A salivary amylase transgene is efficiently expressed in liver but not in parotid gland of transgenic mice

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ABSTRACT

Two distinct mouse amylase cDNAs, corresponding to the genes *Amy-1.1* and *Amy-1.2*, have been isolated from a YBR/Ki parotid cDNA library. A cosmid clone containing the intact *Amy-1.1* gene from strain YBR/Ki, including both the parotid and liver promoters, was transferred to the germ line of C57BL/6J mice. Two independent transgenic lines were produced. The transferred genes are organized as a 4-copy autosomal locus in line Tg518 and an 8-copy Y-linked locus in line Tg2736. Serum of both transgenic lines contained high levels of the AMY1B isozyme encoded by the transferred gene. Transcripts were detected in liver and, at a lower level, in several other tissues including white and brown fat. The anticipated expression in parotid was not observed. Constructs containing 270 or 540 bp of the 5′ flanking region of the parotid promoter, cloned upstream of the chloramphenicol acetyltransferase (CAT) structural gene, were also not expressed in transgenic mice. The results suggest that sequences located more than 5 kb upstream of the *Amy-1* parotid promoter and/or more than 10 kb downstream from the structural gene are required for parotid-specific expression. The results also demonstrate that non-parotid sources can produce a normal level of AMY1 in serum. Liver is the probable source of AMY1 in serum of these transgenic mice.

INTRODUCTION

As a result of its genetic distance from the commonly studied inbred strains, YBR/Ki mice provide a source of regulatory variation which is amenable to genetic and molecular analysis. In the course of our investigation of amylase regulation in this strain, we have isolated the YBR/Ki amylase genes on overlapping cosmid clones (1;2). We also localized pancreas-specific and insulin-responsive elements of the YBR/Ki pancreatic amylase gene by expression of cloned genes in transgenic mice (3;4).

YBR/Ki mice produce a rare electrophoretic variant of salivary amylase, and the total amylase activity in parotid gland is two-fold greater than in other inbred strains (5;6;7). Quantitative analysis of genomic DNA demonstrated an extra gene copy in YBR/Ki (8), compared with the single copy in other strains (5;9;10). If both gene copies were expressed, they could account for the two-fold elevation of amylase activity and mRNA concentration (8). The current studies were undertaken to determine whether more than one salivary amylase gene is active in YBR/Ki parotid. The results indicate that increased gene dosage is responsible for elevated salivary amylase expression in this strain.

As a first step in localization of regulatory elements, a cosmid clone carrying a YBR/Ki salivary amylase gene was transferred to transgenic mice. The cosmid included both the parotid-specific promoter, located 7.5 kb upstream of the first coding exon (9;11), and the 100-fold weaker liver promoter, located 3 kb downstream of the parotid promoter (12).

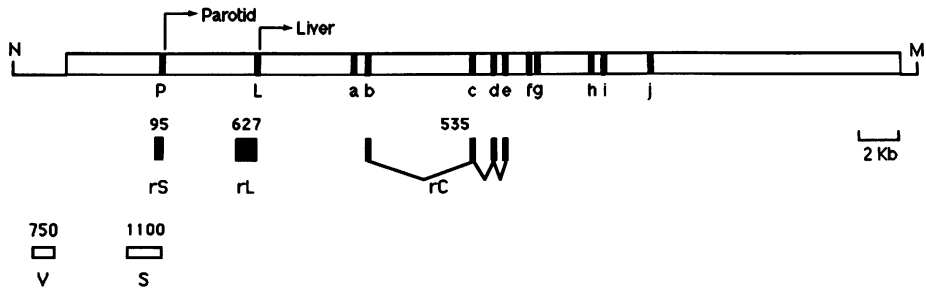

Figure 1. Structure of the transferred *Amy-1* gene. The 44 kb fragment includes a 41 kb insert of genomic DNA from strain YBR/Ki and 3 kb of vector sequence (thin line). Exons are represented by black boxes. Exon P is the parotid-specific non-translated exon; L is the liver-specific non-translated exon. Open rectangles, probes for Southern blots; closed rectangles, riboprobes. N, *Nru* I; M, *Mlu* I.

The liver promoter is known to be expressed at equal levels in liver, parotid and pancreas (12;13). In the transgenic mice, the liver promoter was expressed normally, but expression of the parotid promoter could not be detected.

MATERIALS AND METHODS

Isolation and sequencing of Amy-1 cDNA clones.

A parotid cDNA library from strain YBR/Ki was prepared in the vector pUC9, and screened with a pancreatic amylase cDNA probe, by the methods previously described (2). *Eco*RI fragments from the amylase cDNAs were subcloned into pGEM2 and sequenced by the dideoxynucleotide chain termination method (14) from double-stranded plasmid DNA using the GemSeq K/RT system (Promega). Additional sequence information was obtained using a primer corresponding to bases +740 to +758 of the parotid *Amy-1*^{a} cDNA pMSa104 (15). (Nucleotides are numbered from +1 of pMSa104 throughout this report.)

Preparation of DNA for microinjection.

The 44 kb *Nru* I-*Mlu* I fragment from cosmid clone cSamD4 (1) was purified by agarose gel electrophoresis, electroeluted into dialysis tubing, and dialyzed against 10 mM Tris hydrochloride, pH 7.5, containing 0.1 mM EDTA. The DNA was microinjected into fertilized mouse eggs of strain C57BL/6NCr as previously described (3).

For construction of the CAT hybrid genes, a 552 bp *Rsa* I-*Pst* I fragment and a 302 bp *Alu* I-*Pst* I fragment containing the parotid promoter were isolated from a plasmid containing the 1.1 kb *Pst* I fragment S (Figure 1). These fragments were cloned into the *Sma* I site of the promoterless vector pSV0CAT (16). DNA fragments containing the parotid promoter/CAT hybrid genes were isolated by digestion with *Nde* I and *Bam* HI, followed by electrophoresis and elution as described above. The DNA was microinjected into fertilized eggs of heterozygous C57BL/6J×C3H mice. CAT activity was assayed in tissue homogenates as previously described (4).

Electrophoresis of amylase isozymes.

Amylase isozymes in serum were separated by electrophoresis on 7% polyacrylamide gels at pH 8.1 as described (17;18). Amylase enzymatic activity was visualized with a starch-iodine stain.

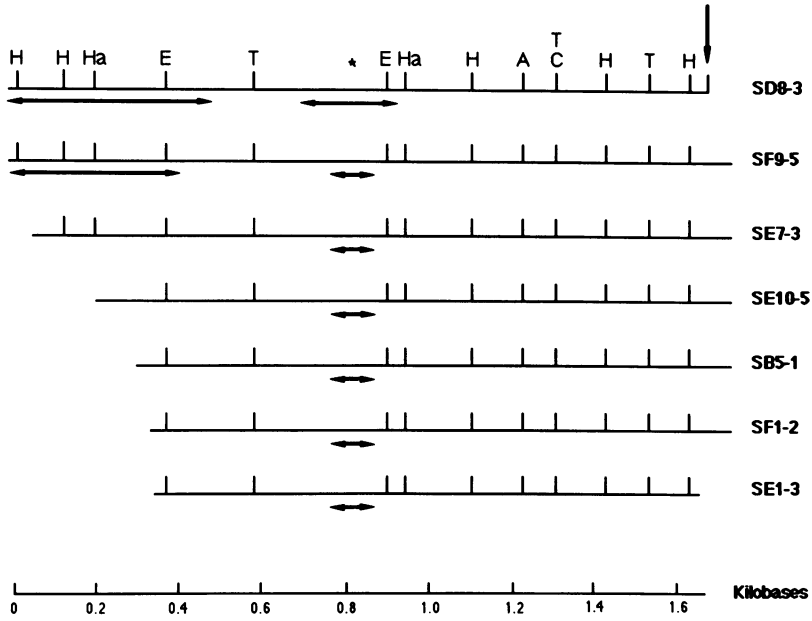

Figure 2. Restriction maps of amylase cDNA clones isolated from a YBR/Ki parotid library. Sequenced regions are underlined. The location of the nucleotide difference between *Amy-1.1* and *Amy-1.2* is indicated by the asterisk. The arrow marks the start of the polyA tract. A, *Ava* I; C, *Cla* I; E, *Eco* RI; H, *Hinf* I; Ha, *Hha* I; T, *Taq* I.

Southern blot analysis.

Genomic DNA was isolated from transgenic animals and analyzed by Southern blot hybridization essentially as described previously (3). Restriction fragments were separated by electrophoresis on 1.2% agarose gels and transferred to nitrocellulose filters. Filters were hybridized with a 750 bp *Eco*RI-*Pst* I fragment purified from pJB8 (V, Figure 1) or with a plasmid carrying the 1.1 kb *Pst* I fragment which includes the parotid promoter of *Amy-1.1* (S, Figure 1). Probes were labeled by the random oligomer-primed method (19).

Table 1. Properties of two *Amy-1* cDNAs from strain YBR/Ki. The sequences of the other five YBR/Ki cDNAs (Figure 2) were the same as clone SF9-5 in this region. The sequence of the parotid cDNA pMSa104 from strain A/J is included for comparison (15). The length (nucleotides) of protected fragments expected after hybridization of each type of transcript with the rC riboprobe from clone SD8-3 are indicated in the last column.

cDNA	Strain	Gene	+807 to +812	Amino acids	Protected fragments
SD8-3	YBR/Ki	*Amy-1.1*	TATCTA	tyr-leu	537
SF9-5	YBR/Ki	*Amy-1.2*	TATCAA	tyr-gln	439 and 97
pMSa104	A/J	*Amy-1^a*	TCCCAA	ser-gln	436 and 97

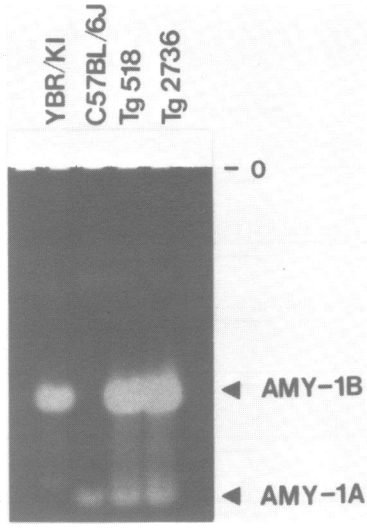

Figure 3. Serum amylase isozymes in transgenic mice. Five μl aliquots of diluted serum (1:1,000) were analyzed by electrophoresis and stained for amylase activity. The positions of AMY1 isozymes A and B are indicated.

Ribonuclease protection assays.
DNA fragments rS, rL, and rC containing parotid promoter, liver promoter, and coding sequence of *Amy-1*, respectively (Figure 1) were cloned into pGEM and transcribed using SP6 polymerase (rS and rC) or T7 polymerase (rL). The *Hae* III-*Pst* I fragment in rS contains the sequence −64 to +34 from the parotid promoter (8). rL contains the *Eco* RI-*Hind* III fragment from −374 to +137 of the liver promoter (20). rC contains the *Eco* RI fragment +372 to +902 of the *Amy-1.1* cDNA clone SD8-3. All riboprobes were gel purified and ethanol precipitated before use.

Total RNA was prepared from mouse tissues by guanidinium thiocyanate extraction and centrifugation over CsCl (20;21). The quality of the RNA was evaluated by electrophoresis on 1% agarose gels and staining with ethidium bromide to detect degradation of the ribosomal RNAs. Assays were carried out as described by Samuelson et al. (20), except that hybridizations contained 40,000 cpm (Cerenkov) of ^{32}P-labeled riboprobe. Protected fragments were analyzed by electrophoresis on 6% polyacrylamide/8M urea gels.

RESULTS

Two types of amylase cDNA clones from YBR/Ki parotid.
Seven amylase cDNA clones were isolated from a YBR/Ki parotid gland cDNA library and characterized. Recognition sites for six restriction endonucleases did not differ among the clones (Figure 2). Sequencing identified a T to A transversion at nucleotide +811 of clone SD8-3, which is not present in the other clones (Table 1). This single base difference results in substitution of glutamine for a leucine residue. The genes encoding the two amylase transcripts have been designated *Amy-1.1* and *Amy-1.2*. Both differ in sequence from the transcript previously isolated from strain A/J (Table 1). Partial sequencing of the cDNA clones (Figure 2) revealed four additional differences from the A/J cDNA sequence (15): a C to G transversion at +16 of the salivary non-translated exon, an A to G transition

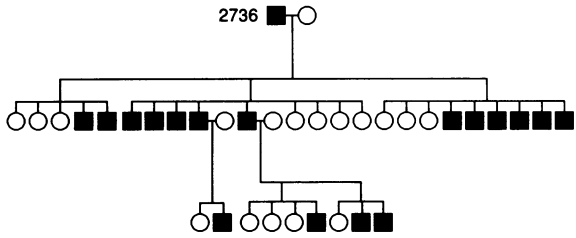

Figure 4. Y-linked transmission of the transferred genes in transgenic line 2736. The founder is individual 2736. Transgenic offspring were identified by electrophoretic analysis of serum. Closed symbols, transgenic individuals; open symbols, non-transgenic individuals; circles, females; squares, males.

at +180 resulting in replacement of isoleucine by phenylalanine, a C to T transition at +710, and a T to C transition at +375. Since none of these changes would account for the slower electrophoretic mobility of the YBR/Ki isozyme, we presume that there are additional differences in the nonsequenced regions of the cDNA.

Production of transgenic mice carrying an intact Amy-1.1 gene.

The intact *Amy-1.1* gene (Figure 1) was previously isolated from a YBR/Ki cosmid library (1). A fragment containing the intact gene with 5 kb of 5′ flanking region and 12 kb of 3′ flanking region was microinjected into fertilized mouse eggs. Two of the forty-eight offspring were identified as transgenic by Southern blot of genomic DNA. The AMY1B isozyme was present at a high level in serum and urine of both transgenic individuals (Figure 3), indicating expression of the transferred YBR/Ki gene. These animals were the founders of transgenic lines Tg518 and Tg2736.

Transgenic lines were generated by crossing the founders to strain C57BL/6J. In line Tg2736, expression of the B isozyme was inherited as a Y-linked locus (Figure 4). Inheritance was autosomal in line 518, which has been maintained as a homozygous stock without any apparent phenotypic effect of the inserted genes.

To determine the number of gene copies transferred, genomic DNAs from the transgenic mice were probed with a vector fragment that does not hybridize with endogenous mouse sequences (V, Figure 1). Comparison with standards indicated that Tg518 and Tg2736 contain approximately four and eight transgene copies per haploid genome, respectively (Figure 5A).

The transgene was also identified by a restriction fragment length polymorphism unique to strain YBR/Ki (8). A probe containing the parotid promoter (S, Figure 1) hybridizes with a 5.4 kb *Eco* RI fragment which is characteristic of the *Amy-1* genes of strain YBR/Ki. Other strains, including C57BL/6J, contain a 4.7 kb hybridizing fragment. Both transgenic lines contain the 5.4 kb fragment (Figure 5B), as well as the endogenous 4.7 kb fragment and two faint bands which are the result of hybridization with vector sequences. The presence of the 5.4 kb *Eco* RI fragment demonstrates that the parotid promoter was not deleted from the transferred genes.

Ribonuclease protection assay for transgene transcripts.

A ribonuclease protection assay based on the nucleotide substitutions in the region +808 to +811 was used to distinguish endogenous and transgenic transcripts. A cDNA fragment containing sequences +372 to +908 from the *Amy-1.1* cDNA clone SD8-3 was subcloned in the vector pGEM2, and transcribed to produce a uniformly labeled riboprobe. YBR/Ki *Amy-1.1* transcripts should protect a 537 nucleotide fragment of this probe (Table 1).

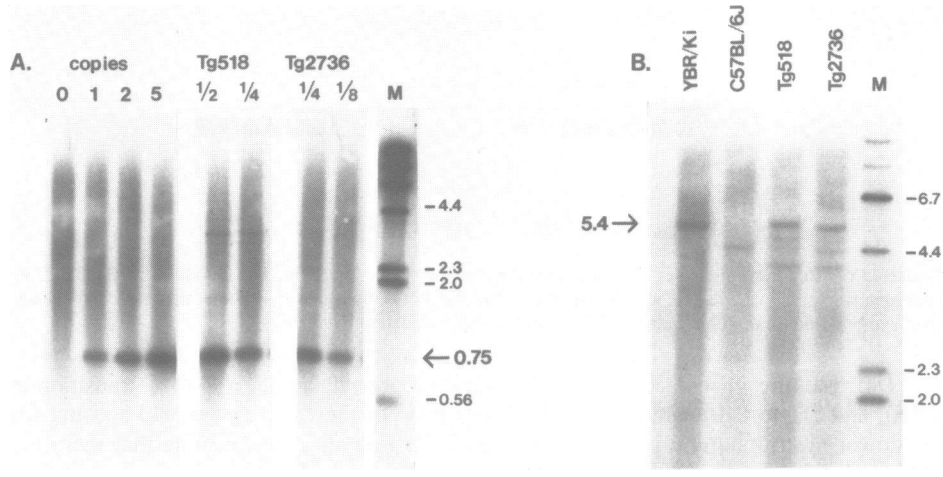

Figure 5. Detection of the *Amy-1* transgene by Southern blot analysis of genomic DNA. *A. Copy number.* Genomic DNA from a homozygous individual of line Tg518 and a male of line Tg2736 was diluted with nontransgenic DNA from strain C57BL/6J in the ratio 1:2, 1:4, or 1:8. Ten μg of the diluted DNA was digested with *Eco* RI and *Pst* I. The blot was probed with a vector fragment, V. The copy standards contained 10 μg of C57BL/6J genomic DNA plus 15, 30, or 75 pg of plasmid pBR322 DNA, corresponding to 1, 2 and 5 copies per haploid genome. *B. Parotid promoter.* Ten μg of genomic DNA was digested with *Eco*RI and the blot was hybridized with the parotid promoter probe S. The 5.4 kb fragment characteristic of strain YBR/Ki is indicated by the arrow.

Hybridization to *Amy-1.2* transcripts results in cleavage of the riboprobe at the mismatch at position +811 to generate two fragments of 439 and 97 nucleotides. The expected fragments characteristic of both transcripts are observed in a protection assay of RNA from liver and parotid of strain YBR/Ki (Figure 6, lanes 4 and 6). Since the abundance of transcripts in parotid gland is approximately 100-fold higher than in liver, we assay 50 μg of liver RNA and 0.5 μg of parotid RNA. RNA from strain C57BL/6J protects the 439 and 97 nucleotide fragments (Figure 6, lanes 1 and 3, respectively). The 537 nucleotide fragment can thus be used as a specific assay for expression of *Amy-1.1* in transgenic mice.

Amy-1 expression in tissues of transgenic mice.

RNA was prepared from fourteen tissues of transgenic mice and analyzed with the ribonuclease protection assay. Data is presented for transgenic line 2736, but all experiments were also carried out on line 518 with virtually identical results. Fifty μg of liver RNA from transgenic mice protected the 537 nucleotide fragment characteristic of the transferred gene (Figure 6, lane 7). The similar intensities of the 537 and 439 nucleotide fragments in liver RNA indicate that the transgene is expressed at a level comparable to that of the endogenous gene. The equal intensity of the 537 nucleotide fragment in lanes 4 and 7 demonstrates that the abundance of the transcript in liver of this transgenic line is essentially equivalent to the abundance of the original gene in strain YBR liver, which can be used as a standard of reference.

Transcripts of the transgene were barely detectable in 0.5 μg of parotid RNA, while the endogenous transcript is readily detected. (Figure 6, lane 9). Protection of the 439 nucleotide fragment by the endogenous transcript in these RNAs demonstrates that the parotid RNA samples were not degraded. Even in 10 μg of parotid RNA (lane 14), the

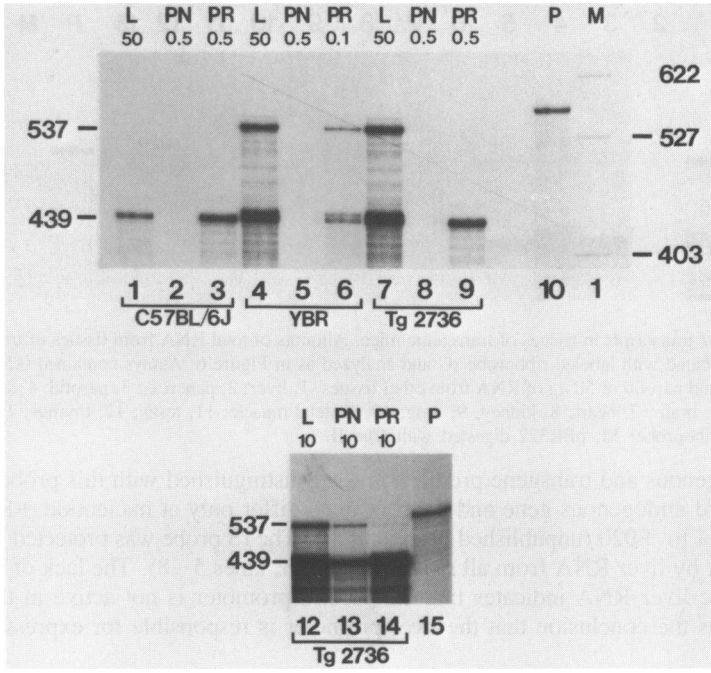

Figure 6. Detection of *Amy-1* transcripts with a riboprobe protection assay. The indicated quantities of total RNA from liver (L), pancreas (PN) and parotid (PR) of the indicated strains was analyzed by hybridization with the rC riboprobe. The approximate length of the protected fragments is indicated at the left. A 200 nucleotide fragment derived from the partially homologous pancreatic amylase transcript was visible in lanes 2 and 5, and a 97 nucleotide fragment was visible in lanes 1, 3, 4, 6, 7, and 9 (not shown). P, undigested probe; M, pBR322 digested with *Hpa* II.

transgenic transcript was less abundant than in 0.1 μg of parotid RNA from YBR (lane 6). This comparison indicates a level of expression in transgenic parotid which is less than 1% of the level of the original gene in strain YBR parotid. Direct comparison of 10 μg aliquots of transgenic liver and parotid RNA (lanes 12 and 14) demonstrates much greater expression in liver. We conclude that the parotid specific promoter is expressed at less than 1% of the expected level in parotid of transgenic mice. This low level of transcript in parotid can be completely accounted for by the low level expression of the liver promoter in parotid gland (12).

A lower level of transgene transcripts was detected in submaxillary gland, brown and white fat, skeletal muscle, and testis (Figure 7). The expression of the transgene parallels that of the endogenous gene, and is the same in line Tg518. *Amy-1* transcripts were not detected in spleen of either strain (not shown). Analysis of 10 μg aliquots of pancreatic RNA (Figure 6, lane 13) revealed the low level of transcripts expected from the expression of the liver promoter in pancreas (12).

Amy-1 promoter usage in transgenic liver.

In order to distinguish between transcripts originating from the liver and parotid promoters of *Amy-1*, RNA from various tissues was hybridized with riboprobes rL and rS (Figure 1). The parotid riboprobe rS includes nucleotides +1 to +34 of the parotid nontranslated

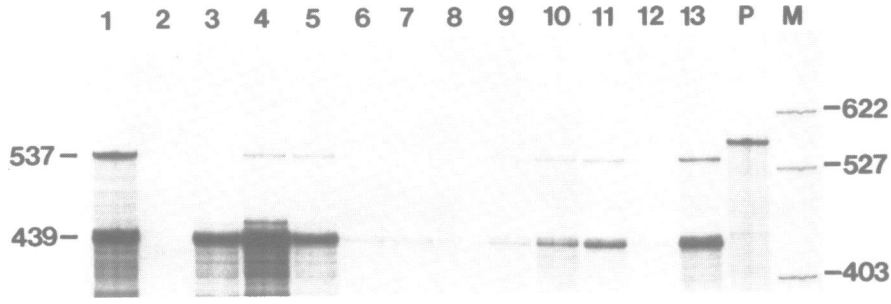

Figure 7. *Amy-1* transcripts in tissues of transgenic mice. Aliquots of total RNA from tissues of transgenic line 2736 were incubated with labeled riboprobe rC and analyzed as in Figure 6. Assays contained 0.5 μg of RNA from pancreas and parotid or 50 μg of RNA from other tissues. 1, liver; 2, pancreas; 3, parotid; 4, submaxillary; 5, brown fat; 6, brain; 7, heart; 8, kidney; 9, lung; 10, skeletal muscle; 11, testis; 12, thymus; 13, white fat. P, undigested riboprobe; M, pBR322 digested with *Hpa* II.

exon. Endogenous and transgene products are not distinguished with this probe, because the C57BL/6J endogenous gene and the transgene differ only at nucleotide +811 within the region +1 to +920 (unpublished observations). The rS probe was protected by parotid RNA but not by liver RNA from all strains (Figure 8, lanes 5–8). The lack of protection by transgenic liver RNA indicates that the parotid promoter is not active in this tissue. This supports the conclusion that the liver promoter is responsible for expression of the transgene.

The liver promoter fragment was protected by 50 μg of RNA from all the tissues positive for transgene expression (data not shown). Because there is no interstrain sequence difference within the liver non-translated exon, this probe does not distinguish between transcripts of the endogenous gene and the transgene. The level of the protected rL fragments was sufficient to account for the transcripts detected with the rC probe in the previous section. The data is consistent with the conclusion that expression of the transgene is the result of transcription from the liver promoter.

Parotid promoter/CAT hybrid genes are not active in transgenic mice.

To test the possibility that removal of negative elements might activate the parotid promoter, we cloned two small promoter fragments from *Amy-1.1* upstream of the CAT structural gene. One construct contained the parotid promoter fragment −520 to +35, while the other contained the fragment −270 to +35. Southern blot analysis identified two founder animals carrying the −520 construct and three with the −270 construct. Transgenic lines were generated from each of these founders. Parotid glands from several individuals of each strain were pooled and CAT activity was assayed by a method previously used to detected expression in transgenic tissues (4). CAT activity was not detectable in parotid homogenates from any of these strains, suggesting that these promoter fragments are incapable of directing efficient tissue-specific expression (data not shown). In one strain carrying the −520 construct, CAT activity was detected in brain. This unexpected expression was probably the result of the specific site of integration in this strain, and was not studied further.

DISCUSSION

The isolation of two distinct amylase cDNAs from a YBR/Ki parotid library demonstrates that two genes are active in this tissue. Transcripts of both genes are abundant in YBR/Ki

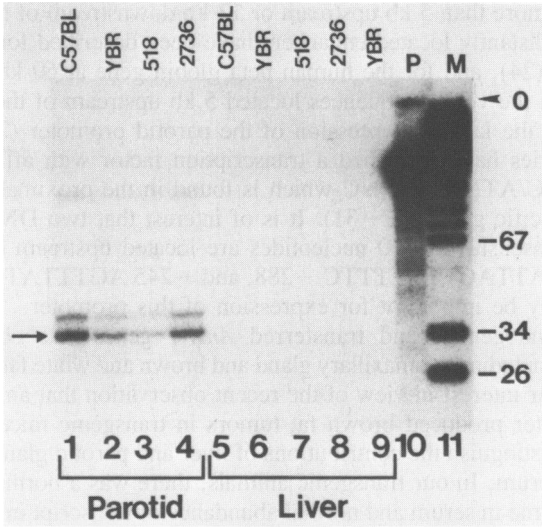

Figure 8. Detection of transcripts from the *Amy-1* parotid promoter. Aliquots of total RNA from parotid (1 μg) and liver (50 μg) were hybridized with riboprobe rS. P, undigested riboprobe; M, pBR322 digested with *Hpa* II.

parotid RNA. Expression of two genes can account for the previously described two-fold elevation of parotid amylase in YBR/Ki mice (6;8). A single *Amy-1* gene has been described for other inbred strains such as DBA/2J, C57BL/6J and A/J (5;10), indicating that a recent duplication occurred in a YBR/Ki progenitor. Recent duplication is also indicated by the near sequence identity of the two YBR/Ki genes. We observed only one difference in 780 nucleotides of the two cDNAs which were compared, and no differences in restriction sites within a 30 kb region (unpublished observation). Gene duplications have occurred repeatedly during the evolution of the amylase gene cluster; another well characterized example is the human salivary amylase gene (22;23). YBR/Ki salivary amylase provides a demonstration of naturally occurring quantitative variation in enzyme level due to gene dosage.

We used sequence divergence and electrophoretic differences to monitor the expression of a YBR/Ki-derived salivary amylase transgene in C57BL/6J mice. The results are summarized in Table 2. In two transgenic lines carrying 41 kb of genomic DNA, YBR/Ki-specific transcripts were detected at the expected abundance in liver but not in parotid gland. The evidence indicates that the liver promoter is active in the transgenic mice. The lack of expression of the parotid promoter suggests that the parotid-specific enhancers of

Table 2. Expression of *Amy-1* promoters in transgenic mice.

Amy-1 Promoter	*Amy-1* Sequences	Reporter	Transgenic Lines expressing/total
Liver	41 kb cosmid	self	2/2
Parotid	41 kb cosmid	self	0/2
Parotid	−520 to +35	CAT	0/2
Parotid	−270 to +35	CAT	0/3

Amy-1 are located more than 5 kb upstream or 12 kb downstream of the structural gene. Examples of such distantly located enhancers have been described for the albumin gene at 10 kb upstream (24), and for the human beta globin gene at 60 kb upstream (25). It is also possible that the vector sequences located 5 kb upstream of the parotid promoter are responsible for the lack of expression of the parotid promoter (26).

Several laboratories have described a transcription factor with affinity for the DNA sequence PuGTTAC/ATNNTPyNNC which is found in the proximal promoter regions of several liver-specific genes (27–31). It is of interest that two DNA elements which conform to this consensus at 9/10 nucleotides are located upstream of the *Amy-1* liver promoter: −275 AATTACTTGTTTTC −288, and −245 AGTTTATCATCTTC −258. These elements may be important for expression of this promoter.

Expression of endogenous and transferred *Amy-1* genes was observed in several unexpected tissues including submaxillary gland and brown and white fat. *Amy-1* expression in fat is of particular interest in view of the recent observation that an oncogene directed by the liver promoter produced brown fat tumors in transgenic mice (32).

It is difficult to distinguish the contributions of liver and parotid gland to the circulating AMY1 in mouse serum. In our transgenic animals, there was a normal concentration of the B amylase isozyme in serum and normal abundance of transcript in liver, but less than 1% of the normal transcript level in parotid. This indicates that liver can produce the major portion of AMY1 in serum. This conclusion is also consistent with our previous detection of AMY1 in serum of fetal mice at a stage of development prior to the activation of the parotid gland (20). Human liver expresses a pancreatic amylase transcript (33), and may be an important source of serum amylase in man.

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