Differential Expression of reg-I and reg-II Genes During Aging in the Normal Mouse

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A cDNA termed reg (for regenerating gene) has been isolated from a rat pancreatic DNA library. Reg expression has been shown to correlate with changes in β cell mass and function. This finding has been recently challenged by studies showing a non-β-cell-dependent regulation of reg expression. All studies to date, however, have neglected the fact that two nonallelic reg genes (reg-I and reg-II) exist in several species. In studying the regulation of each individual copy gene, we investigated reg-I and -II gene expression in a naturally occurring modification of β-cell physiology: normal aging. RNA was isolated from individual pancreata of 1-, 3-, 9-, 20-, and 30-month-old C57BL6J mice (n ≥ 3 per group) and subjected to slot-blot analysis using homologous probes for reg-I, reg-II, insulin, and elastase-I. A progressive age-dependent decrease in total reg mRNA levels (reg-I and -II) was detected. At 30 months of age, total reg mRNA levels were approximately 45% of the level detected in 1-month-old mice (p = .01). This paralleled the decrease in insulin mRNA levels (p = .01), which fell below 50%; by contrast, mRNA levels for elastase-I increased with age (p = .05). Analysis of RNA isolated from purified islets did not reveal any mRNA for reg, suggesting that in the normal mouse, reg is primarily a product of the exocrine pancreas. Reg mRNA were detectable in RNA extracts from stomach, duodenum, and small intestine. By hybridization of total pancreatic RNA with oligonucleotide probes which specifically recognize reg-I or reg-II sequences, we show that reg-I mRNA levels declined with age (p = .001) while reg-II mRNA levels remained unchanged. These data demonstrate that in mouse pancreas the two nonallelic reg genes are differentially expressed during aging and that the decrease in reg-I mRNA levels parallels the decrease in insulin gene expression. Differential regulation of reg-I and reg-II genes may explain the presence of conflicting data in the current literature.

MATURE pancreatic islets of Langerhans are composed of cells that slowly undergo mitosis and that exhibit poor capability to regenerate after injury (Swenne, 1983; Hellerstrom et al., 1988). Administration of poly(ADP-ribose) synthase inhibitors, such as nicotinamide, to rats subjected to 90% pancreatectomy induces regeneration of pancreatic islets and amelioration of diabetes (Okamoto, 1981; Yonemura et al., 1984, 1988). Terazono et al. (1988) isolated and characterized a cDNA from a rat regenerating pancreatic-derived cDNA library, designated reg (for regenerating gene). The 166-amino acid peptide has also been shown to mirror changes in β-cell mass and/or β-cell function. Reg mRNA levels increased markedly in islets induced to proliferate by pancreatectomy and nicotinamide treatment compared to normal islets (Terazono et al., 1988). The increase in reg expression was temporally correlated with the increase in size of the regenerating islets and the decrease in glycosuria. Increased reg gene expression has also been demonstrated in the hyperplastic islets of NON (non-obese) mice after treatment with aurothioglucose (Terazono et al., 1988).

In another model of pancreatic regeneration in which the pancreatic duct of a rat is subjected to nonocclusive obstruction (wrapping), reg expression increased coordinately with the appearance of new islets (Zenilman et al., 1993). Furthermore, subcutaneous implantation of a solid insulinoma tumor into rats caused a dramatic reduction in the volume of the endogenous islets (Chick et al., 1977). This phenomenon was associated with coordinate suppression of both reg and insulin gene expression (Miyaura et al., 1991). Removal of the tumor resulted in a rapid induction of reg gene expression, proliferation of endocrine β cells, and the restoration of endogenous insulin gene expression and secretion (Miyaura et al., 1991).

Although these studies appear to link changes in reg gene expression with changes in β-cell mass, Smith et al. (1994) have shown that reg may be induced even in the absence of proliferative changes. This finding discouraged many laboratories from performing further studies to explore the possible role of reg as a growth and maintenance factor for pancreatic β cells. We believe that a fundamental technical problem may have contributed to the generation of conflicting data. Both proponents and opponents of the view that reg may be important in β-cell physiology have neglected previous reports demonstrating that in several animal species there are two independent, nonallelic reg genes located on different chromosomes (Watanabe et al., 1990; Kaminura et al., 1992; Unno et al., 1993). All studies to date, either in support of or against a role for reg in β-cell physiology, have
been performed using cDNA probes that were not able to distinguish between the two reg mRNAs. We believe that this may have largely contributed to the presence of conflicting results in the literature. One of the aims of this work is to provide experimental evidence for the need to support a revision of those studies, taking into account the two nonallelic reg genes individually.

In this study, we investigated the regulation of pancreatic reg-I and reg-II gene expression during normal aging in the mouse.

MATERIALS AND METHODS

Animals. — One-, 3-, 9-, 20-, and 30-month-old C57BL/6J male mice (n = 3 per group) were housed at the Gerontology Research Center, National Institute on Aging. They were fed a stock diet and maintained in a controlled environment (12-h light/dark cycle; 22 °C). Prior to sacrifice by decapitation, they had free access to water but were fasted overnight. Pancreata were quickly removed and used for subsequent experiments. All procedures were in accordance with National Institutes of Health guidelines and were approved by the Animal Resource Section of the National Institute on Aging.

Oligonucleotide primers and probes. — Oligonucleotides, synthesized on an Applied Biosystems (Foster City, CA) DNA synthesizer were the resin with concentrated ammonium hydroxide at room temperature, deprotected by heating to 55 °C overnight, and purified over Nensorb Prep columns (New England Nuclear, Boston, MA). Polymerase chain reaction (PCR) primers were designed according to the published mouse or rat sequences for reg, insulin, and elastase-I cDNAs (MacDonald et al., 1982; Wentworth et al., 1986; Unno et al., 1993) (Table 1). Homologous cDNAs were generated by reverse transcription (RT)–PCR (see below). To measure total reg mRNA levels, a 246-bp reg-II cDNA probe that hybridizes to mRNAs for both reg-I and reg-II was generated by RT-PCR in addition, two oligonucleotides specific for reg-I or reg-II mRNA were used to detect the relative abundance of each gene individually (Table 1 and Figure 1). Oligonucleotide and cDNA probes were radiolabeled using [γ-32P]ATP (polynucleotide kinase) and [α-32P]dCTP (random priming), respectively (Amershaw, Arlington Heights, IL).

Synthesis of pancreatic cDNA probes by RT–PCR. — One microgram of mouse pancreatic RNA extracted from a 3-month-old male was used as template for RT which was performed at 37 °C in a final volume of 20 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25 °C), 1.5 mM MgCl2, 0.01 mg/ml gelatin, 0.2 mM each dNTP, 40 units/μl RNAse (Promega, Madison, WI), 0.5 μM downstream primer (Table 1), and 7 units/μl avian myeloblastosis virus reverse transcriptase (Promega) (Saiki et al., 1988; Shuldiner and Perfetti, 1993).

The second DNA strand was synthesized during the first cycle of PCR in which 5 μl of the RT reaction mixture was subjected to electrophoresis on a composite gel consisting of 1% agarose (Bethesda Research Laboratories, Gaithersburg, Maryland).
MD) and 2% Nusieve (FMC Byproducts, Rockland, ME). The gel was stained with ethidium bromide and photographed to verify that the amplified products corresponded to the predicted size.

Synthesis of reg-I and reg-II cDNA standards. — A PCR-based cloning strategy was used to obtain partial cDNA sequences of mouse reg-I and reg-II using gene-specific oligonucleotide primers specific for each sequence (Figure 1). Mouse reg-I and reg-II PCR products were ligated into pCR-II plasmid (Invitrogen, San Diego, CA). Plasmid DNA was purified using a Qiagen (Chatsworth, CA) kit. Purified plasmid DNA (1–3 μg) was subjected to dideoxy sequence analysis (Sequenase, United States Biochemical, Cleveland, OH). Reg-I and reg-II cDNAs were used as standards to demonstrate that hybridization with gene-specific oligonucleotide probes was (1) specific, and (2) that the specific activity of the radiolabeled probes was similar.

RNA extraction of solid tissue and Northern blot analysis. — Total RNA was prepared from fresh tissue (pancreas, esophagus, stomach, duodenum, small intestine, colon, heart, lung, brain, kidney, spleen, and testis) by homogenization in guanidinium thiocyanate (Chirgwin et al., 1979). Pancreata were collected from all age groups described above, while the other tissues were obtained only from 3-month-old mice. RNA samples (10 μg) were subjected to electrophoresis on a 1.5% agarose, 2.2 M formaldehyde gel. Ribosomal RNA was visualized by staining with ethidium bromide. RNA was transferred to nylon filters (Schleicher & Schuell, Keene, NH), fixed by baking for 2 h in a vacuum oven at 80 °C, and hybridized with an oligonucleotide probe homologous to the mouse 18S ribosomal subunit (5'-ACCGTATCTGATGCTTCCGA-3'). Prehybridization and hybridization were performed at 42 °C in 5 × Denhardt’s solution containing 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate (SDS), 0.1% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA. Filters were washed twice under stringent conditions (2 × SSPE, 0.05% SDS; 45 °C, 15 min) and then exposed to Kodak (Eastman Kodak, Rochester, NY) XAR-5 film with enhancing screens at -70 °C for 24 h.

RNA isolation from mouse islets of Langerhans. — Total RNA from freshly isolated primary cultures of mouse pancreatic islets from 3-month-old mice was isolated using a modification of the guanidinium thiocyanate-pheno1-chloroform method (Chomczynski and Sacchi, 1987). Briefly, cells were homogenized by passage through 18- or 22-gauge needles in the presence of denaturing solution [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% N-lauroylsarcosine, 0.1 M 2-mercaptoethanol]. Then, 0.1 ml of 2 M sodium acetate (pH 4), 1 ml of phenol, and 0.2 ml of chloroformisooamyl alcohol mixture (49:1) were added to the homogenate. The suspension was shaken vigorously and cooled on ice for 15 min. Samples were centrifuged at 10,000× g for 20 min at 4 °C. After centrifugation, the aqueous phase (1 ml) was transferred to a new tube, mixed with 1 ml of isopropanol, and placed at -20 °C for at least 1 h. RNA was pelleted by centrifugation at 20,000× g for 20 min. The RNA pellet was dissolved in 0.3 ml of denaturing solution, transferred to a new tube, and precipitated with 1 volume of isopropanol at -20 °C for 1 h. After centrifugation for 10 min at 4 °C, the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried, and dissolved in 50 μl 0.5% SDS by heating at 65 °C for 10 min and vortex mixing.

Slot-blot analysis. — Fifteen micrograms of total RNA from pancreas and other tissues were diluted in sterile TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 7.4)] to a final volume of 50 μl. Twenty microliters of 37% formaldehyde and 20 μl 10 × standard saline citrate (SSC) were added, and the solution was placed at 60 °C for 15 min. The samples were then diluted with 1 ml of cold 10 × SSC, and 300 μl of the solution was applied in triplicate to a nylon membrane in a slot-blot manifold. The wells were washed three times with 400 μl cold 10 × SSC, air dried, and baked in a vacuum oven at 80 °C for 2 h. The membranes were hybridized with 0.5–1 × 10⁶ cpm of 32P-labeled probe per ml of hybridization solution. Hybridization conditions were the same as those described for Northern blot analysis. The blots were washed twice in 2 × SSPE and 0.5% SDS for 15 min at 55 °C when hybridized with cDNA probes, or twice in 2 × SSPE and 0.5% SDS for 15 min at 42 °C when hybridized with oligonucleotide probes, then subjected to autoradiography.

Quantification of mRNA levels. — The relative levels of mRNA for reg-I, reg-II, insulin, and elastase-I between mice of different ages were quantified using a Betascope 703 blot analyzer (Betagen, Waltham, MA). After quantification, the blots were stripped by washing twice with 0.01 × SSPE and 0.1% SDS at 80 °C. To correct for possible differences in loading of RNA, data were normalized by rehybridizing the same blots with 32P-labeled oligo-dT₂₀.

Statistical analyses. — The data were expressed as mean ± SEM of three to five replicates and analyzed by one-way analysis of variance (ANOVA). Regression analysis was performed for all data presented.

RESULTS

Subcloning of mouse reg-I and reg-II cDNAs and validation of probe specificity. — To generate cDNA standards for reg-I and reg-II, RT-PCR was performed using oligonucleotide primers specific for each sequence (U and D for reg-I, and Uᵦ and Dᵦ for reg-II) (Figure 1). The identity of the two products was confirmed by their difference in size, as detected by agarose gel electrophoresis: reg-I and reg-II PCR products were 395 and 418 bp long, respectively (data not shown). Both products were subcloned and sequenced to further confirm their identity (data not shown). Figure 2 shows oligonucleotide hybridization of serial dilutions (1:10) of reg-I and reg-II cDNA standards with gene-specific oligonucleotide probes (reg-I probe, Left; reg-II probe, Center) and with an oligonucleotide probe common to both sequences (probe C, Right). The two gene-specific probes recognized exclusively their respective sequences, and the common probe recognized both reg-I and reg-II sequences (Figure 2).
Oligonucleotide probes

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Reg-I and -II

Insulin

Elastase-1

Figure 2. Gene-specific oligonucleotide hybridization. (Upper) Serial dilutions (1:10) of reg-I and reg-II cDNAs were subjected to slot-blot hybridization in triplicate with oligonucleotide probes that specifically recognized reg-I (Left), and reg-II (Center), as well as probe C (Right) that recognized both sequences. (Lower) Sequences of oligonucleotide probes used for gene-specific hybridization.

The same membranes containing serial dilutions of reg-I and reg-II cDNA standards were hybridized with the 246-bp reg-II cDNA probe. With the hybridization and wash conditions employed, this probe recognized both reg-I and reg-II sequences (data not shown).

Northern blot analysis of pancreatic and extrapancreatic RNA. — Northern blot analysis was performed for all samples to verify the integrity of the RNA by ethidium-bromide staining and visualization of the 28S and 18S ribosomal bands. In addition, hybridization of RNA samples with an oligonucleotide probe homologous to the mouse 18S ribosomal subunit revealed that no significant degradation occurred (data not shown).

Reg mRNA in whole pancreas and in islets of Langerhans. — Northern blot analysis of total RNA extracted from 3-month-old mouse islets and from 3-month-old whole pancreas was performed with cDNA probes for mouse insulin, elastase-I, and reg-II (which recognized equally well both reg-I and reg-II sequences). Reg mRNA was detectable in the whole pancreas but not in the isolated islets (Figure 3). This indicated that in the normal mouse, reg is primarily, if not exclusively, a product of the exocrine pancreas. Insulin mRNA was detectable in both samples, and elastase-I was present exclusively in whole pancreas (Figure 3).

Reg gene expression in extrapancreatic tissues. — Slot-blot analysis of total RNA from several extrapancreatic mouse tissues with the 246-bp reg-II cDNA probe that recognized both reg-I and reg-II sequences showed that reg mRNA was detectable in several segments of the gastrointestinal tract. Reg mRNA was present in extracts from duodenum, small intestine, and colon (Figure 4). No reg mRNA was detected in both esophagus and stomach. In addition, reg mRNA was absent in brain, cerebellum, heart, lung, liver, spleen, kidney, and testis (Figure 4). Hybridization of the same membranes with the mouse insulin cDNA probe was negative for all tissues with the exception of the pancreas, demonstrating that the extrapancreatic samples were not contaminated with pancreatic RNA. Finally, hybridization with an oligonucleotide probe homologous to the poly(A) tail of all mRNAs demonstrated that an approximately equal amount of RNA was used for each individual sample (Figure 4).
Figure 4. Detection of reg mRNA in extrapancreatic tissues. Five micrograms of total RNA obtained from a 3-month-old mouse were subjected to slot-blot analysis and hybridized with the 246-bp reg-II probe, which recognizes equally reg-I and reg-II mRNAs (B) and insulin-II cDNA probes (C). The data were normalized by hybridizing the same membrane with oligonucleotide dT20 (A).

Pancreatic reg and insulin gene expression during normal aging. — Total reg mRNA levels (reg-I and reg-II), as detected with the 246-bp reg-II cDNA probe, decreased progressively with age (Figure 5). At 30 months of age, reg mRNA levels were approximately 46.8 ± 7.9% of the level detected in 1-month-old mice (p = .01). This decrease was parallel to the age-dependent decline in insulin mRNA levels which fell by 43.3 ± 10.1% with age (p = .02) (Figure 6). Regression analysis of either reg or insulin mRNA levels showed a constant decrease of mRNA levels for both of them per each month of age (Figures 5 and 6). By contrast, elastase-I mRNA levels showed a modest but significant increase with age. At 30 months of age (p = .05), elastase-I mRNA levels were approximately 124 ± 8.1% of the level detected in 1-month-old mice (p = .05) (Figure 7).

Differential expression of reg-I and reg-II genes during normal aging. — Hybridization with a reg cDNA probe that recognized both reg-I and reg-II sequences showed that total reg mRNA levels decreased markedly with age (Figure 3). In order to investigate if there was any difference in the relative expression of reg-I versus reg-II, we took advantage of a DNA segment (corresponding to a region encoding exon 2) that was substantially different between reg-I and reg-II sequences to design oligonucleotide probes that specifically hybridized to mRNA for either reg-I or reg-II (Figures 1 and 2). Total pancreatic RNA from 3-, 9-, and 30-month-old mice was then subjected to slot-blot analysis in duplicate and hybridized with reg-I and reg-II-specific oligonucleotide probes. We found that with age, the two nonallelic mouse reg genes were differentially regulated. There was an age-dependent decline in reg-I mRNA levels (p = .001), while there was no significant variation in reg-II mRNA levels (Figure 8). At 9 and 30 months of age, reg-I mRNA levels were, respectively, 59.2 ± 16.9% and 39.9 ± 28.1% of the levels detected in 3-month-old mice (p = .01 at 9 months, p = .003 at 30 months). Regression analysis of the data showed a statistically significant decrease in reg-I mRNA levels per each month of age (Figure 8).

**DISCUSSION**

The reg gene was cloned from a regenerating pancreas following surgical subtotal pancreatectomy and treatment with nicotinamide (Terazono et al., 1988). Interventions that cause a reduction in β-cell mass or suppression of β-cell function (i.e., implantation of an insulinoma) (Miyamura et
Insulin

Oligo dT20

Figure 6. Quantitative slot-blot analysis for insulin mRNA. Five micrograms of pancreatic RNA from 1-, 3-, 9-, 20-, and 30-month-old mice were subjected to slot-blot analysis with the insulin-II cDNA probe. The data were normalized by hybridizing the same membranes with oligo-dT20 and were expressed as a percentage of the mean value observed in the 1-month-old group. As evaluated by one-way ANOVA, there was a statistically significant decrease in insulin mRNA levels (p = .005). Regression analysis of the data showed that insulin mRNA levels decreased .07 relative units per month of age (p < .0001). The two blots on the right represent two individual experiments, while the values shown on the graph indicate the mean of three different experiments. For graphic purposes, the age scale on the x axes was drawn using an equal arbitrary spacing between bars.

al., 1991) result in a decrease in reg mRNA levels, while interventions that stimulate islet proliferation (i.e., removal of an insulinoma) (Miyamura et al., 1991) or surgical wrapping/partial occlusion of the pancreatic duct (Zenilman et al., 1993) are associated with marked increases in reg mRNA levels. Watanabe et al. (1994) have shown that administration of recombinant reg to 90% depancreatized rats induces β-cell proliferation. Furthermore, reg protein has been shown to induce DNA synthesis in isolated islets in culture (Watanabe et al., 1994) as well as in islet-derived cell lines (Zenilman et al., 1993).

In this study, we demonstrate that the mouse reg genes are expressed in the exocrine pancreas but not in normal pancreatic islets of 3-month-old mice. We also found that the stomach, the duodenum, and the small intestine exhibit detectable levels of reg mRNA. This is in contrast with previous observations which have shown extrapancreatic expression of reg only in the gall bladder (Terazono et al., 1988), a tissue that we did not analyze. Others have detected increased expression of reg (Satomura et al., 1993) and reg-related genes (Lasserre et al., 1992), respectively, in the diseased pancreas and in neoplastic colonic tissue, further linking enhanced reg expression to the proliferative state. The common embryological origin of the exocrine pancreas and the gastrointestinal tract could represent the biological basis for the detection of reg mRNA in the gut. The negative results presented by other groups may be related to a greater level of sensitivity achieved in our laboratory.

We demonstrated that there was an age-dependent decrease in reg mRNA levels that closely parallels decreases in insulin mRNA levels. Since reg is mainly a product of the exocrine pancreas, we investigated the expression of elastase-I, which is one of the major exocrine gene products, to discern whether the decrease in mRNA levels was a generic phenomenon associated with aging of the whole organ or with a more specific event. Elastase-I mRNA levels showed a moderate but significant increase with age. We did not measure reg protein levels; therefore, we do not have the experimental evidence that changes in reg mRNA are followed by analogous changes in protein levels. While our findings do not allow extrapolation concerning the protein(s) levels, others have extensively showed that changes in reg mRNA levels are followed by parallel variations in the functional status of pancreatic β cells (i.e., increased insulin synthesis is associated with increased reg mRNA levels, while suppression of insulin synthesis is paralleled by decreased reg mRNA levels), implying that these phenomena are likely mediated by the counterpart protein(s) (Terazono et al., 1988; Miyaura et al., 1991).

Interestingly, we found that the two nonallelic mouse reg genes were differentially regulated during aging. While reg-I mRNA levels progressively declined with age, reg-II mRNA levels remained unchanged. This finding may help to further explain why different laboratories reported conflict-
belong to the reg gene family. Therefore, in studying their biology (Drickamer, 1988; Kamimura et al., 1992; Lasserre et al., 1992; Zenilman et al., 1993; Watanabe et al., 1994); all studies to date have neglected to consider each of the two nonallelic genes individually (Terazono et al., 1988; Miyamura et al., 1991; Smith et al., 1994; Watanabe et al., 1994). This may have led to inaccurate data interpretation. It is possible to speculate that if a difference in gene expression or biological activity among the two nonallelic genes exists, this difference may have been either greatly underestimated or artificially magnified, depending on the sensitivity and the specificity of the technique used. Over the last few years, it has been extensively reported that reg belongs to a large gene family that shares a significant degree of nucleotide homology (Drickamer, 1988; Kamimura et al., 1992; Lasserre et al., 1992; Zenilman et al., 1993; Watanabe et al., 1994); despite such high structural similarity, very different biological functions have been claimed for individual proteins that belong to the reg gene family. Therefore, in studying their regulation, it is necessary to prove that the technique used is gene-specific and that it eliminates any possible cross-reactivity with other very similar genes. On the basis of our findings, it is likely that those studies postulating a crucial role of reg in β-cell physiology and/or islet mass may have used assay conditions that measure mostly reg-I mRNA levels. On the other hand, reports challenging this relationship may have mostly detected reg-II mRNA levels that, according to our study, do not parallel the expression of the insulin gene.

The study of recently duplicated generic loci offers a unique opportunity to investigate how two genes, initially with identical patterns of expression and function, gain individual characteristics over time. We have previously shown that in Xenopus laevis the two nonallelic insulin genes (Shuldiner et al., 1991) and the two nonallelic insulin-like growth factor I (IGF-I) genes (Perfetti et al., 1994) are differentially expressed during development. It is hypothesized that duplication may offer a selective advantage to the organism since the duplicated allele may act as a "backup" for its partner (Soares et al., 1985; Graf and Kobel, 1991). Thus genetic alterations that may have been lethal at a nonduplicated locus are no longer lethal. Alternatively, duplicated loci may evolve differential functions and may be the basis for the early events leading to the evolution of gene families (Kobel and Du Pasquier, 1986). We hypothesize that early in the course of the evolution of gene families, progression to differential function first involves alterations in regulatory regions resulting in the differential expression (e.g., developmental and/or tissue-specific) of an otherwise identical or highly similar protein product. Later, further mutations in coding regions may have resulted in protein products with distinct (or overlapping) biological functions. On the basis of sequence analysis, Unno et al. (1993) demonstrated that mouse reg-I and rat reg-I genes are more closely related than mouse reg-I and reg-II genes, suggesting that the ancestral gene for reg may have been duplicated prior to divergence between mouse and rat. On the basis of this hypothesis, it is conceivable that the differential expression of the two reg genes may be associated with a different biological function of the two gene products.

In conclusion, we show that in the normal mouse (1) the two nonallelic reg genes are differentially expressed during aging, and (2) decreases in reg-I expression parallel decreases in insulin expression during aging.

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