α -Amylase gene transcription in tissues of normal dog

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ABSTRACT

We studied the distribution of α -amylase mRNA in normal dog tissues by northern blotting (NB) and reverse transcription-polymerase chain reaction (RT-PCR) with human pancreatic (*AMY2*) and salivary (*AMY1*) α -amylase cDNA-specific primers. Analysis of poly(A⁺) RNA from various normal tissues by NB indicated the presence of detectable levels of α -amylase mRNA transcripts only in pancreas. Dot-blot analysis of DNA amplified with primers common to both (human) isoamylase mRNAs showed presence of α -amylase gene transcripts not only in pancreas but also in liver, small intestine, large intestine and fallopian tube. Traces of amylase gene transcripts were also observed in ovary, uterus and lung. Interestingly, amylase transcripts were not detectable in the parotid gland by NB or RT-PCR. We have also localized α -amylase mRNA transcripts to dog pancreas by in situ transcription and in situ hybridization. Our results suggest that there is high degree of homology between the α -amylase mRNA sequences in dog and human at least in the exon 3–4 regions of the human gene.

INTRODUCTION

α -Amylase (α -1,4-glucan 4-glucanohydrolase, E.C. 3.2.1.1) is a digestive enzyme that hydrolyzes α -1,4 glycoside bonds in starch and glycogen. In humans and rodents, it occurs in two major isozymic forms: salivary (Amy1) and pancreatic (Amy2) amylases which are encoded by the *AMY1* and *AMY2* genes, respectively. In these mammals, salivary gland and pancreas are the primary sources of the enzyme. In dog, on the other hand, pancreas alone has been found to be the major source of the enzyme. α -Amylase genes are highly conserved across and within various species and there is a very high degree of homology between the pancreatic and salivary amylase proteins and also between the cDNAs (1–8).

Despite the long-term interest in the mammalian α -amylases (reviewed in 9–11), there has been no direct evidence for the actual sites of the enzyme production in extrapancreatic tissues of dog. Several investigators, basing their conclusions on biochemical and histochemical analyses, suggested either dog

liver (12–17), small and large intestine (18–23) or lung (24) as possible sources of the enzyme. Since the levels of enzyme in extrapancreatic tissues are very low, the source of the enzyme remains unclear. Several reasons, including serum α -amylase contamination, adsorption of the enzyme to cell surface, increased permeability of the enzyme, false α -amylase activities due to the presence of other enzymes such as maltase, glucoamylase and certain other proteins, have been suggested as causes for the low levels of enzyme activity in these tissues. As there is little or no direct evidence for actual synthesis of the enzyme in various extrapancreatic tissues, we sought such evidence in the distribution of α -amylase mRNA in normal dog tissues. We used both northern analysis and a recently developed gene-amplification technique (25, 26) known as the reverse transcriptase-polymerase chain reaction (RT-PCR). Because of the high degree of conservation of the isoamylase mRNAs across species we used α -amylase mRNA-specific primers common to both the human isozymes. We have also localized α -amylase mRNA to dog pancreas by in situ transcription (IST) and by in situ hybridization (ISH). Finally, we correlated the enzyme activity in various tissue extracts with the biosynthesis of the enzyme.

MATERIALS AND METHOD

Tissues

Normal dog tissues were obtained from the Laboratory Animal Resources Center at Indiana University. Normal human pancreas and parotid were obtained at autopsy of a cardiac patient. Portions of each tissue were washed in ice-cold physiological saline, quickly frozen in liquid nitrogen and stored at -70°C until use.

RNA preparation

Total cellular RNA was isolated from various tissues by the guanidinium-isothiocyanate-CsCl density gradient centrifugation method (27). Quality of RNA was determined by examining 5 μg of each sample by electrophoresis in formaldehyde-agarose gel (28). Gels were stained with Hoechst 33298 (29) to detect DNA and counterstained with ethidium bromide to check the integrity of RNA isolated. No traces of DNA were observed and the two ribosomal RNAs appeared as sharp bands (18S and 28

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S), indicating that the RNA from various tissues was of good quality (data not shown). Poly (A⁺)-enriched RNA was isolated by repeated passage of cellular RNA through oligo-dT cellulose columns (28).

Northern blotting

Northern blot analysis of poly (A⁺) RNA was performed according to the procedure of Davis *et al.*, (28). Filters were prehybridized and hybridized at 65°C as described (5).

Probes

*Pst*I-linearized pBR322 containing a human pancreatic cDNA insert (3) and chicken β -actin cDNA (Oncor) were labeled by the random-priming method with [α]-³²P-dCTP (Boehringer Mannheim Co.). The specific activity of probes ranged from 4.2 to 7.4 $\times 10^9$ cpm/ μ g. PCR primer and ϕ X174 size marker were end labeled with [γ -³²P]-dATP (27) to a specific activity of 0.2 to 1.3 $\times 10^9$ cpm/ μ g.

Dot blot

Dot-blotting of RNA and DNA were performed as described by Davis *et al.*, (28) with ³²P-labeled probes.

Primers for Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Gene-specific oligonucleotide sequences for RT-PCR were designed from the published cDNA sequences of human *AMY1* and *AMY2* genes (3,4). Primers were synthesized by Dr. Steve Larsen on an Applied Biosystems DNA synthesizer Model 381 A. The reverse primer P1 (5'-CAGCAGTCCCATATTCTGGATGG-3') and the forward primer P2 (5'-GCCACATGTGCTTGAAGCATCA-3') were derived from the common exons 3 and 4 of the α -amylase genes (*AMY1* and *AMY2*), respectively and cover a region of 231 bases and the intervening intron.

RT-PCR

Total RNA (5–20 μ g) was reverse-transcribed with M-MLV reverse transcriptase (BRL) and P2 or P3 primers according to the procedure described in the cDNA synthesis manual provided by BRL. Reverse transcribed RNA (cDNA) was amplified (26), with *Taq* DNA polymerase (Cetus), gene-specific primers (64 pM of each P1 and P2) and dNTPs (800 μ M each) during 30–45 rounds on a Perkin-Elmer Cetus PCR DNA Thermocycler. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 63°C for 3 min. After 15 to 20 rounds of amplification additional *Taq* DNA polymerase and dNTPs were added to each tube. [³²P]-labeled PCR products were obtained by substituting [α -³²P]-dCTP (5–10 μ Ci/reaction) for cold dCTP. Five to 10 μ l of the PCR mixture was fractionated on either 5% polyacrylamide gel or 3% Nusieve GTG-1% Seakem LE agarose (FMC) composite gel, stained with EB and analyzed. A *Hae*III digest of ϕ X174 RF DNA was used as a size marker. Human pancreatic and splenic RNA were used as positive and negative controls, respectively.

In situ transcription (IST)

In situ transcription (IST) within the freshly frozen sections (8 μ m) of pancreas fixed in 4% neutral buffered paraformaldehyde was performed as described (30). α -Amylase mRNA was reverse transcribed with M-MLV (BRL) reverse transcriptase in the presence of P2 primer and [α -³²P]-dCTP.

In situ hybridization (ISH)

α -Amylase mRNA was localized to dog pancreas with ³²P-labeled P2 primer by the procedure of Morley and Hodes (31).

α -Amylase activity

Small pieces of tissue were cut, weighed and washed three times for 15 min each in 0.02 M sodium phosphate buffer (pH 6.9); 0.15 M NaCl at 4°C on a rotary shaker. Tissues (100–300 mg) were homogenized in 1 ml buffer at 4°C with a Polytron homogenizer. The extracts were centrifuged and the supernatant solutions assayed. Amylase activity was determined with a chromogenic substrate, Amylochrome (Cibacron Blue F3GA-dyed amylose) as recommended by the manufacturer (Hoffman-La Roche, Inc.).

RESULTS AND DISCUSSION

Northern blot analysis

In search of direct evidence for sites of α -amylase gene transcription, we screened various normal tissue RNAs by dot blotting and probing with the human *AMY2* cDNA. Dot-blot analysis of total RNA failed to detect amylase mRNA in any tissue studied except pancreas (data not shown). We then analyzed poly

Figure 1: Northern blots of poly(A⁺)-enriched RNA (3 μ g) from various normal dog tissues and total RNA (5 μ g) from dog pancreas probed with ³²P-labeled human pancreatic cDNA.

Figure 2: Sensitivity of northern blotting in detection of α -amylase transcripts in dog pancreatic cellular RNA under various washing conditions. Filters were washed in 2 $\times$ SSC-0.5% SDS at room temperature for 10 min prior to washing at 65°C for 1 hr in the following solutions: I, 2 $\times$ SSC-0.5% SDS; II, 0.2 $\times$ SSC-0.1% SDS; III, 0.1 $\times$ SSC-0.05% SDS; IV, 0.05 $\times$ SSC-0.025% SDS; V, 0.025 $\times$ SSC-0.012% SDS; VI, 0.012 $\times$ SSC-0.006% SDS.

(A⁺)-enriched RNA from various tissues by northern blotting and probing with human *AMY2* cDNA (Fig. 1). Surprisingly, α -amylase transcripts were detectable only in dog pancreas even though a number of extrapancreatic tissues have been suggested as producers of small amounts of the enzyme. In contrast, β -actin transcripts were detected in all the RNAs studied (data not shown). We next established the sensitivity of detection of α -amylase gene transcripts in the dog pancreatic total RNA (Fig. 2) as well as mouse liver mRNA (Fig. 3) by northern blotting. We included mouse liver in our studies since it is known (32) to contain a small number of α -amylase transcripts per cell (0.02% of mRNA). We were able to detect α -amylase transcripts in as little as 15–30 ng of total dog pancreatic RNA as compared to a minimum of 1 μ g of poly (A⁺) RNA from mouse liver under the conditions of stringency used routinely (wash I-II).

Detection of α -amylase gene transcripts by RT-PCR

Our results from the northern blot analysis confirmed our assumption, based on the enzymatic analysis (Table 1), that the amount of amylase mRNA in extrapancreatic tissues is much lower than that of rodent liver. Therefore, we decided to use RT-PCR, a highly sensitive, recently developed gene amplification technique. Detection of HIV-1 RNA by RT-PCR has been shown to be 10⁴ times more sensitive than the detection of unamplified material by other techniques (33). We showed that RT-PCR is 3–6 $\times$ 10³ more sensitive than northern blotting for detecting α -amylase gene transcripts in human pancreatic total RNA (unpublished). Thus, due to the relatively low sensitivity of northern blotting and the possible occurrence of low-abundance α -amylase gene transcripts in many of these tissues we screened various cellular RNAs by RT-PCR. We used P1 and P2 primers to detect both *AMY1* and *AMY2* gene transcripts. We included introns between the primers to distinguish PCR fragments generated from the mRNA from those that might result from the amplification of genomic sequences. We found a DNA fragment of expected size generated with exon 3–4 primers (P1 and P2) on agarose gel electrophoresis not only in specimens from dog pancreas but also in other tissues such as small intestine, large intestine and liver (Fig. 4). These amplified DNA fragments were comparable in size (231 bp) to the fragments obtained from the human pancreas or parotid. Dot-blot analysis with human *AMY2* cDNA probe of RT-PCR-generated cDNA from various tissue

RNAs showed α -amylase gene-specific hybridization signals in pancreas, fallopian tube (FT), liver and small and large intestine (Fig. 5). Weak hybridization signals were also obtained with ovary, uterus, and lung. Interestingly, amylase gene transcripts were not detectable in the dog parotid gland by either northern blotting or RT-PCR. The absence of salivary α -amylase gene transcripts in dog is supported by a number of related observations made by others: 1. Reid (15) and Vacikova (35) found only one isozyme (pancreatic) in dog serum by gel filtration and agar gel electrophoresis, respectively, 2. dog liver and small intestinal

TABLE 1: α -amylase gene expression in normal dog tissues.

No.	Tissue	Enzyme activity* (Dye U/g or ml)	RT-PCR <i>AMY</i>
1.	Pancreas	6560.0 $\pm$ 97.0	+
2.	Sm. Intestine	2.2 $\pm$ 0.9	+
3.	Fallopian tube	1.9 $\pm$ 0.4	+
4.	Uterus	1.4 $\pm$ 0.3	+
5.	L. Intestine	1.4 $\pm$ 0.4	+
6.	Parotid	0.9 $\pm$ 0.3	–
7.	Lung	0.4 $\pm$ 0.3	+
8.	Heart	0.4 $\pm$ 0.1	–
9.	Kidney	0.3 $\pm$ 0.4	–
10.	Bladder	0.3 $\pm$ 0.1	–
11.	Liver	0.1 $\pm$ 0.3	+
12.	Ovary	0	+
13.	Adrenal	0	–
14.	Prostate	0	–
15.	Testis	0	–
16.	Spleen	0	–
<i>Controls</i>			
17.	Human Pancreas	7159.0 $\pm$ 120	+
18.	Dog serum	22.4 $\pm$ 1.3	NA
19.	Rat liver	4.5 $\pm$ 1.0	ND
20.	Mouse liver	4.2 $\pm$ 0.1	ND
21.	Human Serum	1.7 $\pm$ 1.0	NA
22.	Human Spleen	0	–

Legend: *, mean $\pm$ s.d.s from three experiments; NA, not applicable; ND, not determined.

Figure 5: Dot blot of RT-PCR-generated amplified DNA fragments from various tissue RNAs of Dog. Two μ l of RT-PCR mixture was applied to the filter, probed with ^{32} P-labeled human pancreatic cDNA (pcHPA) and developed after overnight exposure at -70°C . RT-PCR product obtained from human splenic RNA was used as negative control.

amylases were found to be identical to the pancreatic amylase (14, 19), 3. no amylase activity could be detected in either parotid or submaxillary glands by thin or thick starch film histochemical technique even after prolonged incubation (36) and 4. no amylase activity was observed in dog saliva and a sugar diet did not induce the appearance of salivary α -amylase (18,37). As might be expected on the basis of the lack of homology between human and rodent exon S, which is characteristic of *Amy1*, no transcripts were detected in the dog after priming with human exon S-derived sequences (data not shown). The nature of the extrapancreatic amylase, save for its homology to human *AMY*, remains to be elucidated.

The different distributions found by RT-PCR and northern analysis in the present study are similar to the recently reported findings on the dystrophin gene product (34). Dystrophin mRNA was found only in skeletal muscle, heart and cultured myotubules after northern blotting of poly (A⁺) fractions. In contrast, gene transcripts were found in additional tissues such as liver, placenta and spleen after RT-PCR.

Localization of α -amylase mRNA in dog pancreas by IST and ISH

We localized α -amylase mRNA to acinar cells of pancreas by the recently described IST technique and confirmed the results by the well-established ISH technique with the human α -amylase mRNA- specific primer (P2). As is evident from the autoradiogram shown in Fig. 6, strong signals were observed in tissue section only in the presence of reverse transcriptase and the primer. The dark and bright field IST-photomicrographs of the same section (Fig. 7a and b) exhibited scattered silver grains

Figure 6: Autoradiogram of freshly frozen and fixed cryostat sections of pancreas. In situ transcription was carried out with P2 primer and the sections were exposed to X-ray film (Kodak XAR-5) for 20 min at -20°C before developing the film.

representing the newly synthesized labeled α -amylase cDNA all over the acinar cytoplasm. Our results also indicate that IST was specific for α -amylase mRNA since very weak or no signals above background were observed when primer or reverse transcriptase was omitted from the reaction (Fig. 6). This method may be generally useful for relatively quick screening for mRNA in various tissues. Low background signal due to primer-independent reverse transcription during IST in tissue sections treated with RT in the absence of primer is similar to that observed by Tecott *et al.*, (30). Such priming was attributed to DNA-RNA, RNA-RNA or DNA-DNA hybrids (30,42).

ISH with the α -amylase mRNA-specific primer (P2) showed significantly higher numbers of silver grains over acinar cells than over background (ductal regions) of normal dog pancreas (Fig. 8). Negative control slides treated with a heterologous ^{32} P-labeled plasmid DNA and with hybridization solution only exhibited relatively very low background grain count (data not shown). Thus the results from IST and ISH correlated very well.

Our results correspond to our previous localization of α -amylase mRNA to acinar cells in human pancreas (43) by immunohistochemistry and by ISH in human parotid (31), as well as the work of others with rat pancreas and parotid (44, 45). The presence of amylase mRNA in dog pancreas was first demonstrated by MacDonald (46) by in vitro translation technique. We have now localized the amylase mRNA to acinar cells of dog pancreas by IST and ISH with the aid of a human amylase mRNA probe. The specificity of the primer P2 for α -amylase mRNA in IST and ISH was demonstrated by the 1. pattern of silver grains throughout the acinar cytoplasm similar to the mRNA distribution observed by others in human parotid, rat pancreas and parotid, 2. primer- and RT-dependent strong signals in tissue sections and 3. relatively low background of silver grains in the ductal region of pancreas. Our results from IST and ISH once again suggest the existence of a high degree of homology between the human and dog α -amylase cDNAs in the regions studied.

Figure 7: (A) Dark-field photomicrograph (400 $\times$) of pancreas showing α -amylase mRNA-specific silver grains. The mRNA was localized with P2 primer by in situ transcription; (B) Bright-field photomicrograph of the same field.

α -Amylase activity in various normal tissue extracts

Finally, we correlated the enzyme activity with its biosynthesis in various normal tissue extracts of dog. A comparison of the α -amylase activity in various tissues and sera is shown in Table 1. Also indicated is whether or not α -amylase gene transcripts were detected in these tissues by RT-PCR. In our survey of normal dog tissues for enzyme activity, we found only pancreas with a higher level of enzyme activity than dog serum. No α -amylase activity was observed in ovary, testis, adrenal, prostate or spleen. However, traces of amylase gene transcription were detected in ovary. In the rest of the tissues analyzed including parotid, only low levels of the enzyme activity were found. Among these tissues, small intestine, large intestine, fallopian tube and uterus had approximately one-twentieth of the enzyme activity found in normal serum and 35% to 50% of the activity found in the extract of mouse liver. We found that dog serum has about a 13-fold higher enzyme activity than normal human

Figure 8: Freshly frozen section of normal dog pancreas. In situ localization of α -amylase mRNA to acinar cells with a ^{32}P -labeled P2 primer. Ductal and acinar structures are shown (400 $\times$). Note high density of grains in acinar cells as compared to the background grain count in ductal epithelium which served as a built-in negative control.

serum. This is within the range (4.4 to 18-fold) reported by others (16,47,48). Interestingly, low levels of amylase activity found in the kidney, heart, bladder and parotid, a major producer of the enzyme in humans and rodents, could not be correlated with the presence of amylase mRNA in these tissues. King (49) and Bhoola and Dorey (50) have also observed very low levels of the enzyme activity in dog parotid. The reason for enzyme activity in these tissues without the presence of mRNA is not clear. Though it is highly unlikely, it is possible that the putative amylase gene present in these tissues differs significantly from the human amylase gene and the amylase gene detected in pancreas and a few other tissues of dog.

Canine serum contains maltase and glucoamylase which contribute to the apparent α -amylase activity determined by saccharogenic assays (47,48). In the present study we attempted to increase the specificity of the assay by use of an α -amylase-specific chromogenic substrate (Amylochrome). The presence of proteins, bilirubin, hemoglobin or reducing sugars generated by the other carbohydrases does not interfere with this assay (48,51).

In summary, our results provide direct evidence for α -amylase gene transcription in extrapancreatic tissues of dog which include small and large intestines, liver, FT, ovary, uterus and lung. The α -amylase gene expressed in these tissues was obviously detected because of its homology to human *AMY2* cDNA. However, as in the case of the dystrophin gene, the physiological significance of very low amounts of amylase transcripts in nonpancreatic tissues is not yet clear. In a similar survey of normal human tissues by RT-PCR, we found α -amylase gene transcripts in female reproductive tissues, lung and intestinal tissues (unpublished).

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