRNA is expressed rapidly and specifically in response to cellular pancreatitis-induced injury; its induction occurred almost similarly in edematous and necrohemorrhagic pancreatitis, indicating that p8 mRNA is maximally activated even in response to a mild pancreatic injury. Furthermore, in vitro studies suggest that p8 mRNA is induced in pancreatic and non-pancreatic cells in response to some apoptotic stimuli. p8 acts as a promoter of cellular growth factor when its cDNA is transfected into COS-7 and AR4-2J cells. Although we failed to identify p8-related sequences, analysis of its primary and secondary structure suggests that p8 is a basic helix-turn-helix-containing gene with slight homology to several homeotic genes and sufficient signal to be targeted to the nucleus. We therefore propose p8 as a putative transcriptional factor which can regulate pancreatic growth.

Acute pancreatitis is the most frequent disease of the pancreas. The spectrum of acute pancreatitis can range from mild edematous to severe necrotizing (1). Autodigestion of the gland is one pathogenic theory which proposes that hydrolytic enzymes, especially trypsin, not only digest pancreatic and peripancreatic tissues but can also activate other enzymes such as elastase and phospholipase. The active enzymes then digest cellular membranes and cause proteolysis, edema, interstitial hemorrhage, vascular damage, coagulation necrosis, fat necrosis, and parenchymal cell necrosis.

Living organisms, including mammalian cells, respond to stress or pathological aggression by altering their normal pattern of protein synthesis (3–7). Such a changeover is characterized by dramatic increase in synthesis of stress proteins with a concomitant decrease in production of the normal array of cellular proteins. Stress proteins are not novel components of the stressed cells since most of them are expressed in cells grown under normal conditions (8, 9). Because most attacks of acute pancreatitis are mild and self-limiting, it is possible that the pancreatic cells can rapidly adapt their phenotype to this pathological situation, in which in turn could stop the progression of the pancreatitis. However, in 10–20% of the cases, a severe disease with multiple local and systemic complications develops (10). In these later cases, we can speculate that the pancreatic defense mechanisms fail to protect the gland and then the organism.

Studies in animals and humans performed during the acute phase of pancreatitis demonstrated that content and secretion of pancreatic enzymes, which are potentially harmful, were generally reduced by the cells (11, 12), as part of a defense mechanism. Conversely, other genes were strongly activated during the acute phase of the disease. Most of these genes can act to prevent evolution of the acute pancreatitis, whereas others can participate in pancreatic regeneration following pancreatitis. Among expression of these new genes, those of the structurally related PAP I, II, III, and lithostathine/reseg (13–16), are rapidly induced. In addition, c-myc and H-ras oncogenes, the cytostkeletal villin and actin (17), the growth factors IGF-I (18) and II, IGF-I receptor and IGF-binding proteins 1 and 3 mRNAs (19), the hepatocyte growth factor (20) and its receptor (c-met) (21), and the anti-apoptotic clusterin gene (22) were also activated during acute pancreatitis. These results support the view that during acute pancreatitis there is not a mere decrease of pancreatic function but a well defined response of the gland characterized by specific alterations of proteins (e.g. trypsinogen, chymotrypsinogen, proelastase, and phospholipase A) are activated within the pancreas rather than into the intestinal lumen (reviewed in Ref. 2). Activated enzymes, especially trypsin, not only digest pancreatic and peripancreatic tissues but can also activate other enzymes such as elastase and phospholipase. The active enzymes then digest cellular membranes and cause proteolysis, edema, interstitial hemorrhage, vascular damage, coagulation necrosis, fat necrosis, and parenchymal cell necrosis.
protein synthesis, as previously proposed (12). Such specific responses may suggest that the pancreas, like the liver (23) or other organs (5, 6), exhibits a stringent emergency program that enables the survival of the gland or the organism under the situation of acute stress.

The objective of our research is therefore to characterize the pancreatic emergency program in response to pancreatitis at the molecular level. We therefore developed a strategy in which the phenotype of the pancreas with acute pancreatitis has been established by characterization of a large number of its transcripts. Such a cDNA collection should therefore contain all potential candidates and represent a reservoir from which mRNAs with appropriate patterns of expression can be eventually selected. In this report, we describe the cloning, sequence, and expression of a new gene, named p8, which was identified from a pancreas with pancreatitis; the gene is strongly and rapidly induced in the inflammed pancreatic gland.

MATERIALS AND METHODS

Pancreatic cDNA Library

Acute pancreatitis was induced in rats by retrograde injection of 200 μl of 1% sodium taurocholate in the main pancreatic duct, as described by Lankisch et al. (24). Total RNA was prepared as recommended by Chirgwin et al. (25) from rats killed 24 h after the induction of pancreatitis. Polyadenylated RNAs were purified by affinity chromatography on oligo(dT)-cellulose (26). Two micrograms of poly(A)+ RNA were used for the first strand cDNA synthesis with an oligo NotI-dT18 as primer in the presence of reverse transcriptase. DNA polymerase was used in conjunction with RNase H to synthesize the complementary cDNA strand. Enzymes and reagents were obtained as a kit from Pharmacia Biotech Inc. and used according to the manufacturer’s recommendations. The double-strand cDNA was ligated into the plasmid pT7T3D (Pharmacia Biotech Inc.) into the EcoRI/NotI restriction sites. A small portion of the ligation mixture was transformed into competent Escherichia coli NM522 by Hanahan’s method (27) and spread over LB plates supplemented with 100 μg/ml ampicillin. The library contained a total of 4.1 × 1010 independent recombinant clones. 4800 fresh transformant colonies were picked, grown in microwell plates, and stored at −80 °C in the presence of 15% glycerol.

Sequencing and Data Base Analysis

The cDNA inserts of 288 phagemids were sequenced with a Sequenase Version 2.0 DNA Sequencing kit (U. S. Biochemical Corp.) using the synthetic oligonucleotide 5′-CCCTCATCAAAGGGATAAGC-3′ as primer. Sequencing reactions were run on a 6% acrylamide, 7.5% polyacrylamide sequencing gel and autoradiograms were read manually on an IBI reader gel system (IBI, New Haven). About 200 nucleotides were read for each sequence. Sequences from the library were compared with each other to determine their frequency of appearance. Each sequence was also compared with all sequences available in GeneBank using the BLAST network service (28). Only the 10 best reports, sorted from most statistically significant (lowest Poisson P-value) to least statistically significant (highest Poisson P-value) were taken in account.

Sequencing of the Rat p8 mRNA

DNA inserts of clones 1H11, 2A9, and 2H10 were completely sequenced using the Sequenase Version 2.0 DNA Sequencing kit (U. S. Biochemical Corp.) following the manufacturer’s recommendations and with appropriate synthetic oligonucleotides as primers.

Rat p8 mRNA Expression—Sprague-Dawley rats were used in all experiments. Animals were housed with free access to food and water. Experiments were performed according to the standard ethical and legal guidelines and with the permission of the local committee for the inspection of animal experiments.

Acute Experimental Pancreatitis—Acute necrotizing pancreatitis was induced in rats weighing 200–250 g by retrograde injection of either 1 or 4% sodium taurocholate as described above. Edematous pancreatitis was induced by two intraperitoneal injections of cerulein (40 μg/kg) at 30-min intervals as described by Yamaguchi et al. (29). The animals were killed after 3, 6, 12, 24, 48, and 72 h and after 5 and 10 days. Pancreas, liver, kidney, salivary glands, testes, stomach, duodenum, jejunum, ileum, colon, heart, lungs, brain, spleen, skeletal muscle, and thymus were processed according to Chirgwin et al. (25) for RNA preparation.

Subtotal Pancreatectomy—Rats weighing 100–110 g were used in these experiments. The splenic, gastric, and duodenal segments of the pancreas were removed by gently swabbing the tissue away from its vascular and peritoneal attachments with a cotton swab, as described by Foglia (30). Only one well defined portion bounded by the common bile duct and the duodenum was left in place. Fed rats were sacrificed at 12 h and 1, 2, 3, 6, 14, and 90 days post-pancreatectomy and the remnant pancreas was removed and RNA extracted as described above.

Ontogeny—19- and 21-day-old rat fetuses, newborn, and 3, 5, 7, 9, 13, 15, 19, 21, 23, 25, 35, 45, and 90-day-old rats were used. Fetal pancreata and adult pancreas were obtained from timed pregnant rats 19 and 21 days after appearance of vaginal plug, and pancreas and livers from several animals (male and female) were pooled. Rats were sacrificed by decapitation, and their pancreas and livers were rapidly removed and submerged in liquid nitrogen before processing.

Preparation of Anti-p8 Antibodies and Western Blot

A peptide sequence corresponding to amino acids 61 to 80 of rat p8 was chemically synthesized (Neosystem, Strasbourg, France). The purified peptide was conjugated to ovalbumin and used to immunize New Zealand White rabbits at the recommended intervals. Antiserum was collected by puncture of the ear vein 10 days after the last injection. Pancreas from a control and a rat with pancreatitis was homogenized in 100 mM Tris, pH 8.0, and 5 mM benzamidine, submitted to 5 freeze-thaw cycles using liquid nitrogen and a waterbath at 37 °C, and centrifuged in Microfuge for 15 min at 10,000 × g. 200 μl of each sample were submitted to SDS-polyacrylamide gel electrophoresis (15% acrylamide, 0.5% bisacrylamide) and transferred to a nitrocellulose membrane. The first antibody was used at a 1:2500 dilution and the second antibody was a peroxidase-labeled anti-rabbit IgG antibody provided with the ECL-kit (Amersham). Immunoblotting was performed using the ECL kit and membranes were exposed to a Kodak Biomax film for 1 min.

In Situ Hybridization Analysis for Expression of Rat p8 mRNA

Preparation of the Probes—The rat p8 complementary RNA (cRNA) probe was transcribed from the 602-base pair NotI-EcoRI rat p8 cDNA fragment (clone 2H10), inserted into the plasmid vector pT7T3D (Pharmacia). The templates were linearized with EcoRI and T3 RNA polymerase was used for in vitro antisense transcription, or NotI and T7 RNA polymerase for the sense cRNA transcription. After linearization, cRNA probes were labeled with DIG-UTP (Boehringer Mannheim).

In Situ Hybridization—The in situ hybridization method was described by Komnitho et al. (31) with modifications. Briefly, duplicate 5-μm thick, formalin fixed, paraffin-embedded tissue sections were placed on Superfrost Plus-treated slides (Fisher Scientific). Tissue sections were then deparaffinized, hydrated, and permeabilized with 2 μg/ml proteinase K (Amersham, Arlington Heights, IL), and a 1-glutaminemodified hybridization buffer containing 50% formamide, 50 mM HEPES, 5 × Denhardt solution, 4 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate), 250 μg/ml denatured herring sperm DNA, and 5 μl EDTA, pH 8.0, for 2 h at 42 °C. Hybridization was performed overnight at 56 °C in a humidified chamber (5–10 ng of digoxigenin-labeled RNA probe in 200 μl of hybridization buffer per slide). As a negative control, the sections were treated with RNase A prior to prehybridization, and a second negative control was performed using the rat p8 sense RNA as a probe. After hybridization, unbound probe was digested with RNase A (20 μg/ml) for 30 min at 37 °C. The post-hybridization washes were all performed at 37 °C, and consisted of two washes in 2 × SSC, two in 0.2 × SSC, and one in 0.1 × SSC (each for 15 min). The hybridization signal was detected according to the instructions of the Dig Nucleic Acid Detection kit (Boehringer Mannheim). The slides were counter-stained with Harris solution (Sigma), and mounted with glycerol/phosphate-buffered saline (50% v/v).

Cell Culture

AR4-23 pancreatic acinar cells were used after 48 to 55 passages. The cells were routinely cultivated at 37 °C in a 5% CO2, 95% air atmosphere in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum (Life Technologies, Inc.), 4% antibiotics (50 units/ml penicillin, and 50 μg/ml streptomycin). COS-7, Rat2, and IEC6 cells were maintained under the same culture conditions as AR4-23 cells except that fetal calf serum was 5% for Rat2 cells, and insulin (10 μg/ml) was added to IEC6 cells. When cells reached 80–90% confluence, they were dissociated with 0.05% trypsin and 0.02% EDTA in Puck’s saline A and replated into 100-mm Petri dishes.
Fig. 1. Sequence of rat p8 mRNA and deduced sequence of the encoded protein. The open reading frame encoding rat p8 is given in lower case letters. Noncoding sequences are in lowercase letters, and the polyadenylation site AATAAA is underlined.

AR4-2J cells were incubated in medium with indicated doses of cycloheximide (20 μg/ml), phorbol 12-myristate 13-acetate (1 μM), dexamethasone (1 μM), and staurosporine (0.1 μM) (Sigma), TNFα (500 units/ml), epidermal growth factor (2 ng/ml), IL-1 (250 units/ml), IL-4 (1 unit/ml), and IL-6 (500 units/ml) (Boehringer Mannheim), ceramide (20 μg/ml) (Euromedex) or cells were serum starved for the indicated times. Treatments with H2O2 (0.1 mM) were performed as 1-h incubations in serum-free medium and heat-shock treatment was achieved by a 1-h incubation at 42 °C; the cells were then grown for an additional 12 or 24 h under standard conditions. After the different treatments, approximately 2.5 × 10^6 cells were lysed for RNA extraction (32). Genomic DNA was prepared from approximately 5 × 10^6 cells. Cells were collected in 500 μl of buffer (10 mM Tris-HCl, pH 7.4, 0.4 mM EDTA). When cells were lysed by the addition of 0.1% SDS and frozen at −20 °C. As thawing, the cells were incubated 2 h with 20 μg/ml pancreatic DNase I at 37 °C and then washed four times for 5 min at room temperature in 2× SSCE. DNA pellets were resuspended in 200 μl of TE buffer and submitted to electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

**Northern Blot**

Twenty μg of RNA were submitted to electrophoresis on a 1% agarose gel and vacuum blotted onto Hybond-N membranes (Amersham). The filters were hybridized with the corresponding 32P-labeled probes for 16 h at 65 °C in 5 × SSPE (1 × SSPE is 180 mM NaCl, 1 mM EDTA, 10 mM NaH2PO4, pH 7.5), 5 × Denhardt solution, 0.5% SDS, and 100 μg/ml single-stranded herring sperm DNA. Then, the filters were washed four times for 5 min at room temperature in 2 × SSC, 0.2% SDS, twice for 15 min at 50 °C in 0.2 × SSC, 0.2% SDS, and once for 30 min in 0.1 × SSC at 50 °C.

**Transfection of the Rat p8 cDNA and Cell Proliferation**

The full-length rat p8 cDNA, clone 2H10, was subcloned into the EcoRI-NoI restriction sites of the mammalian expression vector pcDNA3 (Invitrogen), downstream from the cytomegalovirus promoter. This plasmid also contains the aminoglycoside phosphotransferase-3' gene that confers resistance to the antibiotic geneticin (G418 sulfate). The recombinant plasmid was named pcDNA3/rp8. As control, we have used the pcDNA3/CAT plasmid in which the CAT gene was also under the control of the cytomegalovirus promoter. The plasmids were transfected into COS-7 and AR4-2J cells using the calcium phosphate-DNA co-precipitation as described previously (33). To select for stable expression of the pcDNA3/rp8 and pcDNA3/CAT, the cells were cultivated over 2–3 weeks in media containing G418 (600 μg/ml) starting 48 h after transfection. Surviving cells were maintained in standard culture medium supplemented with G418 (400 μg/ml). Cells transfected with p8 cDNA (COS/p8 and AR/p8) or control (COS/CAT and AR/CAT) were plated in 12-well culture dishes at 5 × 10^3 cells/well. After 1, 2, 3, 4, 5, and 6 days, cells were counted on a hemacytometer. The proliferative response of the p8/COS and p8/CAT cells was also estimated by [3H]thymidine incorporation. Cells (10^6 cells/well) were plated onto 96-well plates and cultured overnight under standard conditions. The media was then removed and the cells were incubated for 6 h in the presence of 1 μCi of [methyl-3H]thymidine (25 Ci/mmol, Amersham). The cells were then lifted off the plate with trypsin, and cellular DNA was collected on glass filters (GF/C glass microfilters, Whatman) with a vacuum filtration cell harvester. Incorporation of tritium was measured with a scintillation counter.

**RESULTS**

Cloning of the Rat p8 mRNA—A cDNA library of 4.1 × 10^6 clones was constructed from the polyadenylated RNA fraction purified from a rat pancreas with acute pancreatitis. From this cDNA library, 288 randomly selected clones were partially sequenced and the resulted sequences were compared with GenBank database. Among these clones, 201 corresponded to fully characterized rat mRNAs and 28 were homologous to human, mouse, or dog proteins. Finally, 59 clones could not be related to any sequence in the database. Among them, 48 clones were represented only once in the library, 4 were represented twice, and 1 clone was represented three times (clones 1H11, 2A9, and 2H10). Then, expression of the unrelated mRNAs during acute pancreatitis was systematically analyzed by Northern blot. Expression analysis of the clone 2H10 showed an interesting pattern. Indeed, this mRNA is strongly and rapidly activated after the induction of an experimental acute pancreatitis (see below). A more complete work concerning the analysis of all these clones will be published elsewhere.

**Sequence Analysis of the Rat p8**—Sequence of cDNA inserts of clones 1H11, 2A9, and 2H10 was completed. The size of each clone was 591, 566, and 602 base pairs, respectively. Overlapping sequences between these cDNA inserts were identical. Clone 2H10 contained all sequences of the 1H11 and 2A9 clones. A single open reading frame was found in the corresponding mRNA sequence (Fig. 1). Two methionines at position 52 and 55 are potential initiation sites for translation. In vertebrates, however, protein synthesis typically starts from upstream methionine (34) and the initiation codon is surrounded by a well conserved sequence motif. The most important features of that consensus sequence are an A in position −3 and a G immediately after the ATG codon (35), which are actually present around position 55 only. Hence, synthesis of p8 very probably starts at nucleotide 55. The 3' region consisted of a TGA stop codon at position 295 followed by 292 nucleotides on untranslated message (excluding the poly(A) stretch). A canonical polyadenylation recognition site (AATAAA) was found 18 nucleotides upstream of the poly(A) tail. The 2H10 clone contains 54 nucleotides of 5'-untranslated region.

The primary structure of the rat p8 was deduced from the single open reading frame. The p8 polypeptide is 80 amino acids long and has a theoretical pI of 9.45. The predicted molecular weight is 8,955. Using the presumed initiation site, the deduced p8 primary structure cannot be aligned with another known protein present in GeneBank or Swissprot data bases. Using the PROSEARCH data base (36), we have observed only a slight degree of homology with the invariant
amino acids present in the homeodomain of several homeobox genes (Fig. 2). A hydrophobicity plot demonstrates a marked hydrophilicity throughout the length of the protein, except for a mildly hydrophobic region in the middle (data not shown).

Using the PSORT program (Prediction of Protein Localization Sites) version 6.4 at the www.expasy.hcuge.ch site, we have calculated, with high probability, that p8 has no signal peptide (score of 26.82), suggesting that it is not a typical secretory protein (37). Also, p8 does not have transmembrane regions, and it is with low probability a mitochondrial protein (score of 0.100), a lysosomal protein (score of 0.100) or an endoplasmic reticulum anchored protein (score of 0.000). However, p8 has a potential bipartite signal for nuclear targeting at position 63 (RKLLTKFQNSERKKAWR) (38), suggesting that the p8 function is in the nucleus (Fig. 2). Even more interesting, analysis of the predicted secondary structure showed that p8 has a putative basic helix-turn-helix motif (Fig. 2). However, this motif has not significant homology with the helix-turn-helix contained in other known genes.

**Regulation of p8 mRNA Expression during the Course of Acute Pancreatitis**—Pancreatic RNA was obtained from rats at different times after induction of an experimental acute pancreatitis by retrograde injection of 1% sodium taurocholate. As shown in Fig. 3, Northern blot analysis of these RNAs with the p8 cDNA probe reveals a low level of expression in control pancreas but a strong signal 3 h after the induction of pancreatitis. p8 mRNA reaches its maximal expression between 6 and 12 h, and then decreased progressively to control levels after 24 h.

We also evaluated the p8 mRNA expression as a function of pancreatitis severity by comparing p8 mRNA expression in response to edematous cerulein-induced or necrohemorrhagic 4% sodium taurocholate-induced pancreatitis. As shown in Fig. 4, p8 mRNA expression is equally activated with similar kinetics in both models suggesting that severity of the disease does not play an important role in the p8 gene expression.

**Identification of p8 in the Pancreas with Pancreatitis**—The induction of p8 during acute pancreatitis was studied by Western blot using a specific anti-p8 antibody. Reactivity of the antibody against p8 was confirmed by using E. coli extracts containing GST-p8 fusion protein (data not shown). As shown in Fig. 5, the pancreas specimen from the rat with acute pancreatitis strongly expresses p8, whereas the protein cannot be found or detected in the gland of a control rat. The consistency
of calculated molecular mass and the actual molecular mass of the immunodetected p8 suggest that there was not post-translational glycosylation for p8. This is in agreement with the absence of glycosylation sites into the p8 sequence.

**p8 mRNA Expression in Developing and Regenerating Rat Pancreas and Liver**—As shown in Fig. 6, high p8 mRNA levels are observed in fetal pancreas. High expression is still observed in the newborn with a progressive decrease until day 35. At day 45, p8 mRNA levels drop dramatically and remain constant until day 90.

In regenerating pancreas, in response to pancreatectomy (Fig. 7), p8 mRNA is strongly activated with levels of p8 mRNA already very high at 12 h; following subtotal pancreatectomy, they decrease at 24 h and remain constant during the first 14 days. Three months later, p8 mRNA concentration decreases drastically to reach near control values.

Similar to pancreas, fetal liver express p8 mRNA at high levels. These levels remain unchanged until the 25th postnatal day, and then decrease progressively (Fig. 6). Contrary to pancreas, in liver regeneration after partial hepatectomy we do not observed changes in expression of the p8 mRNA (data not shown).

**Extrapancreatic p8 mRNA Expression**—Total RNAs extracted from stomach, duodenum, jejunum, ileum, colon, liver, salivary glands, kidney, lungs, heart, brain, spleen, testes, thymus, and skeletal muscles obtained from control rats (Fig. 8) or at different times after pancreatectomies were probed with p8 cDNA in Northern blot analysis. p8 mRNA was highly expressed in control salivary glands, moderately expressed in stomach, colon, liver, and kidneys, and slightly expressed in control lungs, heart, duodenum, jejenum, and ileum. The message was absent in control brain, spleen, testes, thymus, or skeletal muscles. Expression in extrapancreatic tissues was not affected in animals with acute pancreatitis (data not shown).

**In Situ Hybridization of p8 mRNA in the Rat Pancreas with Acute Pancreatitis**—In situ hybridization was performed to identify which cell types in the pancreas express the p8 transcript. Hybridization with the digoxigenin-labeled antisense RNA for p8 shows specific labeling only in the acinar cells (Fig. 9). In contrast to acinar cells, p8 remains undetectable in the islets of Langerhans, ducts, inflammatory infiltrate, and stromal tissue. The degree of expression detected by in situ hybridization parallels that obtained from Northern blot analysis (Fig. 9). Current hybridization with a digoxigenin-labeled sense RNA probe, as well as with an antisense RNA in a previously RNase A-treated tissue sections shows no specific hybridization (Fig. 9).

**p8 mRNA Expression in AR4-2J Cells**—AR4-2J is a rat pancreatic tumor cell line derived from an azaserine-induced tumor of the rat exocrine pancreas (39), which has conserved some of the acinar cells characteristics. This cellular line was then used to evaluate the mechanisms of p8 mRNA activation in the pancreas. The AR4-2J cells were treated with several factors (see “Materials and Methods”), and the expression of p8 mRNA was monitored at different times by Northern blot analysis. Results are shown in Fig. 10. p8 mRNA is expressed at low but detectable level in untreated AR4-2J cells, similarly to normal healthy rat pancreas. Incubation of these AR4-2J cells with cycloheximide, TNFα, dexamethasone, ceramide, and staurosporine, or in cells grown in the absence of serum, p8 mRNA was strongly induced. Maximal p8 mRNA expression was observed at different times in response to each treatment, suggesting, as expected, different pathways for the p8 gene activation. However, cytokines IL-1, IL-4, and IL-6, the epidermal growth factor, the phorbol ester and an oxidative stress as well as the heat-shock did not stimulate p8 gene expression (data not shown). Taken together, these results suggest that most, but not all cellular injuries, activate p8 gene expression in the pancreatic cells. It is worth noting that the following
Cloning and Expression of the Rat p8 mRNA

We have previously reported that gene expression is strongly altered in pancreas during the acute phase of pancreatitis (12, 40). These phenotypic changes could enable the pancreas to protect itself against the acute attack of pancreatitis. Then, identifying the genes involved in this beneficial response and understanding their function could open new therapeutic strategies for pancreatitis treatment. Thus, with a systematic approach to reach that goal we identified a new mRNA, provisionally named p8, which showed a strong, but transient, induction in the pancreas in response to acute pancreatitis. Furthermore, in vitro studies suggest that p8 mRNA is induced in pancreatic and non-pancreatic cells undergoing apoptosis and that its overexpression promotes cellular growth.

Search for homologies in the data base failed to demonstrate the presence of p8-related proteins. Moreover, an analysis of its primary structure suggests that p8 has no signal peptide nor transmembrane regions; it is also quite improbable that p8 is targeted to mitochondria, lysosome, or anchored into the endoplasmic reticulum. However, the presence of a conserved bipartite motif of nuclear targeting in its COOH-terminal region (Fig. 2) suggests that p8 may function into the nucleus. In addition, the presence of a putative basic helix-turn-helix motif (Fig. 2) and its slight homology with most homeodomains (Fig. 2) suggests that p8 could be a DNA-binding protein, probably a transcriptional factor, as described for other helix-turn-helix-containing genes (41). Moreover, p8 has the potential to be phosphorylated by various kinases (i.e. three potential phosphorylation sites for protein kinase C (42) TKR46–48, TNR54–56, and SER73–75, and one site for casein kinase II (43) TKRE46–49. These observations are compatible with a role in some phosphorylation/dephosphorylation signaling pathways involving translocation to the nucleus and specific binding to DNA. However, although these findings suggest that p8 could be a transcriptional factor, further investigation is needed to confirm this hypothesis.

Like p8, other genes are activated during the acute phase of pancreatitis (40), however, p8 is of particular interest because of its rapid induction (Fig. 2). It is also interesting to note that p8 mRNA is activated almost similarly in edematous and necrohemorrhagic pancreatitis (Fig. 4). This indicates that a moderate pancreatic injury is sufficient to induce maximal p8 mRNA expression. After reaching a certain induction threshold, p8 mRNA does not correlate anymore with the severity of the pancreatitis. In our in vitro studies p8 mRNA is induced in several cell lines (pancreatic, intestinal, and fibroblast) in response to various agents, especially by those inducing apoptosis (Figs. 10 and 11). In contrast, we showed by in situ hybridization that in vivo only pancreatic acinar cells express p8 mRNA during pancreatitis (Fig. 9). These results show that acinar cells express p8 mRNA in a rapid and specific manner in response to cellular pancreatitis-induced injury. The fact that p8 mRNA can be activated in vitro in several cell types but its in vivo expression during pancreatitis is limited to the acinar cells suggests that p8 is induced by intracellular signals in the acinar cells rather than by circulating or locally produced factors. Indeed, we hypothesize that p8 mRNA is induced in response to intracellular signals in cells undergoing apoptosis. Our hypothesis is put forward from two major observations. First, only pancreatic acinar cells enter in apoptosis during acute pancreatitis (44, 45). Second, in our in vitro studies (Fig. 10) showed that the factors which induce a p8 mRNA expression (cycloheximide, TNFa, dexamethasone, ceramide, staurosporine, or serum starvation) also induce apoptosis in the AR4-2J cells (Fig. 10). In contrast, we showed by in situ hybridization that in vivo only pancreatic acinar cells express p8 mRNA during pancreatitis (Fig. 9). These results show that acinar cells express p8 mRNA in a rapid and specific manner in response to cellular pancreatitis-induced injury. The fact that p8 mRNA can be activated in vitro in several cell types but its in vivo expression during pancreatitis is limited to the acinar cells suggests that p8 is induced by intracellular signals in the acinar cells rather than by circulating or locally produced factors. Indeed, we hypothesize that p8 mRNA is induced in response to intracellular signals in cells undergoing apoptosis. Our hypothesis is put forward from two major observations. First, only pancreatic acinar cells enter in apoptosis during acute pancreatitis (44, 45). Second, in our in vitro studies (Fig. 10) showed that the factors which induce a p8 mRNA expression (cycloheximide, TNFa, dexamethasone, ceramide, staurosporine, or serum starvation) also all induced apoptosis in AR4-2J cells (Fig. 10). In contrast, the cytokines IL-1, IL-4, IL-6, the epidermal growth factor, phorbol ester, and the heat-shock do not induce p8 mRNA expression, as they failed to induce detectable apoptosis in these cells. One result, however, remains unclear as incubation of the AR4-2J cells with 0.1 mM H2O2-induced apoptosis but did not activate the p8 mRNA expression. The above mentioned apoptosis-inducing factors mediate their effect through different intracellular pathways (reviewed in Refs. 46 and 47). Therefore, the signaling cascade by which H2O2 induces apoptosis may differ from the signals by which...
the other apoptosis-inducer agents activate p8 mRNA expression. However, we cannot formally exclude the possibility that cycloheximide, TNFα, dexamethasone, ceramide, staurosporine, or serum starvation activate p8 mRNA expression through an apoptotic-independent pathway.

p8 mRNA is overexpressed in pancreas during the acute phase of pancreatitis as well as during pancreas development and regeneration after subtotal pancreatectomy, three situations in which pancreatic cellular proliferation is strongly activated. These observations led us to test whether the p8 gene can act as a promoting growth factor in vitro. Interestingly, growth of COS-7 and the pancreatic derived AR4-2J cells was strongly increased after their transfection with the rat p8 cDNA, demonstrating for this gene a cellular growth-promoting function. The mechanisms by which p8 elicits its growth-promoting effects in vitro are not yet known and remain to be investigated. It is interesting to note that p8 mRNA is apparently induced in tissues undergoing apoptosis. However, how can it be possible that opposite signals of apoptosis and cell growth occur simultaneously in the same organ? It has been previously demonstrated that most of the molecular events leading cells to proliferate or to go to apoptosis are similar. For example, in both phenomena, increases in expression of ornithine decarboxylase, CD45, proto-oncogenes coding for transcription factors as c-fos, c-jun, and c-myc, as well as the activation of transcription factors AP-1 and NFκB occur (48–50).

Some of our preliminary data indicate that p8 overexpression has no anti-apoptotic nor apoptosis promoting effects as evi-
denced by the cellular resistance to some apoptotic inducers or by estimation of spontaneous apoptosis, respectively (data not shown). Hence, it remains conceivable that an anti-apoptotic response may promote cellular growth.

p8 mRNA is not the only growth-promoting factor activated in the pancreas after an experimentally induced pancreatitis. During acute pancreatitis, the expression of several well-characterized growth factors and their receptors was induced including IGF-I and II, IGF-I receptor, hepatocyte growth factor, and c-met among others. These growth factors are probably induced to promote pancreatic regeneration. Interestingly, as p8, most of these genes were also expressed in developing and regenerating (18, 20, 21), but strongly down-regulated in the adult healthy pancreas.

The presence of high levels of p8 mRNA in pancreas of 19 days fetus coincides with the developmental period in which

FIG. 10. p8 mRNA expression in AR4-2J cells. Northern blot analysis shows the hybridization pattern of total RNAs extracted from AR4-2J cells treated for different times with several factors (see “Materials and Methods”). A, TNFα (500 units/ml); B, dexamethasone (1 μM); C, ceramide (20 μM); D, staurosporine (0.1 μM); E, serum starvation; and F, cycloheximide (20 μg/ml). The blots were probed with the 32P-labeled p8 cDNA (top panel). Filters were washed and hybridized with 32P-labeled 28 S ribosomal RNA (bottom panel). G, genomic DNA was prepared, submitted to electrophoresis and stained with ethidium bromide (see “Materials and Methods”) from control AR4-2J cells (lanes A and C) and after treatment with: lane D, TNFα (24 h); lane E, dexamethasone (24 h); lane F, ceramide (12 h); lane G, staurosporine (12 h); lane H, serum starvation (48 h); and lane I, cycloheximide (12 h). Lanes B and J were molecular weight markers (λ/EcoRI/HindIII and pBR322/HaeIII, respectively).

FIG. 11. p8 mRNA expression in Rat2 and IEC6 cells. Northern blot analysis shows the hybridization pattern of total RNAs extracted from Rat2 (A) and IEC6 (B) cells treated for different times with ceramide (20 μM). The blots were probed with the 32P-labeled p8 cDNA (top panel). The filters were washed and hybridized with 32P-labeled 28 S ribosomal RNA (bottom panel).
mRNA expression is strongly activated in vitro in pancreatic as well as non-pancreatic cells undergoing apoptosis. p8 acts as a promoting cellular growth factor when its cDNA was transfected into the COS-7 and AR4-2J cells. We failed to identify p8-related sequences, but analysis of its primary and secondary structure suggests that p8 is a basic helix-turn-helix-containing gene with slight homology to several homeotic genes and sufficient signal to be targeted to the nucleus. Therefore, p8 is a putative transcriptional factor which can regulate pancreatic growth.

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