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Expression of the human amylase genes: recent origin of a salivary amylase promoter from an actin pseudogene

Linda C.Samuelson, Karin Wiebauer[1], Deborah L.Gumucio and Miriam H.Meisler*

Department of Human Genetics, University of Michigan, Ann Arbor, MI 48109-0618, USA and [1]Friedrich Miescher Institut, Postfach 2543, CH-4002 Basel, Switzerland

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ABSTRACT

The human genes encoding salivary amylase (*AMY1*) and pancreatic amylase (*AMY2*) are nearly identical in structure and sequence. We have used ribonuclease protection studies to identify the functional gene copies in this multigene family. Riboprobes derived from each gene were hybridized to RNA from human pancreas, parotid and liver. The sizes of the protected fragments demonstrated that both pancreatic genes are expressed in pancreas. One of the pancreatic genes, *AMY2B*, is also transcribed at a low level in liver, but not from the promoter used in pancreas. *AMY1* transcripts were detected in parotid, but not in pancreas or liver. Unexpected fragments protected by liver RNA led to the discovery that the 5' regions of the five human amylase genes contain a processed γ-actin pseudogene. The promoter and start site for transcription of *AMY1* are recently derived from the 3' untranslated region of γ-actin. In addition, insertion of an endogenous retrovirus has interrupted the γ-actin pseudogene in four of the five amylase genes.

INTRODUCTION

The mouse genome contains two classes of amylase genes which differ in tissue-specificity (reviewed in 1-3). *Amy-2* is expressed at a high level in the pancreas, and *Amy-1* is expressed at a high level in the parotid gland. Both genes are expressed at very low levels in mouse liver (4).

The major human tissues which produce amylase are the pancreas and the salivary glands. The human amylase genes have recently been cloned in several laboratories (5-9). Genomic clones have been classified by comparison of exon sequences with three human amylase cDNA clones previously isolated from pancreas and from salivary gland (10, 11). By this criterion, the human haploid genome contains two pancreatic amylase genes (*AMY2A* and *AMY2B*), three salivary amylase genes (*AMY1A*, *AMY1B* and *AMY1C*), and two truncated pseudogenes (*AMYP1* and *AMYP2*) (5). The three cloned salivary amylase genes did not differ within the 950 nucleotides which were sequenced (5). However, evidence for variation among salivary amylase gene copies has been obtained from genetic studies of electrophoretic isozymes (12).

In the mouse, there is no sequence similarity between the pancreas specific promoter of *Amy-2* and the parotid specific promoter of *Amy-1*. However,as a result of the concerted evolution of the human amylase genes, their 5' flanking regions are nearly identical in sequence and structure (5, 6). A region of 92% homology extends for at least 700 bp upstream of exon a (6). One purpose of the present study was to determine whether this sequence similarity in the promoter region results in coexpression of *AMY1* and *AMY2* in the pancreas and parotid gland. We have examined amylase transcripts by ribonuclease (RNase) protection analysis. In spite of the close relationship between their 5' flanking sequences, the *AMY1* and *AMY2* genes were expressed with strict tissue specificity in parotid and pancreas.

A computer homology search revealed the presence of a processed γ-actin pseudogene upstream of exon a in the human amylase genes. The *AMY1* promoter is derived from sequences within this actin pseudogene. While this work was in progress, Emi *et al.* independently described γ-actin related sequences in a cloned human *AMY1* gene in which the actin sequences were interrupted by a retroviral LTR (13). We have extended this observation by determining the distribution of actin pseudogenes and of endogenous retroviral sequences within cosmid clones covering 240 kb of the human amylase gene cluster. The results are consistent with two recent, independent insertions of retroposons.

MATERIALS AND METHODS

RNA isolation

Total cellular RNA was isolated from frozen tissue samples using a modification of the guanidine thiocyanate homogenization-cesium chloride centrifugation method (14, 15). Tissue samples were obtained surgically, frozen immediately in liquid nitrogen, and stored at -70°C. Frozen pancreatic tissue was generously provided by K.R. Marotti (UpJohn Co., Kalamazoo, MI). The frozen tissues were pulverized with a mortar and pestle on dry ice and homogenized in 4 M guanidine thiocyanate, 2 M 2-mercaptoethanol in 0.1 M Tris-HCl, pH 7.5, using a Polytron homogenizer (Brinkmann). The homogenates were layered over 5.7 M CsCl and centrifuged at 36,000 rpm for 21 hours in an SW40 rotor. RNA pellets were dissolved in DEPC-treated sterile H_2O, extracted with phenol-chloroform, and precipitated with ethanol. The RNA was resuspended in DEPC-treated H_2O and quantitated by absorbance at 260 nm.

Construction of riboprobes and RNase protection assay

Restriction fragments containing the 5' ends of the human *AMY1* and *AMY2*

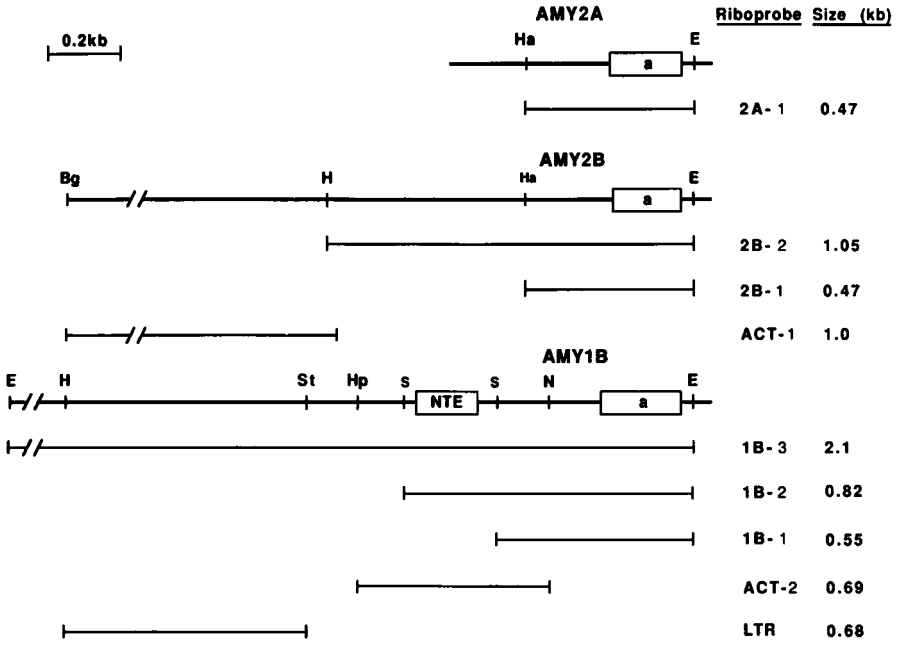

Figure 1. Human amylase subclones for riboprobe synthesis. The indicated restriction fragments were cloned into pGEM vectors. The sites indicated with smaller typeface may occur elsewhere. E, *Eco*RI; Ha, *Hae*III; H, *Hind*III; Bg, *Bgl*II; N, *Nde*I; S, *Sau*3A; Hp, *Hpa*I; St, *Stu*I.

genes were isolated from cosmid clones (Figure 1) and subcloned into pGEM vectors (Promega Biotec, Madison, WI). The *AMY1B* clones were derived from cosmid clone G21, the *AMY2A* clone from cosmid G45 and the *AMY2B* clones from cosmid G6, described by Gumucio *et al.* (5). Single-stranded uniformly labeled probes were generated using ^{32}P-GTP (800 Ci/mM, Amersham) according to the procedure recommended by Promega Biotec. After the transcription reaction was completed, RNA was extracted with an equal volume of phenol-chloroform, and precipitated with ethanol. Full length riboprobe transcripts were isolated from a 6% acrylamide-8M urea gel, precipitated twice with ethanol, and dissolved in H_2O.

RNase protection experiments were performed using a modification of the procedure of Melton *et al.* (16). Aliquots of total RNA from human tissues (80 ng to 50 μg) were mixed with tRNA to a total of 20-50 μg, and 20,000 cpm of labeled riboprobe was added. The samples were dried and redissolved in 10 μl formamide hybridization buffer (16). Overnight hybridization at 45°C was followed by RNase digestion for 45 minutes. Two different RNase digestion

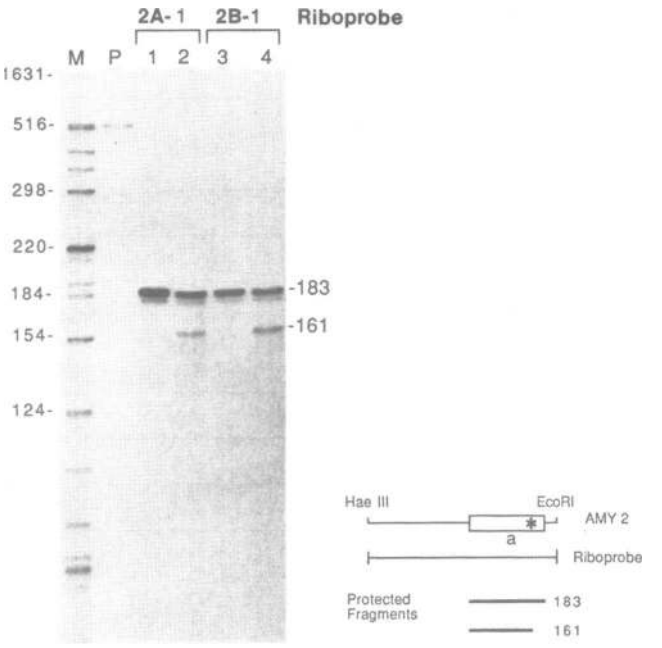

Figure 2. RNase protection of *AMY2* probes by human pancreatic RNA. RNA (200 ng) was hybridized with riboprobes 2A-1 and 2B-1, and tested with mild (Lanes 1 and 3) and extensive (Lanes 2 and 4) RNase digestion conditions, as described in Materials and Methods. M, size markers containing a mixture of pBR322 x *Hinf*I and pBR322 x *Hae*III end-labeled fragments. P, *AMY2A* probe. The expected protected fragments are indicated.

conditions were used. The mild digestion was carried out at 26°C and contained one tenth the concentration of RNase A and RNase T_1 recommended by Melton *et al.*(16). The extensive digestion was carried out at 30°C and contained the recommended RNase concentrations. After RNase treatment, half of each sample was fractionated on a denaturing gel containing 6% acrylamide and 8 M urea.

The predicted sizes of some protected fragments were a few nucleotides larger than the corresponding exons, due to protection of intronic nucleotides which matched the end of the adjacent exon.

Southern blot analysis of cosmid DNAs

Cosmid DNA was isolated as previously described (5) and digested with *Eco*RI and *Hind*III. Restriction fragments were transferred to nitrocellulose by the method of Smith and Summers (17) and hybridized with riboprobes in 50%

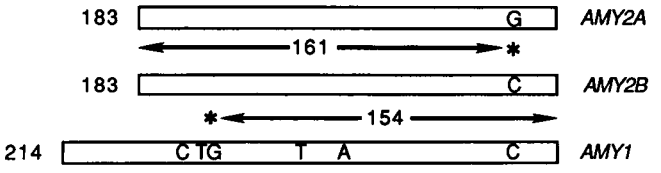

Figure 3. Comparison of exon a from three types of human amylase genes. The residues which differ are marked. The rest of the exon, represented by an open box, is identical for all the genes. Sequence from Gumucio *et al.* (5).

formamide at 50°C (16). The final wash was at 65°C in 0.1 x SSC, 0.1% SDS, and 20 mM pyrophosphate.

RESULTS

AMY2A and *AMY2B* are both expressed in human pancreas

Human pancreatic RNA was analyzed with probes derived from the two human pancreatic amylase genes, *AMY2A* and *AMY2B* (probes 2A-1 and 2B-1, Figure 1). These genes differ at nucleotide +162 within exon a (5). With both probes, pancreatic RNA protected a 183 nucleotide fragment from mild RNase digestion (see Materials and Methods). This fragment corresponds in size to the intact exon a (Figure 2). More extensive digestion resulted in the appearance of an additional fragment of 161 nt. This fragment is consistent with cleavage at the mismatch at nucleotide +162 (Figure 3). Since the 161 nucleotide fragment was observed with both probes, both *AMY2A* and *AMY2B* are transcribed in pancreas.

These data also demonstrate that *AMY1* is not transcribed in pancreas. The sequences of *AMY1* and *AMY2* differ at six nucleotides within exon a (Figure 3). *AMY1* transcripts would be expected to protect several small fragments of the *AMY2* probes from RNase digestion. A predominant fragment of 154 nt would result from cleavage at the dinucleotide mismatch between *AMY1* and *AMY2* (Figure 3). Since fragments of 154 nt or smaller were not observed (Figure 2), we conclude that *AMY1* is not expressed in human pancreas. This was confirmed by the failure of pancreatic RNA to fully protect exon a of an *AMY1B* riboprobe (data not shown).

The sequences of the 5' flanking regions of *AMY2* and *AMY1* are 92% identical for at least 700 bp including the nontranslated exon (NTE) of *AMY1* (6). To determine whether the NTE is transcribed in pancreas, we used a 1.05 kb

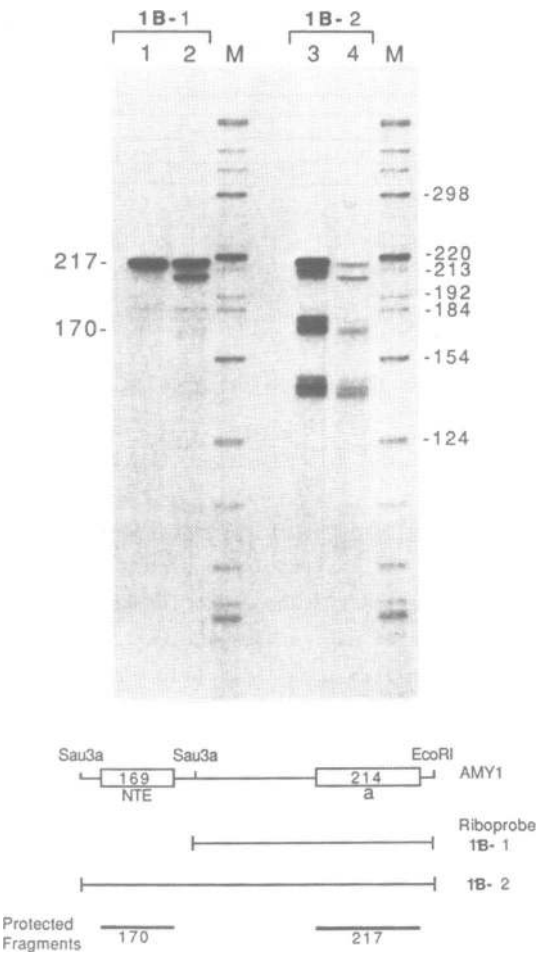

Figure 4. RNase protection of *AMY1* probes by human parotid RNA. RNA (200 ng) was hybridized with *AMY1* riboprobes containing exon a alone (1B-1) or exon a plus the NTE (1B-2). RNase digestion was carried out under mild (Lanes 1 and 3) or extensive (Lanes 2 and 4) conditions. M, pBR322 x *Hinf*I and pBR322 x *Hae*III end-labeled size standards. The expected protected fragments are indicated at the bottom of the figure.

AMY2B riboprobe containing exon a and the NTE region (probe 2B-2, Figure 1). The protection pattern observed with probe 2B-2 was identical to that seen with probe 2B-1, which contains only exon a (data not shown). Since there were no additional bands, we conclude that this upstream region is not included in pancreatic amylase transcripts.

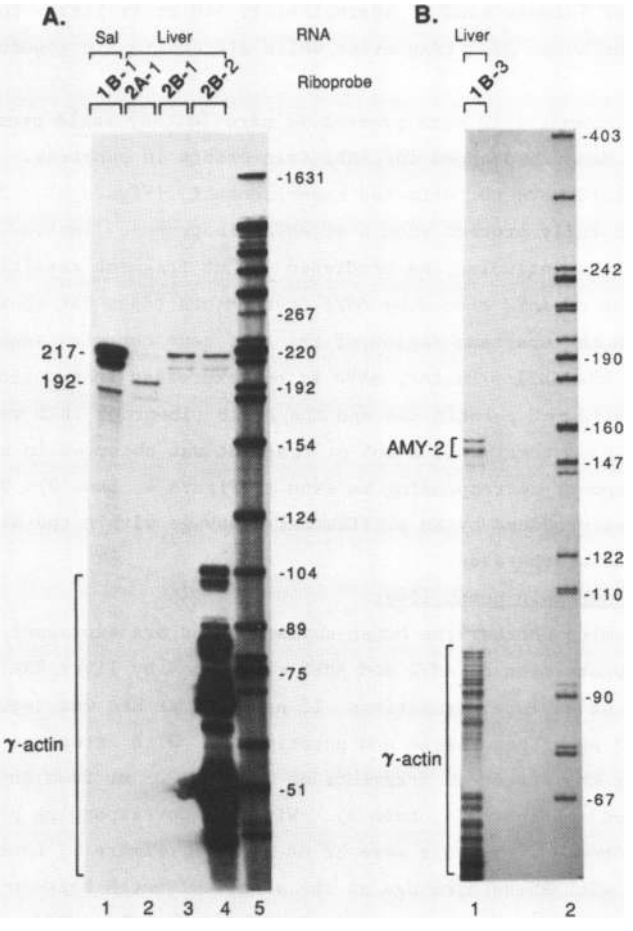

Figure 5. RNase protection by human liver RNA. The riboprobes are indicated at the top of each lane. Extensive RNase digestion conditions were used. A) Lane 1, 80 ng of parotid RNA (Sal); Lane 2, 12 μg liver RNA; Lane 3, 12 μg liver RNA; Lane 4, 12 μg liver RNA; Lane 5, pBR322 x *Hinf*I and pBR322 x *Hae*III end-labeled size standards. B) Lane 1, 50 μg liver RNA; Lane 2, pBR322 x *Hpa*II end-labeled size standard.

AMY1 expression in parotid

Parotid RNA was tested using two probes derived from the 5′ region of *AMY1B* (Figure 4). With probe 1B-1 containing exon a, we detected a 217 nt fragment corresponding to the intact exon. With the larger probe 1B-2 which contains exon a and the NTE, two additional sets of protected fragments were observed (Figure 4, Lane 4). The 170 nt fragment corresponded in size to the

NTE. The other intense band of approximately 140 nt is likely to be derived from protection by an *AMY1* transcript which differs in NTE sequence from the probe.

If *AMY2* transcripts were present in parotid they would protect smaller fragments, as described above for *AMY1* transcripts in pancreas. These smaller fragments were not detected experimentally (Figure 4). Parotid RNA also failed to fully protect exon a of *AMY2* riboprobes. Instead, we observed smaller fragments including the predicted 154 nt fragment resulting from partial protection of *AMY2* exon a by *AMY1* transcripts (data not shown). Therefore, although the upstream region of the *AMY2* gene contains sequences highly homologous to the *AMY1* promoter, *AMY2* is not expressed in parotid.

When hybrids of parotid RNA and the *AMY1B* riboprobe 1B-1 were subjected to extensive RNase treatment, a 205 nt fragment was observed in addition to the 217 nt fragment corresponding to exon a (Figure 4, Lane 2). This fragment most likely was produced by an artifactual cleavage within the AT-rich region near the 5′ end of the exon.

Expression of *AMY2B* in human liver

To determine whether the human amylase genes are expressed in liver, we analyzed the protection of *AMY1* and *AMY2* riboprobes by liver RNA. Due to the low abundance of amylase transcripts, 12 μg of liver RNA was required, compared with 0.2 μg of pancreatic and parotid RNA. With extensive RNase digestion, liver RNA protected fragments of 217 and 205 nt from an *AMY2B* probe containing exon a (Figure 5A, Lane 3). With the corresponding probe from *AMY2A*, the protected fragments were 22 nt smaller (Figure 5, Lane 2), which is consistent with RNase cleavage at the single mismatch between *AMY2B* transcripts and the *AMY2A* probe. These data indicate that the *AMY2B* gene, but not the *AMY2A* gene, is expressed in liver.

The fragments of the *AMY2B* probe protected by liver RNA are not identical to those observed in pancreas. A 183 nt fragment would be expected if the promoter and start site used in liver were the same as in pancreas. The *AMY2B* exon a fragments protected by liver RNA are identical in size to the *AMY1B* exon a fragments protected by parotid RNA (Figure 5A; compare Lanes 1 and 3). This suggests that instead of utilizing the pancreatic transcription start sites, *AMY2B* liver transcripts utilize an upstream splice site which corresponds to the exon a splice acceptor site in *AMY1*.

To determine whether the NTE-related sequences of *AMY2B* are present in liver amylase transcripts, we used riboprobe 2B-2 (Figure 1). In addition to the fragments corresponding to exon a, many small very intense fragments were

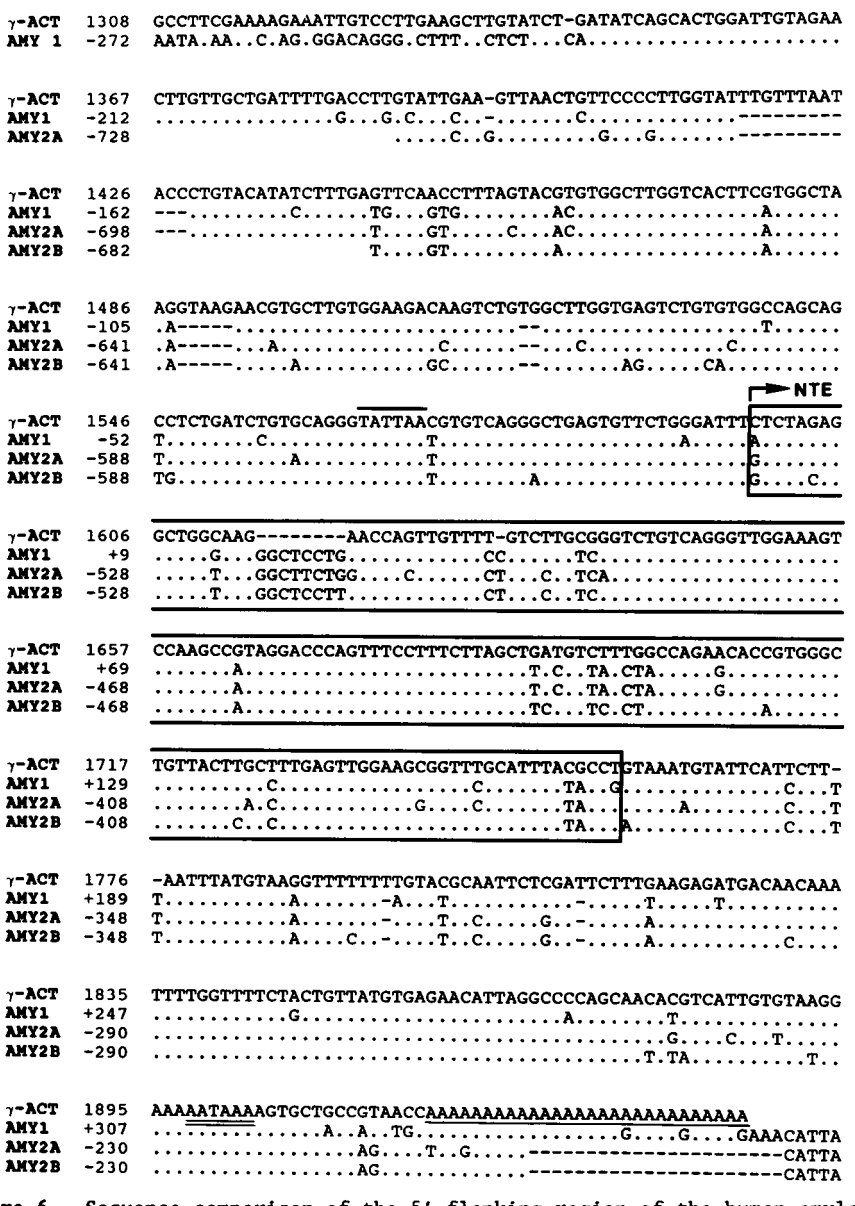

Figure 6. Sequence comparison of the 5′ flanking region of the human amylase genes with the 3′ untranslated region of the human γ-actin cDNA. Dots represent nucleotides which are identical; dashes represent deletions. The TATA box of the parotid promoter is overlined, and the NTE region of *AMY1* is boxed. The polyA addition sequence AATAAA is double underlined, and the polyA stretch at the 3′ end of the homology is underlined. The *AMY1* sequence is from Gumucio *et al.* (5), the *AMY2A* sequence is from Horii *et al.* (6), the *AMY2B* sequence is from Groot *et al.* (9) and the γ-actin sequence is from Erba *et al.* (18).

protected by the liver RNA (Figure 5A, Lane 4). The significance of these fragments is discussed below. We did not observe a 170 nt fragment corresponding to the NTE of *AMY1*.

When *AMY1B* probes were tested with liver RNA, we did not detect fragments corresponding to the intact exon a (217 nt) or the NTE (170 nt) (Figure 5B). This result indicates that *AMY1* is not expressed in human liver. The fragments of approximately 154 nt observed in this experiment correspond to protection by *AMY2B* transcripts.

The AMY1 promoter and NTE are derived from an actin pseudogene

Many small protected fragments were observed when liver RNA was analyzed with *AMY1B* and *AMY2B* probes containing sequences upstream of exon a (Figure 5, Lanes A4 and B1). This suggested that the amylase probes were partially protected by an abundant liver transcript. To determine whether our probes contained sequences related to another liver transcript, we carried out a computer homology search of the Genbank data base. A 564 bp region with 89% sequence identity to the 3' untranslated region of the human γ-actin gene was identified upstream of amylase exon a (Figure 6). The actin-related sequences include the promoter and NTE of *AMY1*. The presence of a polyA sequence at the 3' border of the actin homology suggests that the actin sequences are derived from a processed transcript. The actin-related sequences begin 234 bp upstream of the parotid transcription initiation site, and continue through the NTE into the first intron of *AMY1*. The actin sequences upstream of *AMY1* are derived from the 3' untranslated region of γ-actin.

Distribution of actin sequences

To determine the number and location of actin related sequences within the human amylase cluster, we analyzed cosmid clones spanning a 240 kb region. Cosmid DNAs were digested with *Eco*RI and *Hind*III and hybridized with two actin-related probes derived from amylase cosmid clones (Figure 1). Riboprobe ACT-2, corresponding to the 3' untranslated region of the actin cDNA, was subcloned from *AMY1B*. Riboprobe ACT-1, corresponding to coding sequences +324 to +1332 of the actin cDNA (18), was isolated from the 5' flanking region of *AMY2B*. Sequence analysis has demonstrated a complete γ-actin pseudogene upstream of this gene (manuscript in preparation).

With riboprobe ACT-2, hybridizing fragments were observed in cosmid clones containing *AMY1A*, *AMY1B*, *AMY1C*, *AMY2A* and *AMY2B* (Figure 7A). Each hybridizing fragment contains the 5' end of an amylase gene. With riboprobe ACT-1, there was hybridization only to the *AMY2B* gene (Figure 7B). These re-

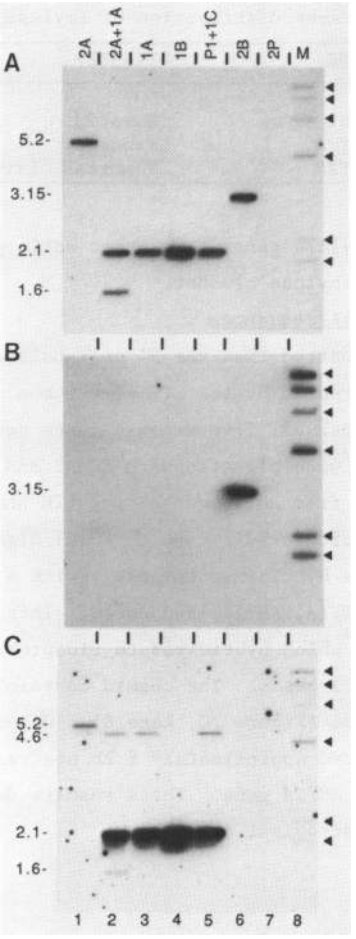

Figure 7. Southern blot of *Eco*RI digested cosmid DNA spanning a 240 kb region of the human amylase gene cluster. Three replicate blots were hybridized with: A) the γ-actin 3' untranslated riboprobe ACT-2; B) the γ-actin coding region riboprobe ACT-1; or C) the retroviral probe LTR, containing LTR and *gag* sequences. The amylase genes contained in each cosmid clone are indicated at the top of the figure. The 1.6 kb fragment observed in Lane 2 (A and C) is derived from the 5' end of the *AMY2A* gene which is truncated in the cosmid clone N1. M, end-labeled molecular weight standard λ x *Hind*III. Lane 1, cosmid G45; Lane 2, cosmid N1; Lane 3, cosmid N7; Lane 4, cosmid G21; Lane 5, cosmid N12; Lane 6, cosmid G6; Lane 7, cosmid G1. Cosmid clones are described in Gumucio *et al* (5).

sults indicate that all of the amylase genes are flanked by the γ-actin 3' untranslated region, but the actin coding sequences are restricted to *AMY2B*. The insertion of the actin sequences evidently preceded the divergence of the

Table 1. Tissue distribution of amylase transcripts

GENE	TISSUE
AMY1	Parotid
AMY2A	Pancreas
AMY2B	Pancreas, Liver

five functional human amylase genes. No other actin pseudogenes were detected elsewhere in the amylase cluster.

Distribution of retroviral sequences

It was recently reported that the 3' untranslated γ-actin sequences upstream of *AMY1* are interrupted by the LTR of a human endogenous retrovirus (13). To determine whether all five amylase genes contain retroviral sequences, our cosmid DNAs were digested with *Eco*RI and *Hind*III and hybridized with a riboprobe derived from *AMY1B* containing LTR and *gag* sequences (LTR, Figure 1). The results of hybridization to *Eco*RI digested DNA is shown in Figure 7C. The LTR probe hybridized intensely with a single fragment from the cosmids containing *AMY1A*, *AMY1B*, and *AMY1C*. This intensely hybridizing fragment is the same one which hybridized to riboprobe ACT-2, and contains the 5' termini of the *AMY1* genes. The cosmid containing *AMY2B* did not hybridize with the LTR probe (Figure 7C, Lane 6). Weakly hybridizing restriction fragments were located approximately 8 kb upstream of the *AMY1* genes, and at the 5' end of the *AMY2A* gene. These results demonstrate that four of the five genes contain retroviral inserts.

DISCUSSION

Using riboprobes derived from exon a of each of the amylase genes, we have evaluated the tissue-distribution of their transcripts. The results are summarized in Table 1. The single base difference between exon a of *AMY2A* and *AMY2B* made it possible to demonstrate that both of these genes are expressed at comparable levels in pancreas. Only *AMY2B* is expressed in liver, at a much lower level than in pancreas and from a different promoter. The difference in liver expression of *AMY2A* and *AMY2B* may be related to the divergent structure of the 5' flanking region of the two genes, discussed below. *AMY1* transcripts were detected only in the parotid gland. These studies demonstrated strong tissue specificity of expression of the *AMY1* and *AMY2* genes, in spite of the extensive sequence identity of their 5'-flanking regions.

On Southern blots of human genomic DNA many fragments which hybridize

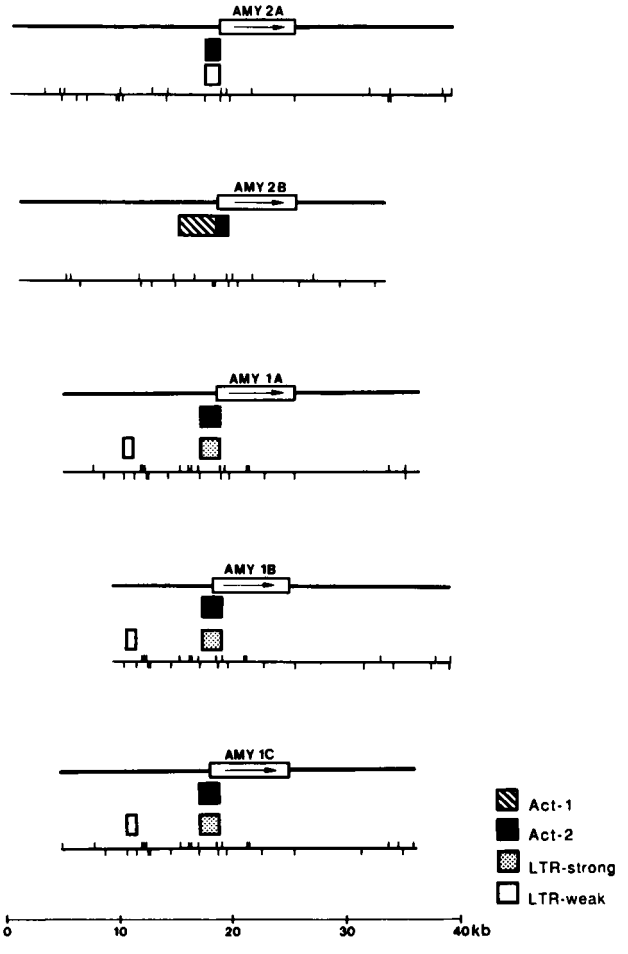

Figure 8. Distribution of γ-actin and retroviral sequences upstream of the five human amylase genes. The restriction map below each gene copy marks the location of *Eco*RI (above the line) and *Hind*III (below the line) sites (5). The boxes represent *Hind*III fragments which hybridized with the riboprobes ACT-1, ACT-2 and LTR (see Figure 7). LTR-strong, intensely hybridizing fragment; LTR-weak, less intensely hybridizing fragment.

with a γ-actin probe have been demonstrated (18). Three of these can now be assigned to the γ-actin pseudogenes located upstream of the amylase genes. The presence of a polyA tract at the 3' terminus of the actin sequences associated with the amylase genes indicates that the mechanism of insertion was by retroposition of an actin transcript. From the 89% sequence identity between the actin pseudogenes and the functional γ-actin gene (18), the time of

origin of the pseudogene can be estimated as approximately 23 million years ago (19).

All five of the amylase gene copies contain actin sequences inserted at the same position upstream of exon a. The actin retroposition therefore preceded the separation of human salivary and pancreatic amylase genes. This observation provides confirmation of previous evidence that these genes are the products of concerted evolution with very recent interactions (5, 20). The distribution of actin-related and retroviral sequences within the amylase gene cluster is summarized in Figure 8. The observation of two LTR-containing fragments separated by 8 kb in the three *AMY1* genes suggests that a complete retrovirus may be present. This is apparently not true for *AMY2A*, which contained only a single LTR-hybridizing fragment. The absence of LTR sequences upstream of the *AMY2B* gene suggests that it separated from the other genes prior to the retroviral insertion. These are the only γ-actin and LTR hybridizing fragments detected in the 240 kb amylase gene region. The pseudogenes *AMYP1* and *AMYP2* are truncated at their 5′ ends (5) and do not contain the γ-actin and retroviral inserts. The actin pseudogenes and the retroviral inserts provide qualitative markers for the study of amylase gene evolution. Further sequence analysis may allow us to elucidate the molecular events which are responsible for the generation of this multigene family.

A remarkable feature of human *AMY1* is the recent origin of a functional promoter from the 3′ untranslated sequence of the γ-actin pseudogene. This promoter may have originally functioned as part of a gene located downstream of the γ-actin gene. Alternatively, insertion of the retroviral LTR 250 bp upstream may have activated the use of a cryptic promoter. Although the retrovirus is inserted in the opposite transcriptional orientation to amylase (13), effects of the enhancer elements in the LTR could be orientation independent. The structure of the *AMY1* genes raises interesting questions regarding the origin of tissue specificity. Salivary amylase expression among mammalian species is limited to primates, rodents and lagomorphs (reviewed in 1). These mammals may share conserved salivary enhancer elements at a distal site which influence transcription from the new human *AMY1* promoter. On the other hand, the retrovirus itself may contribute to expression of human *AMY1* in the parotid gland, independently of conserved sequences. Functional analysis of this complex promoter may provide answers to these intriguing questions.

Analysis of serum amylase is a widely used clinical diagnostic tool. Elevated levels of the salivary amylase isozyme in serum can be caused by ec-

topic production in lung or ovarian tumors (21). The influence of the retroviral LTR on expression of *AMY1* in tumors will be an interesting area for future investigation. Elevated levels of pancreatic amylase isozyme in serum is most often associated with pancreatitis, but in the absence of pancreatitis it presents a diagnostic puzzle (22). The presence of *AMY2B* transcripts in human liver indicates that liver must be considered as a source of elevated pancreatic amylase isozyme in serum.

Many copies of endogenous retroviruses exist in the human genome (23, 24). The effect of these retroviral sequences on the expression of neighboring genes is not known. Endogenous retroviruses have been previously observed 60 kilobases downstream of the β-globin gene cluster (24) and within the first intron of the haptoglobin-related gene (25). The retrovirus associated with the β-globin genes is interesting because it interrupts a *Kpn*I element in a region containing several deletion breakpoints (24). This region, like the human amylase region, may be a target for insertional events. The human amylase genes are located in a chromosome region with an unusually high rate of meiotic recombination, resulting in discordance between the genetic map and the physical map (26, 27). It is tempting to speculate that the frequency of insertion and rearrangement in the amylase gene cluster may be related to some feature of chromatin accessibility which also facilitates recombination.

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*To whom correspondence should be addressed

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