INTERSTRAIN VARIATION IN AMYLASE GENE COPY NUMBER AND mRNA ABUNDANCE IN THREE MOUSE TISSUES

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ABSTRACT

Amylase expression in strain YBR differs in several respects from the standard mouse phenotype. The synthesis of salivary amylase is elevated twofold in YBR mice and the synthesis of pancreatic amylase is reduced to one-half the normal rate. We have compared the concentrations of amylase mRNA in the parotid, liver and pancreas of YBR mice with those in strains A/J and C3H. We observed differences in amylase mRNA abundance which can account for the levels of amylase protein synthesis in the parotid and pancreas of these strains. Unexpectedly, the concentration of amylase mRNA in the liver of YBR mice was also higher than in the other strains. Since liver amylase is transcribed from the same gene as parotid amylase, duplication of the *Amy-1* locus could account for the elevated mRNA concentration in both tissues. Quantitative analysis of genomic DNA by Southern blotting provided direct evidence for duplication of *Amy-1* in strain YBR.

ALPHA amylase is encoded by a clustered multigene family on mouse chromosome *3*. The members of this gene family can be divided into two groups with different tissue-specific expression. *Amy-1* is a single-copy gene in most strains of mice, as indicated by gene titration data (YOUNG, HAGENBÜCHLE and SCHIBLER 1981; SCHIBLER *et al.* 1982). The presence of a single active *Amy-1* gene is also supported by the altered mobility of salivary amylase in a spontaneous mutant, which affects 100% of the enzyme in homozygotes (HJORTH 1982). In strain A/J, the *Amy-1* region can be transcribed from two alternative promoters located 4.5 and 7.5 kb upstream of the structural gene (YOUNG, HAGENBÜCHLE and SCHIBLER 1981; HAGENBÜCHLE *et al.* 1981). One of these promoters is active only in the parotid gland, and the other is active in both the liver and the parotid gland (SCHIBLER *et al.* 1983). The primary transcripts from these promoters are spliced to produce mRNAs that differ only in their 5′ untranslated regions. In inbred mice the *Amy-2* gene region contains multiple gene copies for which the coding regions are approximately 10% divergent from *Amy-1*. The *Amy-2* gene copies are expressed exclusively

in the exocrine pancreas (HAGENBÜCHLE, BOVEY and YOUNG 1980; TOSI *et al.* 1984; GUMUCIO *et al.* 1985; BODARY *et al.* 1985; MIKKELSEN *et al.* 1985).

Several unique characteristics of YBR mice suggest that the amylase gene region differs significantly from that of other strains. Whereas most mice synthesize a single species of pancreatic amylase, two nonallelic genes in strain YBR encode two isozymes that differ in primary sequence (STRAHLER and MEISLER 1982) and can be independently regulated (DRANGINIS *et al.* 1984). We have recently presented evidence indicating that strain A/J carries a null allele for one of these genes (GUMUCIO *et al.* 1985). With regard to quantitative expression, strain YBR produces approximately twice as much parotid amylase as do the typical strains A/J and C3H (HJORTH 1979) but only one-half as much pancreatic amylase (BLOOR, MEISLER and NIELSEN 1981). The molecular basis for these quantitative phenotypes have not been studied previously, although it is known that they are determined by sites linked to the amylase structural genes (BLOOR, MEISLER and NIELSEN 1981; NIELSEN 1982). Finally, the restriction pattern of the YBR amylase gene region is also unique (THOMSEN, HJORTH and NIELSEN 1984).

In the present work, we compared the relative abundance of the messenger RNA in amylase-producing tissues of several strains of mice. In these studies we used a congenic strain, C3H·Amy^{YBR}, in which the amylase gene region from strain YBR is present in a C3H background (NIELSEN 1982). Previous studies demonstrated that the *Amy-1* and *Amy-2* phenotypes of the congenic mice are identical to the YBR phenotypes, indicating that the structural and regulatory sequences are intact. Since background effects of other genetic loci are minimized, we used the congenic strain for measurement of amylase mRNA. We also compared the abundance of amylase sequences in genomic DNA. Our results indicate that mRNA concentrations and gene dosage can account for the unique quantitative phenotypes of amylase-producing tissues in strain YBR.

MATERIALS AND METHODS

Animals: Strain C3H/As and the congenic strains C3H·Amy^{YBR} and C3H·Amy^{CE}, carrying the indicated amylase regions on a C3H/As genetic background (NIELSEN 1982), were kindly provided by J. T. NIELSEN, University of Aarhus. YBR/Ki mice were originally purchased from the Kirshbaum Memorial Laboratory and are maintained in this department. A/J mice were purchased from the Jackson Laboratory.

RNA purification: Total RNA was prepared from pancreas and parotid glands by centrifugation through cesium chloride solution as described by CHIRGWIN *et al.* (1979), except that centrifugation was carried out at 36,000 rpm for 21 hr in an SW41 rotor.

Liver poly(A)$^+$ RNA was isolated as described by SCHIBLER *et al.* (1980). Each preparation was quantitated by measurement of the absorption of triplicate dilutions at 260 nm. The ratio of A_{260}:A_{280} of the RNA before oligo-dT chromatography was $\geq$2.0, indicating little or no contamination by protein or phenol. The yields ranged from 112 to 128 μg of poly(A)$^+$ RNA from three livers.

Dot blots: Aliquots of total RNA from pancreas or parotid were applied to nitrocellulose filters in volumes of 2–10 μl. Filters were hybridized with nick-translated cDNA probes and were washed as described by THOMAS (1980). The filters were cut into squares, 2 × 2 cm, and were counted in a Beckman liquid scintillation counter (LS100) with Aquasol (Amersham) as scintillant.

Northern blot electrophoresis and transfer of RNAs: RNA was denatured with formamide and was subjected to electrophoresis on 1.5% agarose-formaldehyde gels by the method of SEED and GOLDBERG (1982). Deionized formamide was prepared as recommended (SEED and GOLDBERG 1982). Transfer to nitrocellulose filters and hybridization were carried out by the procedures of WAHL, STERN and STARK (1979), as modified by THOMAS (1980), except that salmon sperm DNA concentration was reduced to 25 μg/ml, and dextran sulfate was omitted from the hybridization buffer. Mammalian 18S and 28S rRNAs, and *E. coli* 16S and 23S rRNAs, were included on the gels as size markers. Autoradiographs were scanned with a Biolabs densitometer.

Hybridization probes: Intact plasmids pMPa21 and pMSa104, containing cloned sequences complementary to pancreatic and salivary amylase mRNAs, respectively, from mouse strain A/J (HAGENBÜCHLE, BOVEY and YOUNG 1980) were used to detect amylase mRNAs. The apolipoprotein AI clone 1804 was isolated from a mouse liver cDNA library (MILLER *et al.* 1983). Dot blots were hybridized with probes labeled by nick translation in the presence of [α-32P]dATP (400 mCi/mmol; Amersham).

For quantitation of *Amy-1* in genomic DNA, fragments were isolated from genomic and cDNA clones. A 95-bp *Hae*III-*Pst*I fragment containing the 5′ flanking region of the *Amy-1* salivary promoter, as well as a portion of the first noncoding exon, was subcloned from the YBR cosmid clone cSamD4 (WIEBAUER *et al.* 1985). The other probe used was a subclone of the exon c region of the A/J cDNA pMSa104. This *Fok*I-*Dde*I fragment (nucleotides 438–604) can be used to detect *Amy-1* genomic sequences specifically because it is 28% divergent from the corresponding region of *Amy-2* (HAGENBÜCHLE, BOVEY and YOUNG 1980).

Analysis of genomic DNA: DNA was extracted from mouse liver after homogenization in buffered 0.25 M sucrose using a Dounce homogenizer, lysis with 2% SDS at 60°, digestion with protease (Sigma Type XVI) at 37° for 2 h, nine to 11 extractions with chloroform:isoamyl alcohol (20:1) and precipitation with ethanol. Samples were then treated with ribonuclease, extracted with phenol:chloroform (1:1) and reprecipitated with ethanol. The integrity of the DNA samples was evaluated on agarose gels. Concentrations were determined from the optical density at 260 nm of duplicate dilutions of each DNA sample. The average ratio of OD_{260}:OD_{280} was 1.75, with a range of 1.65–1.85. Restriction enzyme digestions were carried out with three to four units of enzyme per microgram of genomic DNA. Restriction fragments were separated by electrophoresis on agarose gels, transferred to nitrocellulose filters (SMITH and SUMMERS 1980) and hybridized as previously described (TAKEUCHI *et al.* 1986); filters were washed at high stringency: 0.1 × SSC at 65°.

RESULTS

Amylase mRNA in the pancreas of inbred strains: In order to compare the abundance of pancreatic amylase mRNA, total pancreatic RNA from each strain was dotted onto nitrocellulose and hybridized with radiolabeled amylase cDNA. Pairwise comparisons demonstrated that the abundance of amylase mRNA in YBR pancreas is approximately one-half that found in the other strains (Figure 1). This reduction in mRNA concentration can account for the reduced *in vivo* rate of amylase synthesis in YBR pancreas (BLOOR, MEISLER and NIELSEN 1981).

Since strains YBR and CE produce multiple electromorphs of pancreatic amylase, we were interested in determining whether the encoding mRNAs might differ in length. No length heterogeneity was detected in the mRNA from the four strains, which were compared by electrophoresis on agarose gels (Figure 2A).

Amylase mRNA in the parotid glands of inbred strains: The abundance

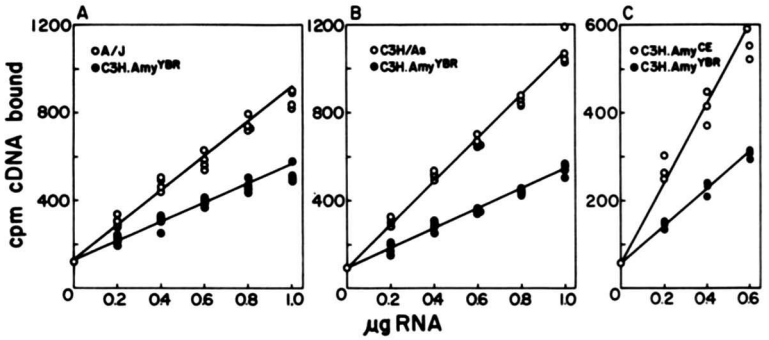

FIGURE 1.—Relative concentrations of amylase sequences in total pancreatic RNA from four strains. Aliquots of RNA were baked onto nitrocellulose filters and hybridized with radiolabeled pancreatic amylase cDNA. The data in each panel is from a single filter; the three filters are from separate experiments. RNA from C3H·Amy^{YBR} was compared with strains A/J (A), C3H/As (B) and C3H·Amy^{CE} (C).

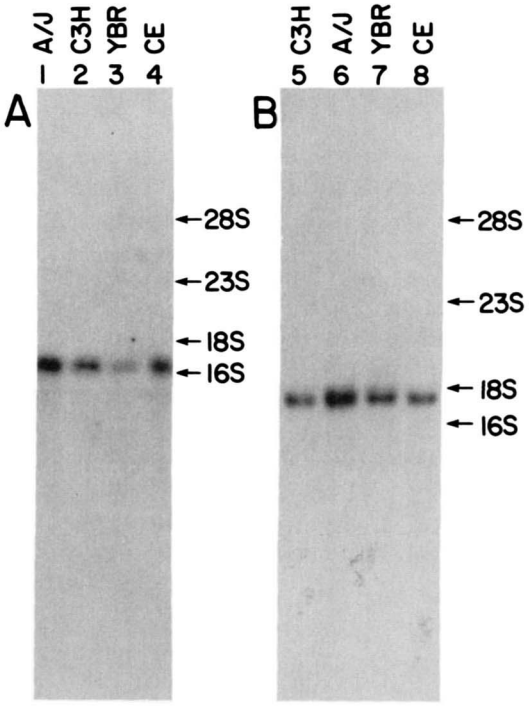

FIGURE 2.—Size homogeneity of amylase mRNA in pancreas and parotid tissues. Aliquots of total RNA were denatured and subjected to electrophoresis on 1.5% agarose-formaldehyde gels, transferred to nitrocellulose filters and hybridized with amylase-specific probes. A, Pancreatic RNA hybridized with pancreatic amylase cDNA pMPa21. B, Parotid RNA hybridized with parotid amylase cDNA pMSa104.

of parotid amylase mRNA in YBR was compared by quantitative dot blot analysis with that found in other strains. Total parotid RNA was isolated and hybridized with mouse parotid amylase cDNA. The abundance of mRNA in

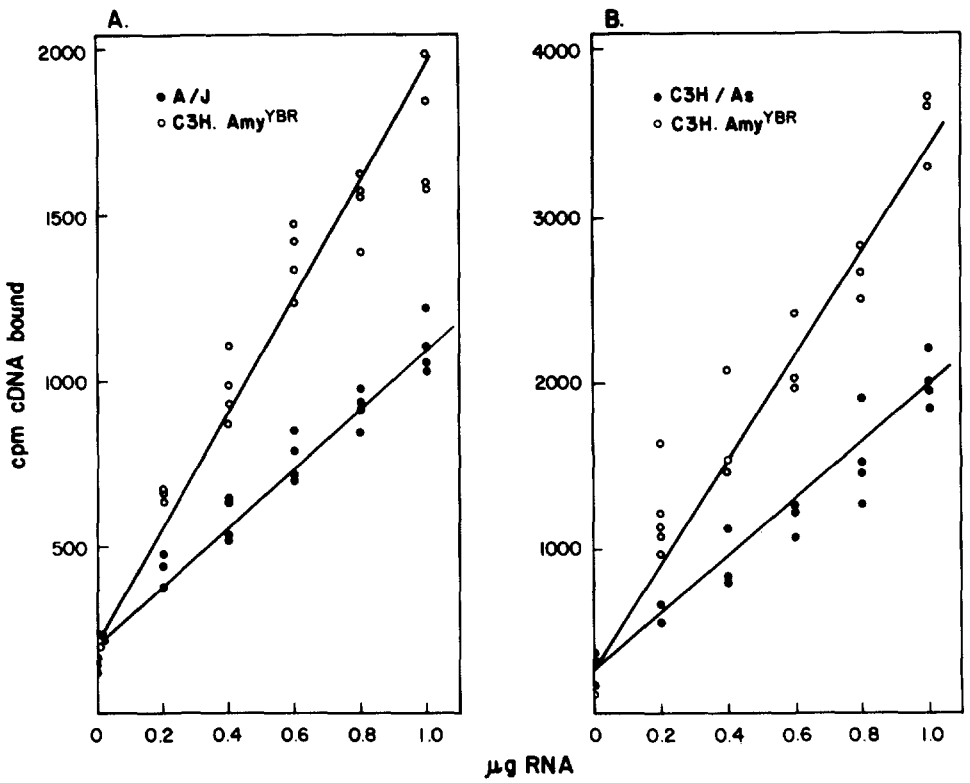

FIGURE 3.—Increased abundance of amylase mRNA in the parotid of YBR mice. Aliquots of total RNA from parotid glands were baked onto nitrocellulose filters and hybridized with ^{32}P-labeled amylase cDNA. The hybridized radioactivity was quantitated in a scintillation counter.

strain YBR differed significantly from that in the other strains. In YBR parotid, amylase mRNA abundance is approximately two-fold higher than in strains A/J and C3H (Figure 3). No heterogeneity in length of parotid amylase mRNA was detected by Northern blot analysis (Figure 2B).

Amylase mRNA in the liver of inbred strains: Since the *Amy-1* locus is expressed in liver as well as in parotid gland, it was of interest to determine whether *Amy-1* expression is also elevated in the liver of YBR mice. Liver poly(A)$^+$ mRNA was analyzed by Northern blot analysis as above, except that the amount of RNA in each lane was precisely determined, and the extent of hybridization was quantitated by densitometry. An autoradiograph of the Northern blot of liver RNA is presented in Figure 4. As expected, the size of the liver mRNA is approximately 200-base pairs (bp) larger than the pancreatic mRNA that was included as a positive hybridization control (YOUNG, HAGENBÜCHLE and SCHIBLER 1981). Poly(A)$^+$ RNA from the liver of C3H·Amy^{YBR} mice hybridizes two- to fourfold more amylase cDNA probe than do the other strains (Figure 4A). To provide an internal control for the concentration and quality of the RNA preparations, the filter was rehybridized with apolipoprotein AI cDNA (Figure 4B, C). Since the apolipoprotein locus is unlinked to

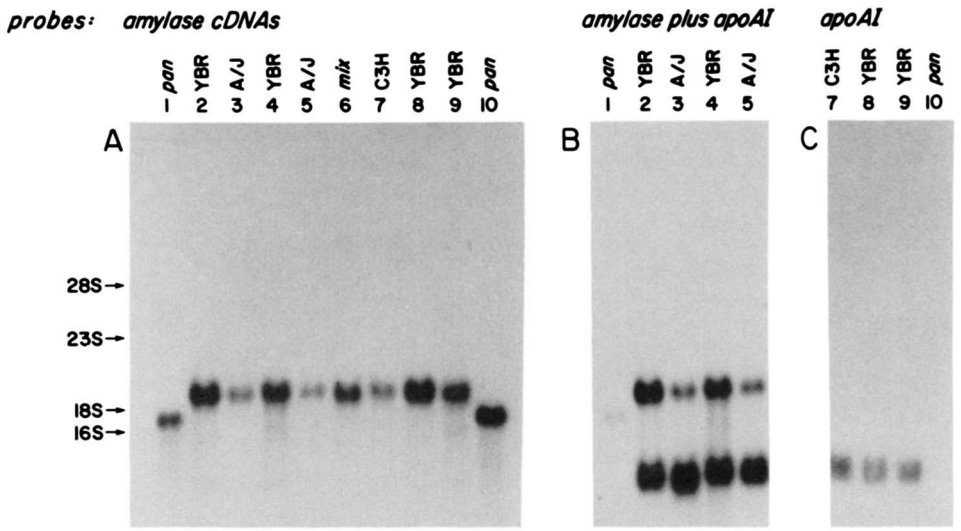

FIGURE 4.—Elevated concentration of amylase mRNA in the liver of YBR mice. Poly(A)$^+$ RNA was isolated from livers of the indicated strains. After electrophoresis on 1.5% agarose, the RNA was transferred by blotting to a nitrocellulose filter. The filter was first hybridized with a mixture of salivary plus pancreatic amylase cDNAs (6.25 ng/ml each) (panel A). After the radioactivity had decayed for 4 months, the filter was divided in two and was rehybridized either with a mixture of salivary amylase cDNA (12 ng/ml) plus apolipoprotein AI cDNA (3 ng/ml) (panel B) or with the apolipoprotein AI cDNA alone (12 ng/ml) (panel C). Each well was loaded with 12 μg of liver poly(A)$^+$ RNA from the indicated strain. (YBR indicates the congenic strain C3H·Amy^{YBR}). Mix = 6 μg RNA from strains YBR and A/J. Two preparations of liver RNA from strain C3H·Amy^{YBR} are included, the first in lanes 2 and 8, and the second in lanes 4 and 9. The specific activity of the probes was $\geq 1 \times 10^8$ cpm/μg DNA.

the amylase genes (ANTONUCCI *et al.* 1984), the concentration of apolipoprotein mRNA should not differ between strains C3H and the amylase congenic C3H·Amy^{YBR}. As expected, the concentration of apolipoprotein AI mRNA did not vary significantly among any of the strains.

Quantitation of *Amy-1* gene copies: The increased mRNA abundance in liver and parotid suggested that there could be an extra copy of *Amy-1* in strain YBR. This possibility was tested by Southern blot analysis of genomic DNA from several strains. Because of the complex hybridization pattern of the amylase gene family, we constructed small probes that hybridize with only one restriction fragment from *Amy-1* and that do not cross-hybridize with *Amy-2* genes. Two small exon-specific subclones that satisfy these requirements were isolated from a genomic clone of *Amy-1* as described in MATERIALS AND METHODS. The first subclone, containing the parotid-specific promoter, hybridizes with a 5.4-kb *Eco*RI fragment in strain YBR and a 4.7-kb *Eco*RI fragment in strain A/J (Figure 5A). DNA from a (YBR/Ki × A/J)F_1 heterozygote contains both restriction fragments (Figure 5A). The intensity of the two parental fragments in the heterozygote was compared by densitometry (Figure 5B). The ratio of hybridization of the YBR fragment to the A/J fragment was 1.9, indicating the presence of twice as many gene copies in strain YBR.

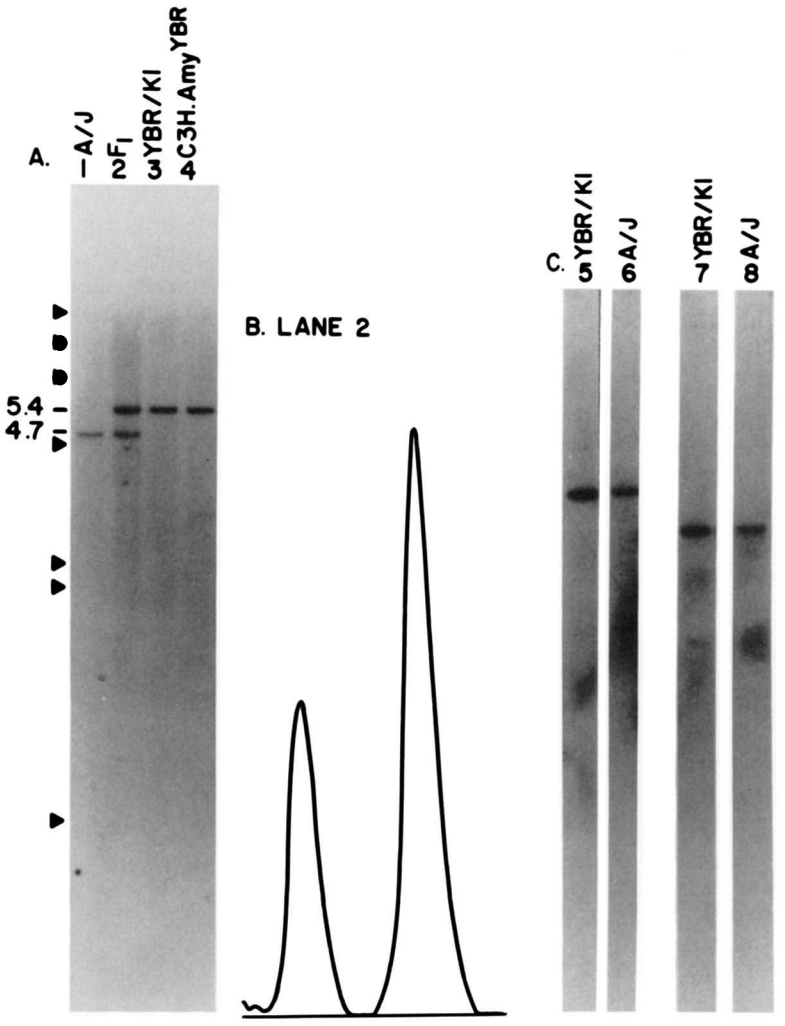

FIGURE 5.—Quantitation of *Amy-1* sequences in genomic DNA. Ten-microgram aliquots of genomic DNA were digested with restriction endonucleases and were analyzed as described in MATERIALS AND METHODS. Restriction fragments were hybridized with probes specific for *Amy-1*. A, *Eco*RI fragments were separated by electrophoresis on an 0.9% agarose gel and were hybridized with a probe containing the parotid promoter. Triangles indicate the positions of size markers: lambda DNA digested by *Hin*dIII. B, Densitometry of lane 2. C, Hybridization of genomic DNA with a probe from exon c, after digestion with *Hae*III (lanes 5 and 6) and *Sau*3A (lanes 7 and 8) and electrophoresis on a 1.7% agarose gel.

This result was confirmed with a 166-bp probe from exon c of *Amy-1*. No variation in fragment length could be detected with this probe using 12 different restriction endonucleases. We therefore compared the intensity of hybridization when equal amounts of genomic DNA were loaded in adjacent lanes. Twofold greater hybridization with DNA from strain YBR/Ki was consistently observed with this exonic probe. Representative data is presented for the 1.4-kb *Hae*III fragment and the 1.1-kb *Sau*3A fragment from strains

YBR/Ki and A/J (Figure 5C). The data indicate that strain YBR carries twice as many *Amy-1* gene copies as do the other strains tested.

DISCUSSION

Until now, it was not known whether the altered rates of amylase synthesis in pancreas and parotid of YBR mice were due to altered rates of translation of amylase mRNAs or to differences in mRNA concentrations. The experiments presented here demonstrate variation in mRNA abundances sufficient to explain the differences in amylase synthesis. Our results are similar to those obtained by OWERBACH, NIELSEN and RUTTER (1981), who demonstrated differences in mRNA abundance in another quantitative amylase variant.

Two different promoters are employed for transcription of the *Amy-1* locus in the liver and parotid of A/J mice (SCHIBLER *et al.* 1983), and both promoters are present in *Amy-1* from strain YBR (GUMUCIO 1986). Our observation that *Amy-1* mRNA abundance is elevated in both the liver and parotid of YBR mice suggested duplication of the entire *Amy-1* gene including parotid and liver promoters. Quantitative Southern blot analysis of genomic DNA, using two probes from separated regions of the gene, provided direct evidence for a twofold excess of *Amy-1* DNA in strain YBR. If the estimated copy number for *Amy-1* in A/J is correct, then YBR/Ki mice must have two nonallelic gene copies. We did not observe more than one hybridizing restriction fragment in YBR genomic DNA with the exon-specific *Amy-1* probes, indicating that the two gene copies share many restriction sites. In addition, we have not detected heterogeneity among the restriction maps of amylase cDNA clones from a YBR parotid cDNA library (L. SAMUELSON, unpublished results). The absence of divergence between the two *Amy-1* gene copies suggests that this strain-specific duplication was a recent event. We conclude that the additional *Amy-1* sequences in strain YBR/Ki can account for the increase of amylase protein synthesis and mRNA abundance in YBR parotid and liver.

The reduction in amylase synthesis and mRNA abundance in YBR pancreas also appears to be a consequence of gene dosage. Our recent characterization of genomic clones from this strain indicates that there are two nonallelic pancreatic amylase genes, *Amy-2.1* and *Amy-2.2* (GUMUCIO *et al.* 1985). Three or four copies of *Amy-2.1* have been detected in the genome of A/J mice (HAGENBÜCHLE *et al.* 1984) in addition to the null allele of *Amy 2.2*. As many as 16 *Amy-2* gene copies have been detected in strain CE/J, and at least five of these are transcribed (BODARY *et al.* 1985; TOSI *et al.* 1984). The increased abundance of amylase mRNA in A/J and CE/J pancreas in comparison with strain YBR is probably related to their increased number of active *Amy-2* genes.

Quantitative variation in gene expression due to variation in gene copy number may be typical of multigene families in which gene copies are tandemly arranged. For example, in the human globin gene family, variation in gene copy number has been described in thalassemic patients. Comparison of the arrangement of amylase gene copies in YBR with other strains of mice may provide information regarding the molecular origin of this type of variation.

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