

Different Tissue-Specific Expression of the Amylase Gene *Amy-1* in Mice and Rats

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We cloned the rat α -amylase gene *Amy-1* and compared its structure and expression with its mouse counterpart. The results showed that the general organization of the transcriptionally active rat *Amy-1* gene was similar to that of its mouse counterpart; i.e., the rat gene also contained two independent transcriptional promoters. The distance between the two promoters in the rat gene was, however, more than double (6 kilobases) that measured in the mouse gene (2.8 kilobases). In addition, the rat genome also contained an independent, orphanlike version of the weaker *Amy-1* promoter, which was transcriptionally silent. In spite of the similar overall organization of the *Amy-1* genes in mouse and rat cells, an interesting difference was observed in the expression of the weak promoter in these two closely related rodents. In rats this promoter was significantly active only in liver cells, while in mice it was utilized with similar efficiencies in parotid, liver, and pancreas cells. Moreover, the transcripts produced in rat liver had a very heterogeneous population of 5' ends, located between 180 and 220 nucleotides upstream of the two homologous start sites observed for this promoter in mouse liver, even though the sequences around this region were strongly conserved between the two species.

The tissue-specific expression of the α -amylase gene *Amy-1^a* of the mouse has been the subject of intensive analysis in our laboratory for the past few years. The mouse *Amy-1^a* gene is a single-copy gene located in chromosome 3 (9). It contains two separate transcriptional promoters whose activity gives rise to two mRNA species that differ in their 5' nontranslated region (11, 34). The upstream promoter is about 30-fold stronger than the downstream one and is active only in cells of the parotid gland (29). In contrast, the weaker downstream promoter is active in cells of all α -amylase-producing tissues of the mouse, i.e., the parotid, liver, and pancreas (10). In parotid cells, the weak *Amy-1* promoter is already active at birth, while the stronger promoter is turned on only during weaning (31). These findings, as well as recent studies on the differential activity of these two promoters upon transfection into heterologous cell types (F. Sierra, A.-C. Pittet, E. Buetti, and U. Schibler, submitted for publication), have led us to propose that the weaker promoter of the α -amylase gene *Amy-1^a* is promiscuous, its activity depending only on the presence of an active chromatin configuration. In contrast, we propose that the stronger, parotid-specific promoter is active only in the parotid gland cells of the mouse, most likely because of its requirement for tissue-specific factors. To determine whether this mode of organization and expression is a general phenomenon, we analyzed the expression of the α -amylase gene in rat tissues, initially by using homologous mouse *Amy-1* DNA probes. We found that, in contrast to the situation in mice, the rat parotid cells did not contain transcripts homologous to the liver-type promoter, although such transcripts were easily detectable in rat liver cells. To further analyze this apparent discrepancy between the two species, we cloned the rat *Amy-1* promoter region and characterized it with respect to structural organization and tissue-specific expression.

MATERIALS AND METHODS

Cloning and sequencing. A rat genomic-DNA library was constructed by the insertion of 15- to 24-kilobase (kb) fragments generated by partial *Mbo*I digestion of rat nuclear DNA into *Bam*HI-digested λ EMBL3 vector arms (7). The ligated molecules were packaged in vitro as described by Frischauf et al. (7). Recombinant bacteriophage were then adsorbed to *Escherichia coli* NM539 cells and plated at near confluency (plates, 22 by 22 cm; Nunc, Roskilde, Denmark).

A library containing 1.5×10^6 recombinant phage was screened by hybridization with various nick-translated mouse probes as described below. Restriction maps of clones were determined by the partial-digestion method of Smith and Birnstiel (32). After subcloning of the appropriate fragments into M13mp10 vectors and growth of the phage in *E. coli* strain WB373 (25), single-stranded DNA was isolated and sequenced by the dideoxynucleotide chain termination method of Sanger et al. (26) or by the quasi-end-labeling technique of Bina-Stein et al. (3).

RNA isolation and analysis. Total cellular RNA from various mouse and rat tissues was isolated by guanidinium isothiocyanate extraction (14). Poly(A)⁺ RNA was isolated by standard oligo(dT) cellulose chromatography, and the RNA was electrophoretically fractionated on glyoxal gels (21). After transfer of the RNA to Gene Screen (New England Nuclear Corp., Boston, Mass.), the membranes were baked for 2 h and hybridized to phage SP6-derived riboprobes as previously described (22).

The location of the cap site of the transcripts was determined by S1 nuclease mapping (4, 29) and primer extension analysis (24). For localization of the cap site for parotid-type transcripts, a synthetic 19-mer (kindly provided by Dr. J. Gysi, Pharmacia, Inc.) was hybridized for 10 min at 65°C in 200 mM NaCl to 1 μ g of poly(A)⁺ RNA. Reverse transcription and labeling of the extended primer were attained under quasi-end-labeling conditions (3, 8), either in the presence or absence of dideoxynucleotides. Elongated primers were

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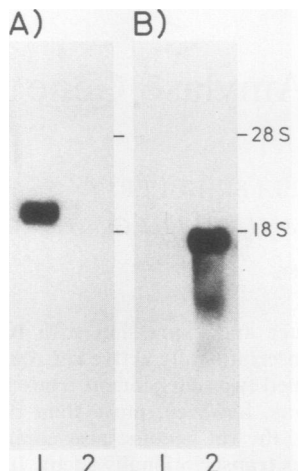


FIG. 1. Expression of *Amy-1* in rat liver and parotid cells. Poly(A)⁺ RNA (5 μ g) from rat liver (lanes 1) or rat parotid (lanes 2) was glyoxylated and electrophoretically separated on a 1.5% agarose gel. RNA was transferred to Gene Screen (New England Nuclear Corp.) and hybridized in formamide to riboprobes complementary to (A) the mouse liver leader region (900-bp *Hind*III fragment from clone λ MChraal⁹/1 [28]) and (B) mouse α -amylase cDNA (1.2-kb *Eco*RI fragment from clone pMSa104 [31]).

then displayed on 8% polyacrylamide gels containing 8 M urea.

RNase mapping was done as described by Melton et al. (22), using ³²P-labeled riboprobes. For these experiments, riboprobes with low specific activity (4 Ci/mmol) were synthesized and used immediately to minimize degradation artifacts resulting from autoradiolysis.

RESULTS

Weak promoter of *Amy-1* is not expressed in rat parotid cells. In mouse parotid cells, both of the transcriptional promoters that control expression of the *Amy-1* gene are active. The upstream, parotid-specific promoter is 30-fold stronger than the downstream liver-specific promoter (29). However, the relative activity of the liver-specific promoter is approximately the same in mouse parotid and liver cells (11, 29). To test whether the same pattern of expression could be observed in rat cells, poly(A)⁺ RNA from both liver and parotid cells was isolated, fractionated on a glyoxal gel, transferred to Gene Screen, and hybridized to a riboprobe complementary to the first exon of the liver-specific mouse *Amy-1* mRNA (Fig. 1A). The results showed that, even though α -amylase transcripts in rat liver cells could easily be detected with the mouse probe, similar transcripts were not present in the rat parotid. As a control, the same blot was washed and hybridized to a riboprobe containing sequences complementary to most of the coding region of the mouse *Amy-1^a* mRNA (Fig. 1B). A strong signal was observed in the lane containing parotid poly(A)⁺ RNA, indicating that the rat parotid cells contain large amounts of α -amylase transcripts, as expected. A closer examination of Fig. 1A indicates that the *Amy-1* mRNA from rat liver cells contains approximately 2,100 nucleotides. Thus, this mRNA appears to be 200 to 300 bases longer than its mouse counterpart (10).

Cloning and isolation of the rat *Amy-1* gene. To determine the structural basis for the difference in tissue-specific ex-

pression of the two promoters in mouse and rat cells, we decided to clone the rat α -amylase type 1 gene(s). For this purpose, a rat genomic-DNA library was constructed in λ EMBL3 (7), and recombinant phage were screened in parallel with mouse genomic-DNA probes containing sequences complementary to either the parotid- or the liver-specific α -amylase leader regions. We detected and isolated two recombinants (λ 794 and λ 795 [Fig. 2]) that hybridized with both probes, as well as four recombinants (λ 70 series) containing sequences complementary only to the mouse parotid probe and five recombinants (λ 90 series) that hybridized only to mouse liver-specific sequences. Restriction site analysis of these clones allowed us to align them into two linear maps, each containing a different liver-specific sequence (Fig. 2). Hybridization to a nick-translated cloned cDNA probe containing most of the mouse α -amylase coding region (the same probe used in Fig. 1B [31]) indicated that only clone λ 96 contains α -amylase protein-coding sequences. This clone appeared to contain a liver-specific sequence which, based on the maps presented in Fig. 2, is linked to the parotid leader region. Furthermore, this hybridization indicated that, as shown in the mouse, the first exon common to both mRNAs is located 4.5 kb downstream of the liver-specific promoter.

Taken together, these results suggest that, in the rat, the overall organization of the *Amy-1* gene is similar to that of its mouse counterpart, in that two tandem promoters precede the body of the gene. As in mouse cells, the parotid-specific promoter was located further upstream than the liver-specific promoter (Fig. 2). In addition, the rat genome contained a second copy of a sequence that cross-hybridized with the mouse liver leader region. The absence of α -amylase protein-coding sequences within approximately 13 kb to either side of this sequence suggests that it is an orphon.

Only the liver-specific leader region linked to the parotid promoter is transcriptionally active. Since the lack of α -amylase-coding sequences in close vicinity to the liver-specific leader region did not by itself prove that this region was an orphon, we tested the degree of homology between each of the two liver-specific leader regions and the transcripts present in rat liver RNA. For this purpose, ³²P-labeled cDNA complementary to liver poly(A)⁺ RNA was hybridized to filter-immobilized dots containing denatured DNA from clones λ 794, λ 91, and λ 76. After hybridization, parallel sets of dots were individually washed at increasing temperatures in 0.5x SSC, (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.25% sodium dodecyl sulfate. As the temperature of the wash increased, the hybridization signals of clones λ 76 and λ 91 disappeared at a considerably faster rate than the signal of clone λ 794 (Fig. 3). This result indicates that sequences present in clone λ 794 share a higher degree of homology with sequences present in rat liver poly(A)⁺ RNA than do the sequences present in clone λ 91. Since the liver-specific leader regions were the only sequences present in clones λ 91 and λ 794 that hybridized to liver poly(A)⁺ transcripts (data not shown), the melting experiment described above indicates that only the leader present in clone λ 794 is transcribed in cells of this tissue. Independent proof of the lack of transcriptional activity from the liver-specific sequences harbored in clones λ 91 and λ 97 was obtained by *in vitro* extension of nascent transcripts present in isolated rat liver cell nuclei (data not shown). These experiments confirmed that the unlinked liver-type sequence present in clones λ 91, λ 93, and λ 97 represented an *Amy-1* orphon, while the liver-specific sequence linked to

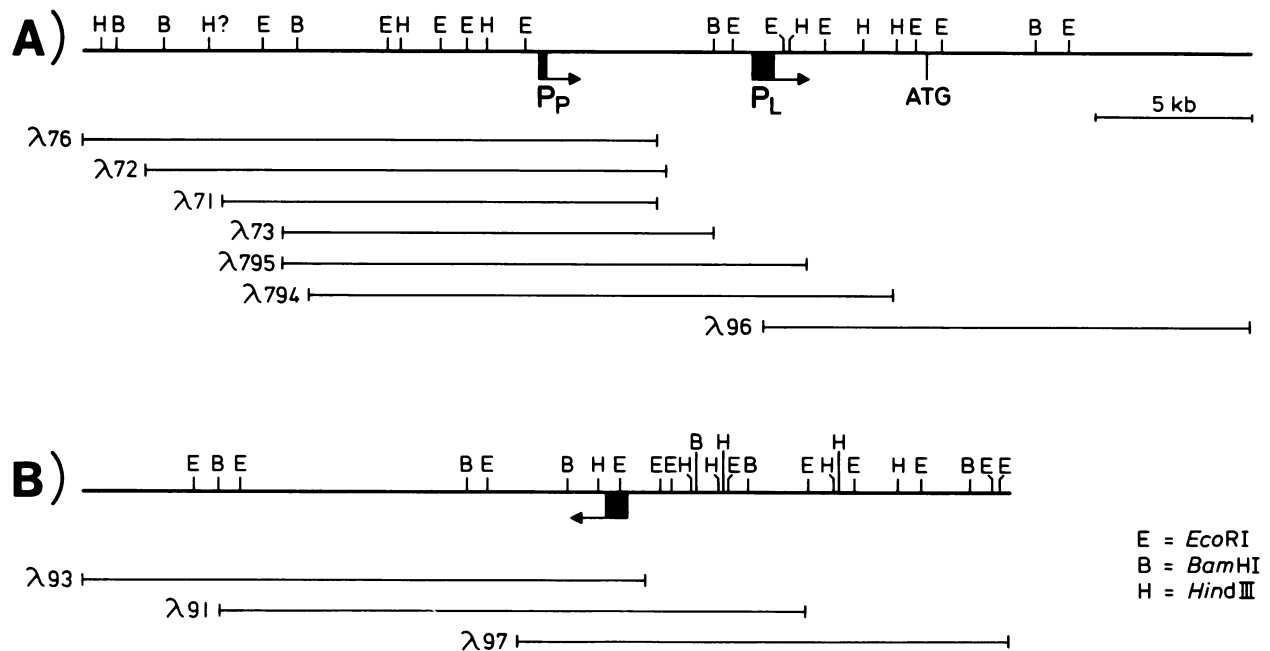


FIG. 2. Restriction maps of rat *Amy-1* genes. Restriction maps were determined by the method of Smith and Birnstiel (32). Locations of promoters and protein coding regions were determined by hybridization to mouse probes as described in the text. P_p, Parotid-specific promoter; P_L, liver-specific promoter; E, *EcoRI*; H, *HindIII*; B, *BamHI*; ■, exons; arrows, direction of transcription.

the parotid-specific promoter was an active α -amylase promoter in rat liver cells.

All the clones we obtained in the original screening of the rat genomic library with the mouse α -amylase promoter probes could be aligned within the maps presented in Fig. 2, suggesting that these are the only regions in the rat genome that contain *Amy-1* promoters. Furthermore, fragments of total nuclear DNA from rat liver, digested with *BamHI*, *EcoRI*, or *HindIII* and hybridized with the same mouse probes, showed homology only with fragments of the sizes expected from the maps in Fig. 2 (data not shown). Thus, it appeared that, in rat cells, as in mouse cells (28, 29), there is only one copy of a transcriptionally active *Amy-1* gene per haploid genome.

Structure of the *Amy-1* promoter region. In spite of the

resemblance in the organization of the *Amy-1* locus in both mouse and rat, an examination of the maps shown in Fig. 4 indicates that the two promoters are approximately 6 kb apart in the rat genome, while they are only separated by 2.8 kb in the mouse genome. To determine whether this difference was caused by a single insertion-deletion event or multiple insertion-deletion events, the 6.7-kb *EcoRI* fragment that contains the rat parotid leader region, as well as most of the region between the promoters, was subcloned for further analysis (clone sRAP-1). This DNA was digested with *EcoRI* in conjunction with either *BamHI*, *HaeIII*, *Sau3A*, or *HhaI*. The DNA fragments were then electrophoretically separated and blotted onto Gene Screen. The blot was hybridized to a nick-translated, 5.7-kb *PstI* fragment from clone λ MChr α 1^a/1 (28), which contains both of the mouse *Amy-1* promoters, as well as the interpromoter 2.8 kb of DNA. The results (not shown) indicated that the mouse probe lacks sequences complementary to a single block of DNA present in the rat clone. This block is approximately equidistant between the two rat promoters (1.5 kb downstream of the parotid-specific promoter and 1.5 kb upstream of the liver-specific promoter). Hybridization of a parallel blot containing digests of sRAP-1 DNA with nick-translated total rat liver DNA (data not shown) indicated that the 3-kb insertion (deletion) contains sequences that are highly repetitive in the rat genome. It is not yet clear whether these extra sequences in the rat gene affects the expression of the two *Amy-1* promoters.

Sequence analysis of the rat *Amy-1* promoters. The sequences surrounding both α -amylase promoters in the rat genome are shown in Fig. 5. For comparison, the corresponding mouse sequences, as well as the sequences around the rat liver orthon leader are included. The sequences around and within the parotid-specific leader region (Fig. 5A) were remarkably conserved between the two species (approximately 92% between nucleotides -380 and +211 in the rat numbering system). Upstream of nucleotide -380,

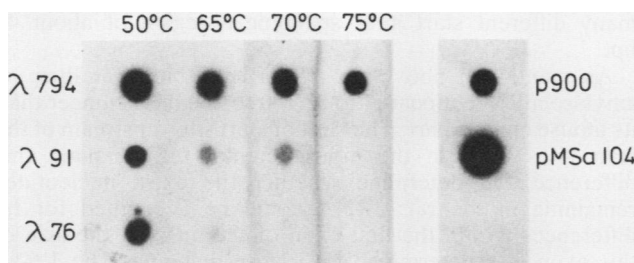


FIG. 3. Transcriptional activity of the two liver specific promoters. A series of dots, each containing 2 μ g of DNA from clone λ 794 (upper row), λ 91 (middle row), or λ 76 (bottom row), as well as controls containing p900 (mouse liver leader region) and pMSa104 (mouse α -amylase protein-coding sequences), was spotted on Gene Screen and hybridized for 4 days at 42°C with ³²P-labeled rat liver cDNA. After hybridization, strips were cut and washed at the indicated temperatures in 0.5x SSC-0.25% sodium dodecyl sulfate. Clone λ 76 does not contain sequences complementary to liver cell transcripts (data not shown); thus, it was used as a control for nonspecific hybridization.

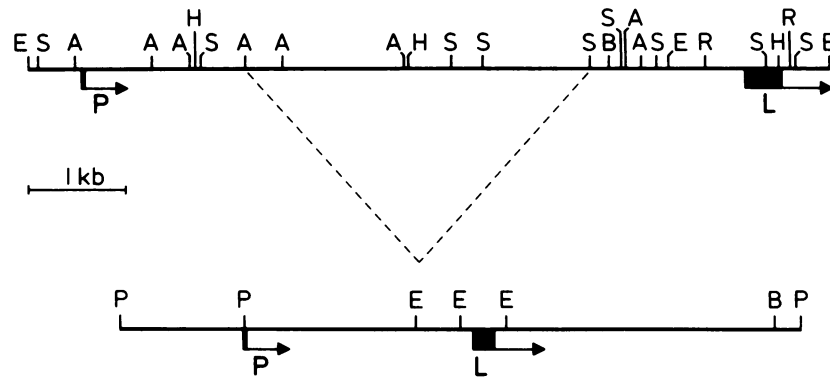


FIG. 4. Relative distances between two *Amy-1* promoters in rat and mouse cells. Schematic representations of the promoter region of the rat (upper line) and mouse (lower line) *Amy-1* genes illustrate that *Amy-1* promoters were further apart in rat cells than in mouse cells. E, *EcoRI*; B, *BamHI*; S, *Sau3A*; A, *HaeIII*; H, *HhaI*; P, *PstI*. Horizontal arrows indicate the locations of promoters and the directions of transcription. For this experiment, clone sRAP-1, containing the 6.7-kb *EcoRI* fragment that includes the rat parotid-specific promoter, was digested with *EcoRI* as well as each of the enzymes shown above. Digested DNA samples were electrophoresed on a 2% agarose gel, transferred to Gene Screen, and hybridized to a nick-translated 5.7-kb *PstI* fragment from clone λ MChroal²/1.

the homology started to degenerate, until upstream of nucleotide -473, significant stretches of homology were no longer found. Since this promoter was used only in the parotid gland cells in both species, it is tempting to speculate that DNA sequences involved in the regulated expression of this promoter might be located downstream from nucleotide -473.

The sequences around and within the liver-specific leader region (Fig. 5B) were also well conserved between the two species (about 84% between nucleotides -302 and +380 in the rat numbering system). Surprisingly, the degree of nucleotide sequence conservation was similar for upstream sequences (84.1%), exon sequences (83.3%), and available intron sequences (85.9%). When the rat orthon sequence was compared with either rat or mouse liver leader regions, reasonably good homology was found up to nucleotide +273 (71% when compared with the rat liver leader region and 73% when compared with the same region in the mouse). However, all homology was abruptly terminated downstream of nucleotide +273, a fact indicating that this sequence may have been duplicated into an unrelated genomic location.

Determination of the mRNA start sites. The sites of transcription initiation for both promoters were determined, by hybridization to ³²P-labeled cDNA and by nuclear run-on experiments (data not shown), to be located approximately within the same regions in which the mouse start sites were found. To further define the exact location of the parotid start site within the available sequences, a 19-mer oligonucleotide complementary to nucleotides +29 to +46 in the rat genome sequences was synthesized for us by Dr. J. Gysi (Pharmacia). By homology with the mouse genome sequences, we expected this oligomer to be contained within the first parotid exon. The oligomer was hybridized to rat parotid poly(A)⁺ RNA, labeled by the quasi-end-labeling method (3, 8), and extended by avian myeloblastosis virus reverse transcriptase both in the presence and absence of dideoxynucleotides. The results of such a primer extension-sequencing reaction are shown in Fig. 6B. It can be observed that the oligomer was extended for either 26 or 29 bases, indicating the presence of two distinct cap sites at positions +1 and +4. It should be noted that, even though the start site used in the mouse corresponds to nucleotide A at position -2, the relative distance between the TATA box and the

cap site is retained (6) because of the presence of four extra nucleotides in the equivalent region of the mouse genome.

The precise transcriptional start sites of the liver α -amylase mRNA were determined by S1 mapping of liver cytoplasmic poly(A)⁺ RNA, as well as liver nuclear RNA, by using a 1,070-base-pair (bp) *Sau3A-EcoRI* fragment labeled at the *Sau3A* site (Fig. 6C). Surprisingly, this approach yielded a very heterogeneous set of bands between 148 and 184 bases long (Fig. 6A). If the start sites homologous to the two mouse start sites had been used in the rat (the sequences are well conserved in this region), two bands of 27 and 58 bases in length would have been obtained. To confirm this unexpected result, the same probe was further digested with *MboII*, and the end-labeled 136-bp fragment was used in a primer extension experiment, with liver cytoplasmic poly(A)⁺ RNA. The primer extension yielded the same pattern that we have obtained by S1 mapping, as shown in Fig. 6A. These results indicate that, in spite of the high level of sequence conservation between the two species, rat liver α -amylase transcripts initiate as far as 156 bp upstream of the major start site found in mouse liver α -amylase mRNA. Furthermore, the experiments indicate that the 5' ends of rat liver α -amylase mRNA are quite heterogeneous, with many different start sites spanning a region of about 40 bp.

As mentioned above, in a Northern blot, rat liver α -amylase mRNA appeared to be 200 to 300 bases longer than its mouse counterpart. The shift of start sites upstream of the start sites used in the mouse cannot fully explain this difference. To determine whether the extra nucleotides remaining in the rat mRNA could be accounted for by differences within the first exon of the mRNA, the size of this exon was determined by RNase mapping. The 1.6-kb *EcoRI* fragment harboring the liver leader region (Fig. 2) was subcloned into pSP65 (clone sRAL-1), and ³²P-labeled complementary RNA was prepared by transcription with phage SP6 RNA polymerase (22). The labeled probe was hybridized to 2 μ g of rat liver poly(A)⁺ RNA, followed by digestion with RNases A and T1. The first liver-specific exon had a heterogeneous size ranging between 355 and 388 bases (Fig. 7A). This finding was in agreement with the presence of heterogeneous start sites, as shown in Fig. 6. The splice donor site, based on this measurement, would be located

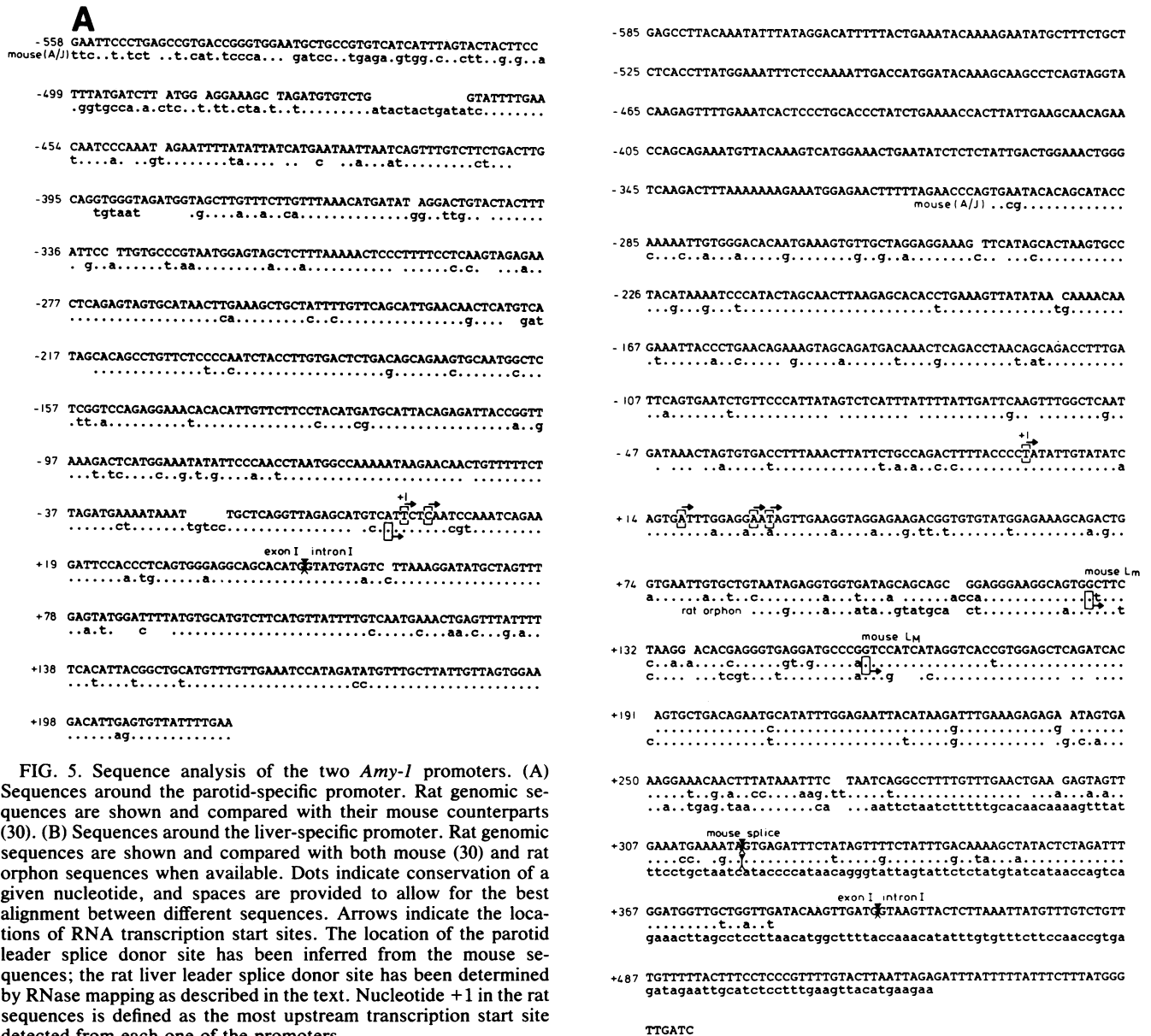


FIG. 5. Sequence analysis of the two *Amy-1* promoters. (A) Sequences around the parotid-specific promoter. Rat genomic sequences are shown and compared with their mouse counterparts (30). (B) Sequences around the liver-specific promoter. Rat genomic sequences are shown and compared with both mouse (30) and rat orphon sequences when available. Dots indicate conservation of a given nucleotide, and spaces are provided to allow for the best alignment between different sequences. Arrows indicate the locations of RNA transcription start sites. The location of the parotid leader splice donor site has been inferred from the mouse sequences; the rat liver leader splice donor site has been determined by RNase mapping as described in the text. Nucleotide +1 in the rat sequences is defined as the most upstream transcription start site detected from each one of the promoters.

around nucleotide +393. In the mouse genome, in contrast, the homologous splice donor site would be located at nucleotide +316 in the rat numbering system (+160 in the mouse numbering system). When an RNase mapping experiment was performed with a probe containing only sequences downstream of the *Hae*III site present within the liver-specific exon, a single major protected fragment of 115 bp was obtained (Fig. 7B). This is the expected size of the

protected fragment if the splice donor site is indeed located at position +393 as previously determined. These results showed that, in rat cells, the liver-specific α -amylase transcripts start heterogeneously about 150 bp upstream from the major liver-specific start sites in the mouse. Similarly, the splice donor site used in rat liver cells was located 77 bp downstream of the site used in the mouse. The size difference in the 5' leader sequence could entirely

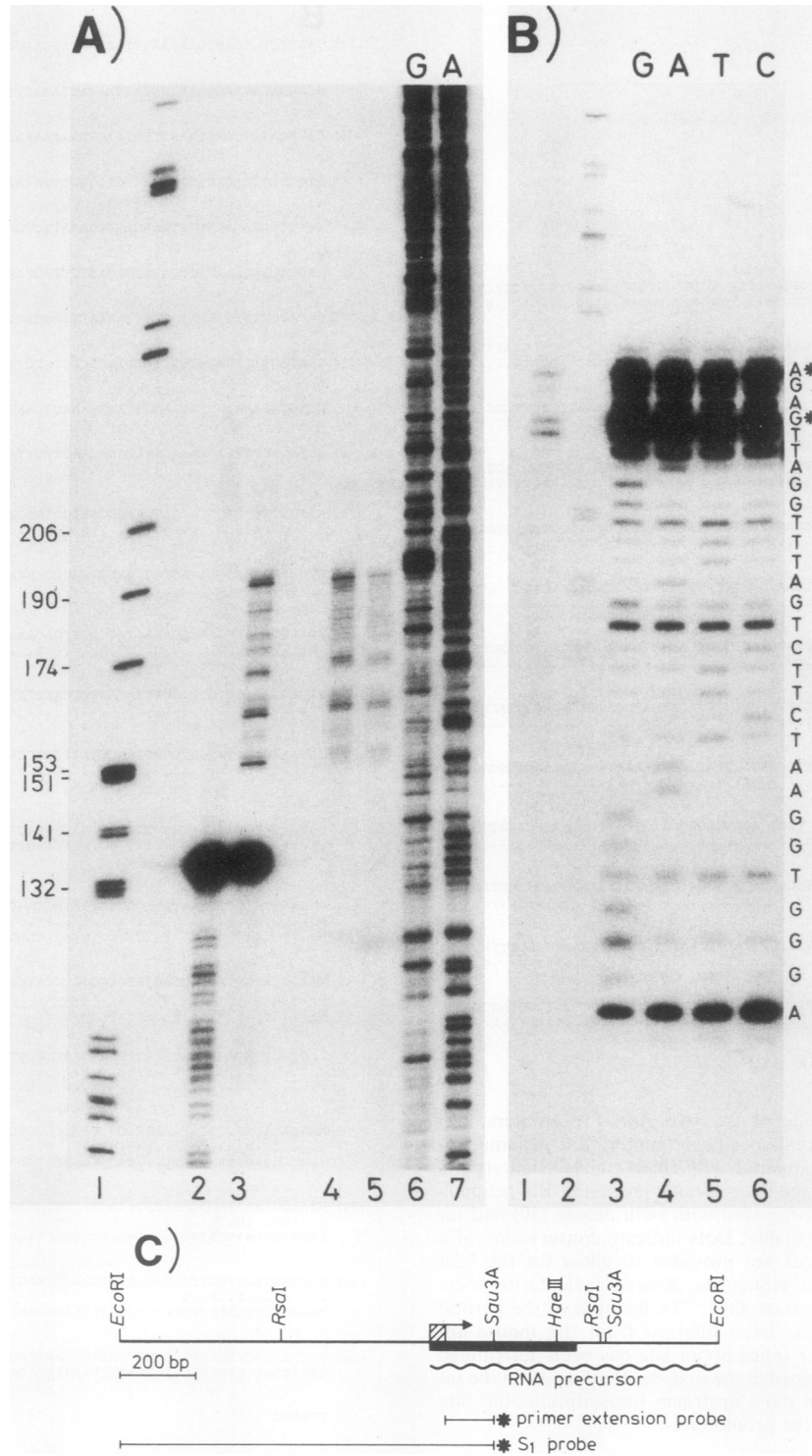


FIG. 6. Determination of the transcription start sites. (A) Liver promoter start sites. The locations of the liver-specific mRNA cap sites were determined by both S1 nuclease mapping and primer extension, using the probes illustrated in panel C. The results were analyzed on a 5% polyacrylamide-urea gel. Lanes: 1, pBR *Hha*I markers; 2, primer extension of 10 μg of tRNA; 3, primer extension of 10 μg of liver cell cytoplasmic poly(A)⁺ RNA; 4, S1 nuclease mapping of 100 μg of liver cell nuclear RNA; 5, S1 nuclease mapping of 1.5 μg of liver cell cytoplasmic poly(A)⁺ RNA; 6 and 7, Maxam and Gilbert sequencing reactions (20) performed on the fragment used for S1 nuclease mapping. (B) Parotid-specific promoter start sites. The locations of the parotid cell α-amylase mRNA cap sites were determined by primer extension sequencing as previously described (3, 8). The products of the reaction were analyzed on a 12% polyacrylamide-urea gel. Lanes: 1, primer extension in the absence of dideoxynucleotides; 2, pBR *Hha*I markers; 3 through 6, primer extension sequencing. (C) Probes used to determine the liver-specific promoter start sites.

account for the previously observed size difference between mouse and rat α -amylase mRNAs.

Weak promoter of rat *Amy-1* is mainly expressed in the liver cells. The weak promoter of mouse α -amylase is expressed in all α -amylase-producing tissues (parotid, liver, and pancreas), but not in other tissues (10). To test whether the weak promoter of the rat α -amylase gene was expressed in any tissue other than the liver, poly(A)⁺ RNA was prepared from several rat tissues, including the α -amylase-producing tissues mentioned above, as well as the other major exocrine glands of the neck (submaxillary and sublingual salivary glands and lacrimal gland). RNA was electrophoretically separated on a glyoxal gel, transferred to Gene Screen, and hybridized with a riboprobe derived from clone sRAL-1. The probe hybridized strongly only with RNA from liver cells (Fig. 8). A considerably weaker band of the correct size was also observed in RNA from rat pancreas, but not in RNA from parotid, submaxillary, sublingual, or lacrimal glands. The heterogeneous RNA species longer than the 28S rRNA observed in parotid cells were probably caused by the presence of polyadenylated precursors of α -amylase mRNA started at the parotid cap site, which contain the liver-specific sequence in their first intron. Two discrete RNA species, one migrating with 28S rRNA (parotid and submaxillary gland cells) and the other faster than 18S (pancreas cells), were observed that yielded weak hybridization signals with the sRAL-1 riboprobe. The larger of the two RNAs could not have been an authentic *Amy-1* transcript since it was unable to protect this riboprobe from ribonuclease digestion (Fig. 8B). It appears likely, therefore, that this RNA contains some fortuitous sequence homology with the liver-specific promoter region of the *Amy-1* gene. The same interpretation may also hold true for the pancreatic-cell transcript migrating faster than 18S rRNA, although the possibility cannot be excluded that this RNA represents a specific degradation product of *Amy-1* RNA. Since neither of the two possible interpretations would modify our conclusions on the tissue-specific expression of the rat *Amy-1* gene, no attempts were made to characterize this RNA species in greater detail.

To examine whether the weak rat *Amy-1* promoter is transiently active during parotid gland cell differentiation, poly(A)⁺ parotid RNA from 4- and 11-day-old animals was analyzed by RNase mapping for the presence of *Amy-1* transcripts initiated at the liver-specific promoter. The riboprobe was derived from clone sRL800, which contains a 745-bp *RsaI-HaeIII* fragment (see Fig. 6C). This promoter was not active in rat parotid cells at any developmental stage tested (Fig. 8). This result indicates that the lack of activity of the weak promoter in rat parotid is not caused by promoter exclusion, since, in younger animals, the upstream strong promoter is silent (31). Further experiments will be required to determine the mechanism by which the weaker promoter of α -amylase is repressed in rat parotid cells.

DISCUSSION

We cloned and characterized the promoter regions of the rat α -amylase gene *Amy-1*. Hybridization of the rat α -amylase clones with mouse probes, as well as with rat liver and parotid cDNAs, indicated that the general arrangements of the genes are very similar in mouse and rat cells. The transcription patterns of the rat and mouse *Amy-1* genes are schematically compared in Fig. 9 with regard to promoter use in cells of the three α -amylase-producing tissues, the

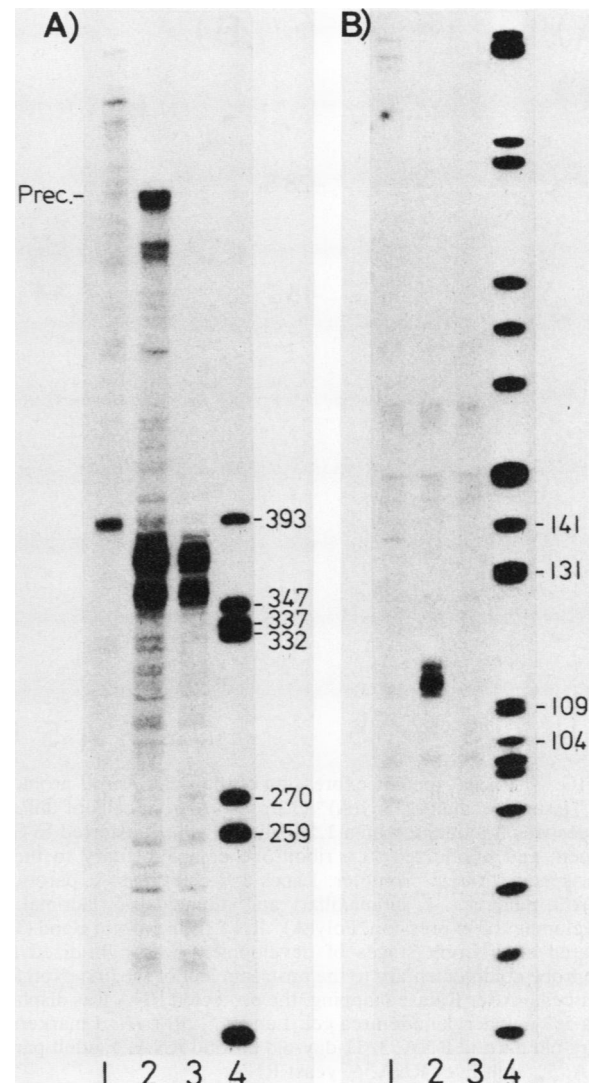


FIG. 7. Sizing of the first exon of the liver-specific transcript. For RNase mapping, probes with low specific activity were prepared as described in Materials and Methods; hybridization and processing were done by the method of Melton et al. (22). (A) Sizing of the whole first exon from liver cells. A riboprobe made from *HindIII*-digested sRAL-1 was hybridized with 60 μ g of parotid nuclear RNA (lane 1), 60 μ g of liver nuclear RNA (lane 2), and 2 μ g of liver cytoplasmic poly(A)⁺ RNA (lane 3). Lane 4, pBR *HhaI* markers. (B) Sizing of the 3' end of the first exon. A riboprobe made from *HaeIII*-digested sRAL-1 (see Fig. 6C) was hybridized with 1 μ g of parotid poly(A)⁺ RNA (lane 1), 1 μ g of liver cytoplasmic poly(A)⁺ RNA (lane 2), or 10 μ g of yeast RNA (lane 3). Lane 4, pBR *HhaI* markers.

parotid gland, liver, and pancreas. In both rodent species, transcription of the *Amy-1* gene was controlled by two tandem promoters that gave rise, through differential splicing, to two distinct mRNAs differing in their 5' untranslated region (34; this paper). In both species, the stronger, parotid-specific promoter of the *Amy-1* gene was located upstream of the weaker promoter. The distance between the two promoters, however, was more than twice as great in the rat gene as in the mouse gene. In contrast, the distances between the downstream promoter and the first common exon (approx-

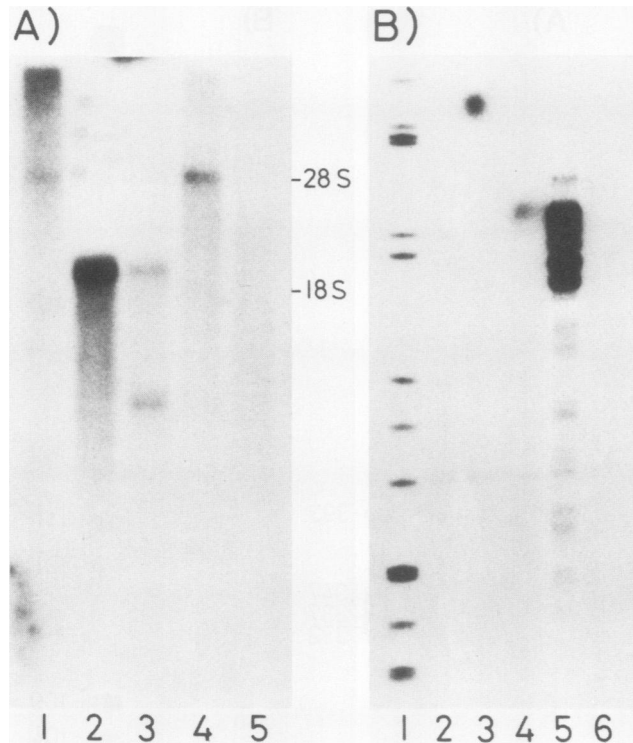


FIG. 8. Tissue-specific expression of the weak *Amy-1* promoter. (A) Tissue specificity. Poly(A)⁺ (3 μ g) RNA from cells of different tissues was fractionated on a 1.5% glyoxal gel, transferred to Gene Screen, and hybridized to a riboprobe complementary to the rat liver-specific *Amy-1* promoter. Lanes and cell types: 1, parotid; 2, liver; 3, pancreas; 4, submaxillary and sublingual; 5, lacrimal. (B) Developmental expression. Poly(A)⁺ RNA from parotid gland (3 μ g) isolated at different stages of development was hybridized to a riboprobe complementary to the upstream half of the first exon from liver cells. After RNase mapping, the protected RNA was displayed on a 5% polyacrylamide-urea gel. Lanes: 1, pBR *Hha*I markers; 2, 4-day-old parotid RNA; 3, 11-day-old parotid RNA; 4, adult parotid RNA; 5, adult liver RNA; 6, yeast RNA.

mately 4.5 kb) were similar in both species. In the rat, but not in the mouse, we also detected a second, transcriptionally silent sequence homologous to the liver-specific promoter. This sequence was not linked to either a parotid-specific promoter or to α -amylase-coding sequences; it is therefore referred to as an orphon.

Sequence comparison of the *Amy-1* promoter regions revealed striking sequence conservation between the mouse and rat genes. It is interesting that the sequences upstream of the strong parotid-specific promoter were well conserved only up to nucleotide -380 . Upstream of this position, homology was gradually lost, indicating that sequences upstream of -380 were not under strong evolutionary pressure. The fact that the degree of homology deteriorated slowly indicates that random mutation, rather than transposition, is the major cause of variations in this region of the sequence. This is in contrast to the situation observed in the α -amylase orphon, where homology with the bona fide liver leader sequences abruptly terminated at position $+273$, a landmark of a single block mutation event.

In spite of the high degree of nucleotide sequence conservation around and within the liver-specific leader sequences, analysis of the mRNA start sites indicates a completely different usage of mRNA cap sites between the two species

(see Fig. 9). In the mouse gene, the liver-specific transcripts initiated at two defined locations (called L major and L minor) at nucleotides $+157$ and $+126$ in the rat numbering system, respectively (34). In the rat liver, however, a series of transcripts with cap sites spanning a 40-nucleotide stretch was observed. The two start sites used in the mouse liver were not used in the rat, even though these sequences were conserved between mouse and rat genes. Conversely, even though the pertinent region was fairly conserved, the mouse did not utilize the liver start sites observed in the rat. At the moment, it is not clear whether the rat or the mouse start sites would be used upon introduction of the rat gene into mouse cells or vice versa. Such DNA transfer experiments would allow an assessment of the relative roles played by DNA sequences and by the cellular environment in controlling liver-specific α -amylase gene expression.

The splice donor site of the liver-specific promoter was also different in the two species, the rat gene splice site being located 77 bp downstream of its mouse counterpart. This difference could be caused by a single point mutation in the rat sequence that changes the G residue immediately preceding the mouse splice site into an A residue. A guanine is located at this position in 73% of splice donor sites (18). The relative displacement of both the cap sites and the splice donor site in the rat gene would account for the observed increase in the length of the liver cell α -amylase mRNA of the rat, when compared with its mouse counterpart.

In the mouse gene, both the parotid- and the liver-specific exons finish in an AUG; however, this AUG is closely followed by an in-frame termination codon present in the first common exon. The AUG that serves as a translational start signal is located a few nucleotides downstream (34). In the α -amylase mRNA from rat liver cells, however, six AUGs were located within the first exon. All but the last one were closely followed by in-frame termination codons (see Fig. 5B). Based on the scanning model of initiation of translation (16), such mRNA would most likely not be efficiently translated. Preliminary experiments suggest, however, that a considerable proportion of liver α -amylase mRNA is associated with polyribosomes (F. Sierra, unpublished observation).

As indicated in Figure 9, in mouse cells, the liver-specific promoter was active in all α -amylase-producing tissues, including the pancreas, whose major sources of α -amylase are the multiple *Amy-2* genes (9, 10). Since *Amy-1* and *Amy-2* genes are closely linked in the mouse genome (23), it is conceivable that, during developmental activation of the *Amy-2* gene, the *Amy-1* gene is included in the active chromatin domain, resulting in the expression of the liver-specific promoter of *Amy-1* in the pancreas cells. In rat pancreas cells, the weak *Amy-1* promoter also appeared to have some residual activity, although this was at least an order of magnitude lower than in liver cells. It is feasible that this reflects a longer distance between the *Amy-1* and *Amy-2* loci in the rat, as compared with these distances in the mouse, resulting in a less active *Amy-1* chromatin structure in the rat pancreas. The concept that the mouse liver-specific promoter requires only open chromatin configuration for its activity is further strengthened by the fact that this promoter, but not the parotid-specific one, is active upon transfection of mouse L cells, which do not express their endogenous *Amy-1* gene (F. Sierra, et al., submitted for publication). Considering this observation, it was all the more surprising to find that in rat parotid cells, the liver-specific promoter was completely silent. Since, as in the mouse, this promoter was also located downstream of the

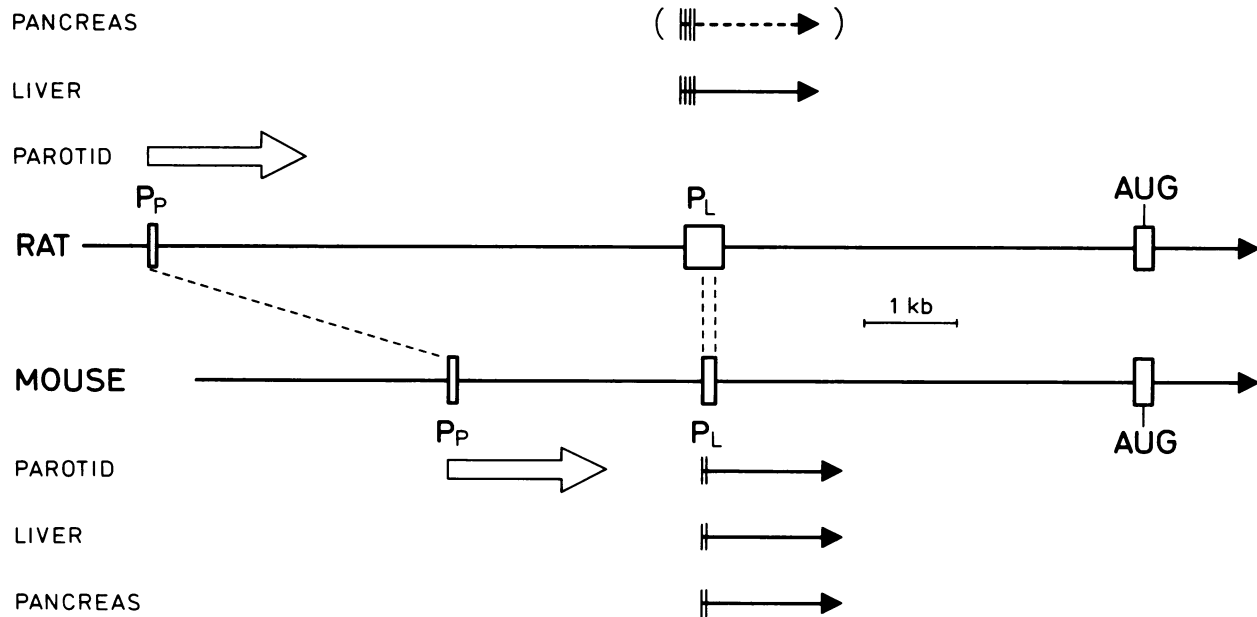


FIG. 9. Summary comparison of the rat and mouse genomic fragments containing the *Amy-1* promoters. The relative expression of the promoters in different tissues is indicated above (for the rat) and below (for the mouse). The thickness of the arrows represents the relative strength of the promoter.

strongly active parotid promoter, it seems obvious that it too has to be in an open chromatin configuration; thus, this promoter would be expected to be utilized in cells of the parotid gland. Its lack of activity can be interpreted in at least two different ways: (i) the rat parotid cells contain factors that specifically suppress the activity of the liver-specific promoter, or (ii) the activity of this promoter in the cells of mouse tissues is caused by a perturbation of the chromatin fine structure, which does not occur or occurs in a different manner in rat parotid cells. While these possibilities have not yet been assessed at the experimental level, it is interesting that the liver-specific promoter (in both animals) was quite unique, in that it did not contain recognizable TATA (except for the mouse minor start site [Fig. 2]) or CAAT boxes. Furthermore, perhaps because of the lack of a TATA box, the transcription initiation sites were heterogeneous. Other promoters whose characteristics correspond to these have been described in the literature. Most notably, they include the late genes of papovaviruses (33) and the sterile transcripts from the immunoglobulin—C μ chain (17). While these transcripts also initiate heterogeneously from TATA-less promoters, their activity might be related to the nearby presence of a strong enhancer (2, 5, 17). Thus, in these cases, it is likely that the activity of the promoters is related to changes in chromatin structure induced by the presence of the enhancer. Interestingly, for both papovaviruses and immunoglobulin sterile transcripts, the promoter is active preferentially at a time when the regular major promoter is silent. In papovaviruses, late transcripts are detectable only after T-antigen production and DNA replication have turned off enhancer-dependent early transcription (33). For the immunoglobulin sterile transcripts, these are most abundant in pre-B and T cells, but less so in mature, immunoglobulin-producing B cells (1, 15). It is therefore possible that the activity (or lack of it) of the liver-specific promoter in the parotid gland cells might be related to the presence of a parotid-specific enhancer in the vicinity of the liver promoter. In this case, the difference in

expression from this promoter in rat and mouse cells could be caused by a subtle difference in the specific chromatin alterations induced by protein binding in the two species. We are currently performing transfection experiments to determine whether a parotid-specific transcriptional enhancer is located near the liver-specific α -amylase promoter. If this is the case, it would be interesting to determine whether the rat orthon also contains enhancer activity and whether it can also induce altered chromatin structures.

The finding that the weak *Amy-1* promoter is used in the liver cells of both mice and rats but in the parotid cells only of mice suggests that this promoter has a physiological significance in hepatocytes but not in parotid cells. Hammetton and Messer have shown that α -amylase produced in hepatocytes is secreted into the bloodstream (12). The physiological role of this enzyme in serum is currently unclear. One attractive possibility is that the serum α -amylase may be involved in the establishment of immunotolerance towards parotid-specific α -amylase. Shaw et al. (31) have shown that significant levels of parotid cell α -amylase in mice are observed only 2 to 3 weeks after birth, at a time when the decision about self and nonself has already been completed by the immune system. Thus, production and secretion of liver α -amylase, which is immunologically identical to the parotid enzyme, may be essential to avoid an autoimmune reaction in the adult animal. The absence of significant levels of pancreatic α -amylase in serum is compatible with this hypothesis, since in pancreas cells, *Amy-2* genes are already induced before birth (13) and thus do not demand a special mechanism to establish immunotolerance.

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